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Departamento de Química Analítica,
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ANALYTICAL STRATEGIES FOR THE CHARACTERIZATION, IDENTIFICATION, AND QUANTIFICATION OF PEPTIDES AND PROTEINS OF INTEREST IN THE PREVENTION AND UNDERSTANDING OF HYPERTENSION

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CERTIFICA:

Que el trabajo descrito en la presente memoria, titulado "Analytical strategies for the characterization, identification, and quantification of peptides and proteins of interest in the prevention and understanding of hypertension", ha sido realizado en este departamento por Dña. Patrycja Anna Puchalska bajo la dirección de las Dras. María Luisa Marina Alegre y María Concepción García López, Catedrática y Profesora Titular, respectivamente, de dicho departamento. Asimismo, autorizo su presentación para que sea defendido como Tesis Doctoral.

Y para que conste y surta los efectos oportunos, firma el presente en Alcalá de Henares a 25 de febrero de 2014.

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Que el trabajo descrito en la presente memoria, titulado "Analytical strategies for the characterization, identification, and quantification of peptides and proteins of interest in the prevention and understanding of hypertension", ha sido realizado bajo su dirección por Dña. Patrycja Anna Puchalska en el laboratorio de Química Analítica de la Universidad de Alcalá. Parte del trabajo experimental recogido en el apartado III.1.3 ha sido realizado en la Universidad de Utrecht, Facultad de Ciencias, Grupo de espectrometría de masas de biomoleculas y proteómica (Holanda). Asimismo, autorizan su presentación para que sea defendido como Tesis Doctoral.

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"When a person is sick, the doctor should first regulate the patient's diet and lifestyle"

Sun Si- Miao, 7 A.D.

"Let food be the medicine and medicine be the food"

Hippocrates, 4 B.C.

TO MY PARENTS ANNA AND JERZY PUCHALSCY

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RESUMEN

La hipertensión constituye un serio problema de salud y está considerada como una de las causas de las enfermedades cardiovasculares y renales. A pesar de la prevalencia de la hipertensión, la mitad de las personas afectadas desconocen que la padecen. Además, los estudios realizados acerca de las causas más importantes de muerte en el mundo predicen un aumento de la contribución de las enfermedades cardiovasculares. Debido a que los síntomas de la hipertensión pueden permanecer inadvertidos y raramente se ponen de manifiesto, la prevención y tratamiento de la hipertensión se consideran de gran importancia en la sociedad moderna.

El conocimiento general tanto del sistema cardiovascular como de las causas de la hipertensión es muy amplio. Sin embargo, aún se desconocen algunos aspectos relacionados con los mecanismos moleculares que intervienen en el sistema cardiovascular. Entre los diferentes sistemas que contribuyen a controlar la presión arterial, el sistema renina-angiotensina es el más importante. En efecto, la enzima convertidora de la angiotensina I (ACE), pieza clave en el sistema renina-angiotensina, convierte la angiotensina I en angiotensina II (vasoconstrictor), desactivando al mismo tiempo la bradiquinina (vasodilatadora), y jugando, por tanto, un papel muy relevante en los niveles de la presión arterial. Ello ha motivado el empleo de fármacos antihipertensivos sintéticos con capacidad para inhibir la actividad de la ACE. Otras proteínas importantes con efecto regulador en el sistema cardiovascular son la PKA, PKG y CaMKII que poseen diferentes isoformas con diferente relevancia. Sin embargo, los niveles exactos en los que se expresan estas proteínas en los órganos todavía no se conocen lo que limita a su vez el conocimiento de su importante función en el sistema cardiovascular.

El tratamiento de la hipertensión ha disminuido la incidencia de accidentes cardiovasculares. Sin embargo, aunque la utilización de fármacos sintéticos ha sido decisiva en este descenso, estos fármacos poseen habitualmente efectos secundarios. Una alternativa interesante a estos fármacos la constituyen algunos péptidos que se encuentran de forma natural en determinados alimentos. De hecho, una estrategia básica para mejorar la salud cardiovascular es modificar la dieta y el estilo de vida ya que la dieta es uno de los factores que más influencia tienen en la salud humana. Entre los compuestos bioactivos que se encuentran en los alimentos, los péptidos bioactivos están recibiendo una gran atención en los últimos años. En particular, los péptidos antihipertensivos y antioxidantes son los más estudiados y han demostrado su efecto positivo sobre la salud cardiovascular. En efecto, los péptidos antihipertensivos pueden inhibir la actividad de la ACE y disminuir la presión

arterial. Por su parte, los péptidos antioxidantes previenen el estrés oxidativo que puede iniciar y promover la aparición de la hipertensión. Los péptidos bioactivos pueden ser ingredientes naturales de los alimentos o bien originarse a partir de proteínas alimentarias de las que forman parte ya sea por procedimientos *in vivo* o *in vitro*. La digestión gastrointestinal constituye en sí misma un procedimiento *in vivo* mientras que el procedimiento *in vitro* implica la hidrólisis de las proteínas por la acción de enzimas o microorganismos adicionados a los alimentos. En el caso de productos procesados, los péptidos bioactivos se liberan a partir de las proteínas durante el procesado del alimento (queso, yogurt, kéfir, *etc*.). Hasta la fecha, la investigación relacionada con péptidos antihipertensivos y antioxidantes se ha centrado principalmente en alimentos de origen animal como la leche, los productos lácteos y la carne. Sin embargo, los péptidos bioactivos de origen vegetal, aunque menos estudiados, a menudo poseen actividades más altas. El maíz y la soja, son ejemplos de fuentes de péptidos bioactivos de elevada actividad.

Las fórmulas infantiles de soja (SBIFs) constituyen una alternativa muy interesante a la leche y los productos lácteos para niños con intolerancia o alergia a algunos constituyentes de la leche, con problemas de alimentación o pertenecientes a familias vegetarianas. Sin embargo, en comparación con la leche y los productos lácteos, las SBIFs han sido poco investigadas en lo que al contenido de péptidos bioactivos se refiere. Estas fórmulas infantiles se elaboran a partir de aislados de proteína de soja que contienen alrededor de un 90% de proteínas. Durante su preparación, se someten a calor intenso o a hidrólisis proteica. Así, las SBIFs pueden contener de forma natural péptidos potencialmente bioactivos con efectos beneficiosos sobre la salud a parte de sus beneficios nutricionales.

En este trabajo, las SBIFs se han elegido como una fuente potencial de péptidos bioactivos. Se han propuesto cuatro métodos diferentes para extraer péptidos de las SBIFs. La capacidad antioxidante de los péptidos se determinó mediante tres ensayos diferentes mientras que la capacidad antihipertensiva se evaluó midiendo la capacidad para inhibir *in vitro* la ACE. La ultrafiltración directa a través de filtros de corte de 10 kDa permitió obtener el extracto con la concentración más alta de péptidos y la capacidad antioxidante más elevada. Los extractos así obtenidos se fraccionaron a continuación utilizando distintos filtros para ultrafiltración y se estudió la capacidad antioxidante y antihipertensiva de las fracciones obtenidas. Se obtuvieron fracciones con pesos moleculares comprendidos entre 5-10 kDa, 3-5 kDa, y por debajo de 3 kDa. La capacidad antioxidante más alta se obtuvo en la mayor parte de los casos en la

fracción peptídica 5-10 kDa. Aunque se propuso un posterior fraccionamiento de este extracto peptídico por isoelectroenfoque en OFFGEL, los anfolitos necesarios para establecer el pH en isoelectroenfoque interferían en los ensayos para evaluar la capacidad antioxidante. Por ello, se probaron varias estrategias para eliminar los anfolitos una vez llevada a cabo la separación por isoelectroenfoque. La separación por cromatografía de líquidos con una columna monolítica permitió eliminar los anfolitos de las muestras investigadas. Sin embargo, las fracciones separadas por OFFGEL mostraron una capacidad antioxidante mucho menor que la muestra inicial sugiriendo un efecto sinérgico entre los péptidos antioxidantes. En consecuencia, se eliminó el paso de separación por isoelectroenfoque en OFFGEL de las muestras. Por otra parte, la mayor capacidad para inhibir la ACE se observó en las fracciones 3-5 kDa y por debajo de 3 kDa. En estos casos, no se consideró ninguna separación adicional de estas fracciones por isoelectroenfoque. Las fracciones peptídicas con capacidad para inhibir la ACE se identificaron mediante HPLC acoplada a espectrometría de masas de quadrupolo tiempo de vuelo (Q-ToF-MS) y el programa PEAKS. El análisis de los datos mostró una baja selectividad en el fraccionamiento por ultrafiltración. Las fracciones que presentaban una mayor capacidad para inhibir la ACE (3-5 kDa y por debajo de 3 kDa) y antioxidante (5-10 kDa) se sometieron a un proceso de digestión gastrointestinal simulada con pepsina y pancreatina. Los resultados mostraron que la capacidad antioxidante prácticamente no se modificaba mientras que la capacidad para inhibir la ACE disminuía. Los péptidos obtenidos tras la digestión gastrointestinal simulada se identificaron también. De los más de 120 péptidos identificados en cada fracción antioxidante, 42 péptidos eran comunes a todas las fórmulas infantiles analizadas. El péptido VAWWM fue identificado en todas las fracciones antioxidantes. Este péptido es parte de la secuencia de la soistatina (VAWWMY) que es un péptido de la soja previamente descrito como un potente inhibidor de la absorción de colesterol y con capacidad de enlazarse a ácidos biliares. En el caso de las fracciones con capacidad inhibidora de la ACE, se encontraron 13 péptidos en la fracción 3-5 kDa y 20 péptidos en la fracción por debajo de 3 kDa que resistieron la digestión gastrointestinal simulada. Es interesante remarcar que el péptido RPSYT se encontró en todas las fórmulas infantiles analizadas y demostró tener capacidad antioxidante e inhibidora de la ACE. Tras sintetizar este péptido y caracterizarlo, se puso de manifiesto su resistencia a la acción de las enzimas gastrointestinales y a las altas temperaturas de procesado, su moderada actividad antihipertensiva y su potente actividad antioxidante.

Como ya se ha mencionado previamente, el maíz y la soja constituyen fuentes atractivas de péptidos bioactivos. Entre ellos, destacar los péptidos con potente actividad inhibidora de la ACE: LRP (IC_{50} = 0.29 μM), LSP (IC_{50} = 1.7 μM) y LQP (IC_{50} = 2.0 μM), obtenidos por digestión con termolisina a partir de α -zeínas del maíz y el péptido VLIVP (IC_{50} = 1.69 μM) que se encuentra en el hidrolizado de la glicinina 11S de la soja con proteasa P. De hecho, la capacidad para inhibir la ACE de estos péptidos es mucho mayor que la de los conocidos péptidos VPP (IC_{50}= 9.13 μM) o IPP (IC_{50}= 5.15 μM) de la leche. Teniendo en cuenta que la actividad de los péptidos inhibidores de la ACE depende en gran medida de la dosis empleada y dadas las diferencias en el contenido proteico que se pueden observar entre distintos cultivos de maíz, queda clara la necesidad de disponer de metodologías analíticas para la determinación cuantitativa de estos péptidos. Sin embargo, a pesar del elevado interés que presenta la determinación de péptidos inhibidores de la ACE, los trabajos publicados en relación a este tema son escasos.

En este trabajo, se ha desarrollado una metodología analítica para la determinación simultánea de los péptidos LRP, LSP y LQP de las α-zeínas presentes en granos de maíz. Los métodos de extracción recogidos en la bibliografía para obtener las α-zeínas se limitaban a trabajar con concentrados de proteínas de maíz tales como el gluten de maíz y no se ultilizaban los granos de maíz que presentan un contenido de proteínas mucho menor. En consecuencia, se desarrolló un método empleando ultrasonidos focalizados de alta intensidad para la extracción de las α-zeínas de los granos de maíz. Además, también se propuso la purificación de los extractos de α-zeínas realizando una precipitación con acetona lo que permitió obtener recuperaciones próximas al 100 % para las proteínas extraídas de diferentes cultivos de maíz. Se seleccionó un medio de separación que permitía la solubilización de las proteínas del maíz y, al mismo tiempo, era compatible con la actividad de la termolisina lo que permitió optimizar un procedimiento de digestión de los extractos de α-zeínas utilizando la enzima termolisina. La presencia de los tres péptidos antihipertensivos en los extractos digeridos se confirmó utilizando HPLC-Q-ToF-MS y utilizando patrones de los péptidos investigados. Se desarrolló un método de separación utilizando una fase estacionaria *fused-core* lo que permitió evaluar la actividad de diferentes cultivos de maíz por HPLC con detección UV.

Adicionalmente, se desarrolló una metodología analítica por HPLC-Q-ToF-MS para la determinación de los tres péptidos mencionados en maíz. Se estudió tanto la estabilidad de las disoluciones de patrones como de muestras y se optimizaron diferentes parámetros del

espectrómetro de masas con el fin de evitar la fragmentación espontánea de los péptidos en la fuente de ionización. La optimización de estos parámetros no solo disminuyó dicha fragmentación en la fuente ESI sino que también dio lugar a una mejora en la sensibilidad. Se propusieron dos estrategias basadas en FASP (filter aided sample preparation) y SPE (solid phase extraction) para eliminar la urea de los extractos digeridos evitando así su interferencia en la detección por espectrometría de masas. Las señales EICs (extracted ion chromatogram) monitorizadas para llevar a cabo la cuantificación de los péptidos de interés fueron las siguientes: 193.1315 *m/z* y 385.2558 *m/z* en el caso de LRP, 316.1867 *m/z* para LSP y 357.2132 *m/z* para LQP. Se evaluaron las siguientes caracteristicas analíticas del método: linealidad, límites de detección y cuantificación, repetibilidad, precisión intermedia y la recuperación de los péptidos. También se investigó la existencia de interferencias de matriz. El método desarrollado se aplicó a la cuantificación de los péptidos LRP, LSP y LQP en diferentes líneas de maíz utilizando el método de calibración de adiciones patrón. Los resultados obtenidos pusieron de manifiesto importantes diferencias en los contenidos de los tres péptidos antihipertensivos en las distintas líneas de maíz analizadas. En general, el péptido más abundante fue el LSP seguido del LQP mientras que el péptido LRP fue el que se encontraba en menor proporción aunque era el que poseía una mayor actividad antihipertensiva.

Con fines a llevar a cabo la determinación del péptido VLIVP en habas de soja, en este trabajo se desarrolló también una metodología analítica utilizando HPLC capilar acoplada a un sistema de espectrometría de masas de trampa de iones (HPLC capilar-IT-MS). La extracción de las proteínas a partir de las habas de soja se hizo inicialmente utilizando un método previamente desarrollado. Sin embargo, este método requería un tiempo considerable e implicaba una posterior precipitación de la proteína glicinina 11S a su punto isoléctrico. El empleo de ultrasonidos focalizados de alta intensidad en condiciones optimizadas permitió reducir el tiempo de extracción de las proteínas de 2 h a 2 min. Dado que la posterior precipitación isoeléctrica de la glicinina 11S resultó no ser cuantitativa, ésta no se llevó a cabo. El péptido VLIVP se identificó, por tanto, en el hidrolizado completo de las proteínas de soja mediante HPLC-IT-MS tanto en modo MS como en modo MS/MS. La inyección del patrón del péptido en el modo MS/MS permitió observar que la transición 540.4 → 425.3 era la principal. La optimización de diferentes variables cromatográficas (gradiente de elución, agente formador de pares de iones y temperatura de separación) permitió separar el péptido VLIVP en 7 min. Además, el hidrolizado de proteínas de soja fue diluido con el fin de

eliminar las interferencias de ionización. El proceso de hidrólisis con la enzima proteasa P se optimizó también con el fin de incrementar su rendimiento y reducir el tiempo de digestión. Se optimizaron diferentes parámetros del espectrómetro de masas para mejorar la sensibilidad del método y se evaluaron las características analíticas del método desarrollado mediante la evaluación de la linealidad, límites de detección y cuantificación, interferencias de matriz, precisión y recuperación del péptido investigado. Se analizaron cinco cultivos diferentes de soja observándose que la variedad procedente de Polonia era la que tenía el mayor contenido en VLIVP.

Finalmente, en este trabajo se ha desarrollado un método SRM (selected reaction monitoring) utilizando detección por espectrometría de masas de triple cuadrupolo (QqQ) para evaluar el contenido de las isoformas de las proteínas kinasas PKA, PKG y CaMKII en distintos tejidos de rata. La simulación *in-silico* de la digestión, los resultados previos existentes y las búsquedas en bases de datos (PeptideAtlas y BLAST) permitieron seleccionar de forma preliminar un grupo apropiado de péptidos proteotípicos. Para ello, se utilizó un espectrómetro de masas Orbitrap-Velos MS con un sistema de fragmentación HCD (higher energy collision dissociation) y se analizaron los tejidos digeridos enriquecidos en las proteínas kinasas mencionadas. Los resultados permitieron confirmar un gran número de los péptidos proteotípicos seleccionados de forma teórica así como definir la lista más probable de transiciones. Este grupo de péptidos y transiciones se verificaron en el QqQ lo que permitió el diseño final del método SRM. El análisis de los péptidos marcados con isótopos pesados en tejidos de riñón digeridos permitió seleccionar y validar un grupo de péptidos y transiciones. La sensibilidad se mejoró programando cada transición durante la separación de los péptidos por HPLC y optimizando la energía de colisión para cada péptido. La aplicación del método SRM a los lisados digeridos de corazón, hígado y riñón permitió observar un rango dinámico que no era lo suficientemente amplio como para determinar todas las isoformas de las proteínas investigadas. La separación previa de los lisados por electroforesis en gel con dodecilsulfato de sodio (SDS-PAGE) permitió reducir la complejidad de los lisados de corazón. Sin embargo, esta estrategia no permitió obtener resultados reproducibles. Por ello, se considera que son necesarios estudios adicionales con el fin de poder disminuir la complejidad de la muestra.

A modo de resumen, en este trabajo se ha investigado por primera vez la presencia de péptidos bioactivos nativos presentes en SBIFs. Estos estudios han permitido obtener una

visión amplia del potencial de los péptidos bioactivos presentes en estos alimentos para bebés y al mismo tiempo observar grandes diferencias entre ellos contribuyendo a incrementar el conocimiento del valor nutricional real de estos alimentos así como de sus efectos fisiológicos y biológicos. Por otra parte, también se han desarrollado metodologías analíticas para la determinación de péptidos inhibidores de la ACE altamente potentes en cultivos de maíz y soja. Estos métodos se han caracterizado y se han aplicado al análisis de diferentes variedades de estos cultivos. Los resultados obtenidos tienen un importante potencial en el campo de la ciencia de los alimentos íntimamente relacionada con el área de la biomedicina. Finalmente, otro aspecto investigado en este trabajo ha sido la determinación de isoformas de proteínas de alto interés cardiovascular en diferentes tejidos de rata. Aunque se desarrolló un método SRM apropiado, la complejidad de la muestra no permitió la cuantificación fiable de las isoformas de PKA, PKG y CaMKII por lo que son necesarios más estudios para superar esta dificultad. Una vez superada, esta estrategia podría tener un impacto enorme en la investigación de los mecanismos moleculares que intervienen en el sistema cardiovascular.

SUMMARY

Hypertension is recognized as a serious worldwide health problem and it is considered as a leading cause of various cardiovascular and kidney diseases. Despite the prevalence of hypertension, around half of affected individuals are unaware of their condition. Additionally, forecasts concerning major causes of worldwide deaths predict the growing participation of cardiovascular disease. Together with the fact that symptoms of hypertension are hidden and rarely occur, the prevention, treatment, and mitigation of hypertension receive a high priority in modern society.

General knowledge on cardiovascular system and hypertension is quite well established. However, some pieces of information related to some molecular mechanisms in cardiovascular system are absent. It is well known that among various systems controlling blood pressure level, the renin-angiotensin system plays the pivotal role. Indeed, angiotensin I converting enzyme (ACE), main player in the renin-angiotensin system, converts angiotensin I into the vasoconstrictor angiotensin II and, at the same time, deactivates the vasodilator bradykinin. Therefore, this enzyme plays a significant role in the control of blood pressure level. Consequently, synthetic antihypertensive drugs with capacity to inhibit ACE activity have been mostly used. Other important regulatory proteins in cardiovascular system are PKA, PKG, and CaMKII. These proteins present different isoforms and various scientific reports have suggested great differences among them. Nevertheless, their exact expression levels in body organs is still unknown, which limits the knowledge on their important function in the cardiovascular system.

The treatment of hypertension has shown to decrease the occurrence of various cardiovascular events. Nevertheless, although the use of synthetic drugs has been decisive for this decrease, they usually cause side effects. An interesting alternative to synthetic drugs are peptides found naturally in some foods. In fact, the basic strategy to improve cardiovascular health is to change diet and lifestyle since nutrition is one of the main factors influencing human health. Among food bioactive compounds, bioactive peptides are attracting great attention. Specifically, antihypertensive and antioxidant peptides are the most commonly reported and have proved to positively contribute to cardiovascular health. Indeed, while antihypertensive peptides can inhibit ACE activity and effectively decrease blood pressure level, antioxidant peptides prevent oxidative stress that can initiate and promote the development of hypertension. Bioactive peptides can be natural ingredients of foods or can be released from parent food proteins by *in vivo* or *in vitro* approaches. The *in vivo* strategy is the gastrointestinal digestion itself while the *in vitro* approach involves protein hydrolysis by non-

specific enzymes or microorganisms added to foods. Special attention deserves processed products. In this case, bioactive peptides are released from proteins during food processing (*e.g.* cheese, yoghurt, kefir). Until date, the investigation concerning antihypertensive and antioxidant peptides was mainly devoted to animal origin foods like milk, dairy products, and meat. However, less studied vegetable origin bioactive peptides have frequently shown more potent activities. Maize and soybean are exceptional examples of sources of highly potent bioactive peptides.

The presence of soybean based infant formulas (SBIFs) in the market is significant since they constitute an alternative to dairy products and milk for infants with intolerance or allergy to some milk constituents, with feeding problems or coming from vegan families. Nevertheless, in comparison with milk and dairy products, they have not been much explored for their content in bioactive peptides. Modern SBIFs are based on soybean protein isolate that contains around 90% of proteins. During manufacturer preparation, SBIFs are subjected to intense heat and/or protein hydrolysis. Thus, SBIFs can naturally contain potential bioactive peptides that might exert specific health effects apart from their nutritional benefits.

In this research work, SBIFs were selected as a potential source of bioactive peptides. Four different methods were proposed to extract peptides from SBIFs. The antioxidant peptide capacity was determined using three different antioxidant assays while antihypertensive capacity was evaluated by measuring the capacity to inhibit ACE *in vitro*. The direct ultrafiltration through 10 kDa Mwco filters provided the extract with the highest peptide concentration level and antioxidant capacity. SBIF extracts were next fractionated using different Mwco filters and the antioxidant and antihypertensive capacity of these fractions were evaluated. Fractions from 5-10 kDa, 3-5 kDa, and below 3 kDa were obtained and studied. The highest antioxidant capacity, in most cases, was detected in the 5-10 kDa peptide fractions. A further fractionation of this fraction was proposed by OFFGEL isoelectrofocusing. Nevertheless, ampholytes necessary to establish pH gradient for the isoelectrofocusing separation interfered with the antioxidant assays employed in this work. In order to remove these ampholytes various strategies were proposed. Chromatographic separation with a monolithic column enabled to remove the ampholytes from the investigated samples. However, individual OFFGEL fractions showed much lower antioxidant capacities than output sample, suggesting a synergistic effect among antioxidant peptides. Therefore, the OFFGEL separation step was removed from the investigation workflow. On the other hand, the highest ACE inhibitory capacity was observed in fractions from 3-5 kDa and below 3

kDa. In these cases, no additional separation of fractions by isoelectrofocusing was considered. ACE inhibitory peptide fractions were next identified by HPLC coupled to quadrupole time of flight mass spectrometer (Q-ToF-MS) and PEAKS software. The analysis of data showed a poor selectivity in the ultrafiltration fractionation. Then, fractions presenting the highest ACE inhibitory (3-5 kDa and below 3 kDa) and antioxidant (5-10 kDa) capacities were submitted to a simulated gastrointestinal digestion with pepsin and pancreatin. Results showed that the antioxidant capacity changed negligibly after the simulated gastrointestinal digestion while the ACE inhibitory capacity of peptide fractions decreased. Peptides obtained after the simulated gastrointestinal digestion procedure were also identified. More than 120 peptides were identified in every antioxidant fraction where 42 peptides were common for all SBIFs. The peptide VAWWM was found in all the studied SBIF antioxidant fractions. This peptide is a part of the sequence of soystatin (VAWWMY), a soybean peptide previously reported as a strong cholesterol absorption inhibitor and bile acid binder. In the case of the ACE inhibitory fractions, there were 13 peptides in the fraction from 3-5 kDa and 20 peptides in the fraction below 3 kDa that could stand the gastrointestinal digestion process. Interestingly, RPSYT peptide appeared in all infant formulas and showed both antioxidant and ACE inhibitory capacities. Therefore, this peptide was synthesized and further characterized. These studies revealed its resistance to gastrointestinal enzymes and high processing temperatures, its moderate antihypertensive activity, and its potent antioxidant activity.

As previously stated, maize and soybean are attractive sources of bioactive peptides. A special attention deserves exceptionally potent ACE inhibitory peptides LRP (IC_{50} = 0.29 μM), LSP (IC_{50} = 1.7 μM), and LQP (IC_{50} = 2.0 μM), obtained by the thermolysin digestion of maize α-zeins, and peptide VLIVP (IC_{50} = 1.69 μM) found in the protease P hydrolysate of 11S soybean glycinin. ACE inhibitory capacity of these peptides is much higher than that of known peptides VPP (IC_{50}= 9.13 μM) or IPP (IC_{50}= 5.15 μM) from milk. Taking into account the highly dosage dependence of ACE inhibitory peptides and the differences in protein content observed among maize crops, it is clear the need for analytical methodologies for the quantitative assessment of these peptides. Nevertheless, despite the huge interest existing to determine ACE inhibitory peptides in foods, the literature concerning this area is quite scarce.

In this work, an analytical methodology for the simultaneous determination of LRP, LSP, and LQP peptides from α-zeins contained in whole maize kernels was developed. Existing extraction methods to obtain α-zeins were focused on maize protein concentrates such as corn

gluten meal and not on maize kernels with much lower protein concentration. Therefore, a method using high intensity focused ultrasounds for the extraction of α-zein proteins from whole maize kernels was developed. Furthermore, the purification of α-zein extracts by acetone precipitation was also proposed. The recoveries of proteins extracted from different crops were close to 100%. A suitable buffer enabling the solubilization of maize proteins and that was compatible with thermolysin activity was selected. The digestion procedure of α-zein extracts by thermolysin was optimized. The presence of the three antihypertensive peptides in the digested extracts was confirmed using HPLC-Q-ToF-MS analysis and by comparison with peptide standards. Separation conditions in a novel fused-core stationary phase were optimized and the antihypertensive capacity of maize crops was evaluated by HPLC-UV.

The determination of the above-mentioned peptides in maize kernels by HPLC-Q-ToF-MS was also carried out in this research work. The stability of the standard and sample solutions was studied. Different MS parameters were optimized to avoid spontaneous in source fragmentation of peptides. The optimization of these parameters not only decreased the spontaneous fragmentation in the ESI source but also enabled to increase sensitivity. Two different strategies based on FASP (filter aided sample preparation) and SPE (solid phase extraction) were proposed to remove urea from digested extracts due to its interference with MS detection. Appropriate EIC (extracted ion chromatogram) signals (at 193.1315 m/z and 385.2558 *m/z* for LRP, at 316.1867 *m/z* for LSP, and at 357.2132 *m/z* for LQP) were monitored for the quantification of targeted peptides. The developed method was characterized by evaluating linearity, limits of detection and quantitation, repeatability, intermediate precision, and recovery. A study on the existence of matrix interferences was also performed. The developed method was applied to the quantification of LRP, LSP, and LQP peptides in different maize lines using the standard additions calibration method. Results demonstrated great differences in the three peptides contents among the studied maize lines. In general, most abundant peptide was LSP followed by LQP while LRP peptide showed the lowest content despite being the most antihypertensive.

In order to evaluate VLIVP content in soybean crops, an analytical methodology using capillary HPLC and ion trap mass spectrometry (capillary HPLC- IT-MS) was developed. A previously developed method was firstly implemented to extract proteins from soybean crops. Nevertheless, the selected extraction method was time consuming and involved a further precipitation of 11S glycinin at its isoelectric point. Application of high intensity focused ultrasounds and the optimization of conditions permitted to decrease the extraction time from

2 h to 2 min. Further isoelectric precipitation of the 11S glycinin fraction resulted non quantitative and, thus, this step was rejected. Peptide VLIVP was identified in the protease P hydrolysate of whole soybean proteins using capillary HPLC-IT-MS in MS and MS/MS modes. The injection of the peptide standard using MS/MS showed that the transition 540.4 → 425.3 was the dominant. Various chromatographic conditions (elution gradient, ion-pairing reagent, and separation temperature) were optimized being possible the separation of VLIVP peptide within just 7 min. Moreover, the protease P hydrolysate of soybean proteins was diluted to remove sample ionization interferences. The digestion with protease P enzyme was optimized to obtain a better digestion performance and to reduce the digestion time. In order to improve sensitivity, various MS parameters were also optimized. The methodology was characterized by the evaluation of linearity, limits of detection and quantification, matrix interferences, precision, and recovery. The developed method was applied to the analysis of five different soybean crops showing the highest peptide content in the soybean variety from Poland.

Finally, an SRM (selected reaction monitoring) assay using triple quadrupole (QqQ) was developed to estimate the content of PKA, PKG, and CaMKII kinase isoforms in different rat tissues. *In-silico* digestion simulation, previous results, and database searches (PeptideAtlas and BLAST) enabled the preliminary selection of an appropriate set of proteotypic peptides. A new strategy using an Orbitrap-Velos MS with a HCD (higher energy collision dissociation) fragmentation system was employed. Digested tissues containing enriched targeted protein kinases were analyzed. Results allowed to confirm a great number of theoretically selected proteotypic peptides and to define the most probable list of transitions. Such set of peptides/transitions was verified on the QqQ enabling to create a final SRM assay. Analysis of heavily labeled peptides in kidney digested tissues allowed to validate selected set of peptides and transitions. Sensitivity was increased by scheduling the method over the HPLC run and by the optimization of collision energy for every peptide. SRM assay was applied to heart, liver, and kidney digested lysates showing a dynamic range not high enough to determine all targeted proteins isoforms. Previous lysate separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) allowed decreasing heart lysate complexity. Nevertheless, such approach showed a lack of reproducibility when applying to a big set of heart lysates. Additional studies to low down sample complexity were suggested.

Summarizing, this work has evaluated for the first time the presence of native bioactive peptides in SBIFs. Native peptides present in SBIFs have been studied for the first time.

These studies showed a broad view of potential bioactive peptides in SBIFs. These results have improved the knowledge on real nutritional value and physiological and biological effects of SBIFs. On the other hand, methods to determine highly potent ACE inhibitory peptides in maize and soybean crops were developed. These methods were successfully characterized and applied to the analysis of different crop varieties. The results have a great potential importance in food research strictly connected with the biomedical field. Finally, the quantification of protein isoforms of high cardiovascular interest in various tissue samples was also investigated. Although an appropriate SRM assay was developed, the complexity of sample did not enable a reliable quantification of targeted PKA, PKG, and CaMKII isoforms. Additional studies to overcome this issue must be conducted and, when successful, this approach could have a huge impact on the overall knowledge on molecular mechanisms of the cardiovascular system.

ACRONYMS, ABBREVIATIONS, AND SYMBOLS

ACRONYMS AND ABBREVIATIONS:

AA- acetic acid

ABTS - 2,2'-azino bis-(3-ethylbenzothiazoline-6-sulfonic acid)

ACE- angiotensin I converting enzyme

ACN- acetonitrile

ADP- adenosine-5'-diphosphate

Ang- angiotensin

B-ME- β-mercaptoethanol

BP- blood pressure

CAM- calmodulin protein

CaMKII- Ca^{2+}/calmodulin-dependent kinase II

cAMP- cyclic adenosine 3',5'-monophosphate

CGM- corn gluten meal

cGMP- cyclic guanosine 3',5'-monophosphate

CID- collision induced dissociation

CVD- cardiovascular disease

DBP- diastolic blood pressure

DC- direct current

DPPH - 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity

DTT- dithiothreitol

ECD- electron capture dissociation

EDTA- ethylenediaminetetraacetic acid

EIC- extracted ion chromatogram

ESI- electrospray ionization

ET- electron transfer

ETD- electron transfer dissociation

EtOH- ethanol

FA- formic acid

FASP- filter aided sample preparation

FOSHU- food for special health use

GDP- guanosine-5'-diphosphate

GTP- guanosine-5'- triphosphate

HA- hippuric acid

HAT- hydrogen atom transfer

HCD- higher energy collision dissociation

HHL- hippuryl-histidyl-leucine

HIFU- high intensity focused ultrasounds

HPLC (LC)- high performance (pressure) liquid chromatography

I.D.- internal diameter

IEF- isoelectrofocusing

IPA-isopropanol

IPG- immobilized pH gradient gel

IT- ion trap

LC (HPLC)- high performance (pressure) liquid chromatography

LIT (LTQ)- linear ion traps

LTQ-Ortbitrap- commercial name of LTQ- Fourier transform technology

MALDI- matrix assisted laser desorption

MeOH- methanol

MS- mass spectrometry

Mwco- molecular weight cut off

pI- isoelectric point

PKA- cAMP dependent kinase

PKA-C- catalytic subunit of PKA

PKA-R- regulatory subunit of PKA

PKG- cGMP dependent kinase

PTP- proteotypic peptide

Q- quadrupole

QqQ- triple quadrupole

Q-ToF- quadrupole time of flight

RAS- renin-angiotensin system

RF- radio frequency

ROS- reactive oxygen species

RP-LC- reversed phase liquid chromatography

RT- room temperature

SBP- systolic blood pressure

SDS- sodium dodecyl sulfate

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHR- spontaneously hypertensive rats

SIM- single ion monitoring

SPE- solid phase extraction

SPI- soybean protein isolate

SRM- selected reaction monitoring or multiple reactions monitoring (MRM)

TFA- trifluoroacetic acid

ToF- time of flight

Tris-HCl- tris (hydroxymethyl) aminomethane-hydrochloride

UV- ultraviolet

SYMBOLS:

IC_{50}- inhibitory concentration

m/z- mass per charge

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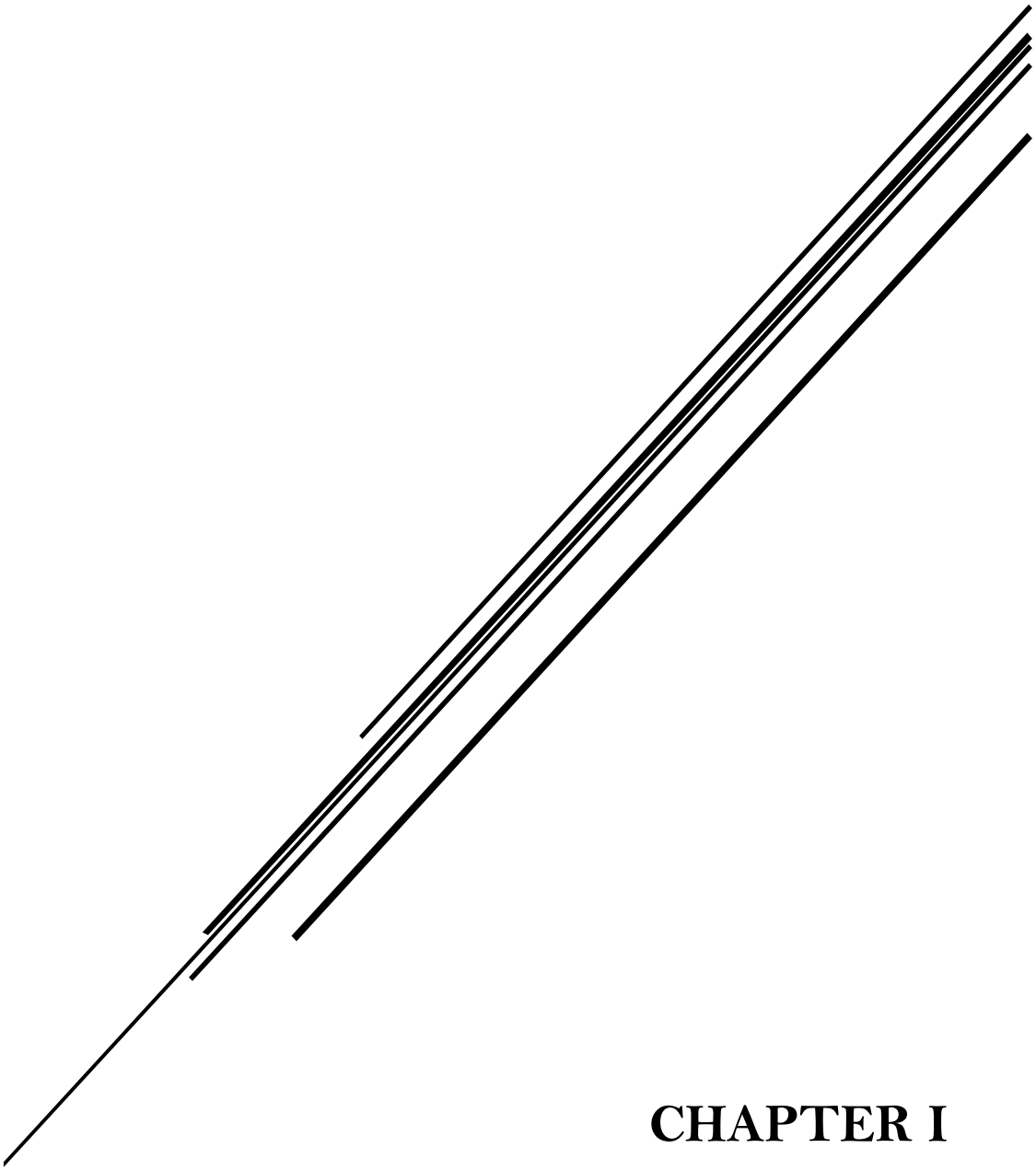
CHAPTER I. INTRODUCTION



CHAPTER I

INTRODUCTION

I.1. Hypertension and cardiovascular disease

Blood is distributed to all parts of the body through blood vessels creating a force against their walls, commonly named blood pressure (BP). BP is expressed as millimeters of mercury (mmHg) [1]. A state of persistently raised BP is defined as high blood pressure or hypertension. According to the European Society of Hypertension, an optimum adult BP is 120 mmHg of systolic blood pressure (SBP) (heart contraction or beat) and 80 mmHg of diastolic blood pressure (DBP) (heart relaxation) although these values can be extended to 129/84 mmHg of SBP/DBP, respectively [2]. Hypertension can be classified based on levels indicated in Table I.1.

Table I.1. Classification of BP levels (mmHg) [1]. Source: [2].

Category	SBP (mmHg)	DBP (mmHg)
Optimum	*120*	*80*
Normal	*120-129*	*80-84*
High normal	*130-139*	*85-89*
Grade 1 hypertension (mild)	*140-159*	*90-99*
Grade 2 hypertension (moderate)	*160-179*	*100-109*
Grade 3 hypertension (severe)	*>180*	*>110*
Isolated systolic hypertension	*>140*	*90*

The permanent high BP level is a serious worldwide health problem related with many health complications. Hypertension, can cause a heavier work of heart and, when uncontrolled, can lead to heart attack, expansion of heart muscle, and, finally, heart failure. It may cause permanent organ damage and development of bulges (aneurysms) or weak spots through blood vessels, making them more likely to clog and burst. Hypertension has been recognized as a cause of stroke, which is a rupture of a blood vessel and leakage of blood into the brain, causing permanent brain damage, problems with speech or vision, weakness or even paralysis. Kidney failure, blindness, and cognitive impairment are also some effects of hypertension [3]. Indeed, hypertension is considered a leading cause of several cardiovascular (CVD) and kidney diseases [4, 5] and has been recognized as a risk factor for myocardial infarction, renal failure, congestive heart failure, progressive atherosclerosis, and dementia [6].

[1] Classification for adults (≥ 18 years old), not taking any antihypertensive drug, and not ill at the moment.

According to the World Health Organization, around 17 millions of deaths per year are associated to CVD, being hypertension responsible for 9.4 millions of these deaths [3]. Kearney *et al.* [7] reported that in 2000, 26.4 ± 0.2% of the worlds' adult population suffered hypertension (26.6 ± 0.2% in men and 26.1 ± 0.1% in women). Although hypertension occurs frequently, it has been reported that over a half of hypertensive population is unaware of its condition [4]. Estimated total number of adults with hypertension in 2000 was 972 ± 15 millions from which 333 ± 3 millions were in economically developed countries and 639 ± 15 millions in economically developing countries [7, 8]. Pereira *et al.* [9] reported that although the prevalence of hypertension in developing countries is 32.2% for men and 30.5% for women, just 9.8% of men and 16.2% of women are aware of the ill and control hypertension. Similar results were also obtained for developed countries. In addition, the prevalence of hypertension increases with age, from < 10% in younger adults (30-59 years) to > 65% in older adults (aged ≥ 60 years) [10]. It is predicted that in 2025, the number of adults with hypertension will increase by about 60% to a total number of 1.56 billion individuals [7, 8]. The projected trend for major causes of deaths expects an increase in mortality rates due to CVD (see Fig. I.1). This is related to the fact that hypertension rarely causes symptoms and, if so, they are headache, shortness of breath, dizziness, chest pain, palpitations of heart or nose bleeding. For this reason, hypertension is commonly called 'invisible' or 'silent killer' [3].

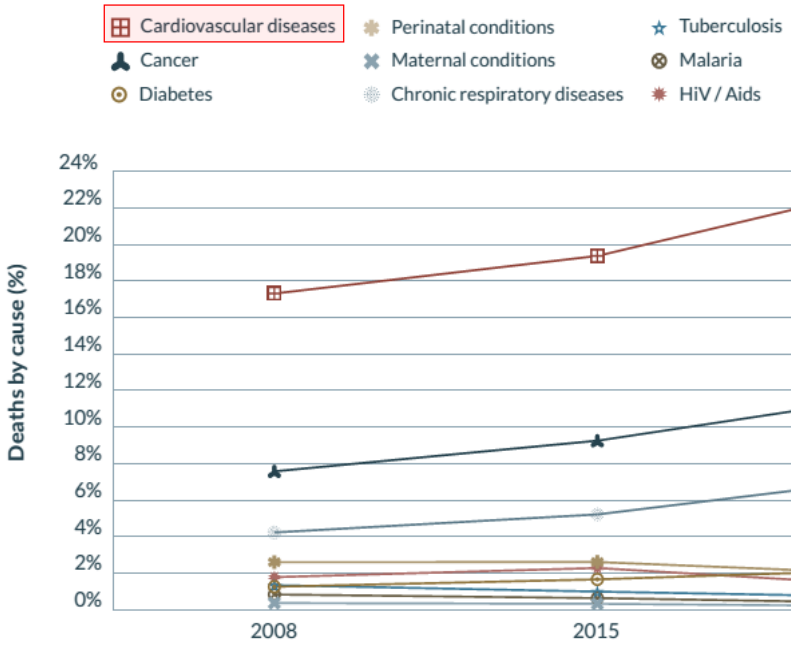


Fig. I.1. Projected mortality trend from 2008-2030 for major non-communicable and communicable diseases. Source: [3].

A scheme summarizing the most relevant factors leading to the development of CVD is depicted in Fig. I.2. On the first place are social determinants and drivers like education or income that influence the second group of factors consisting of an unhealthy diet, lack of physical activity, or abuse of tabaco or alcohol. All together leads to the development of hypertension and, along with other metabolic factors (obesity, diabetes, raised blood level of lipids, *etc.*), can cause the increase risk of heart disease, stroke, kidney failure, and other hypertension complications [3].

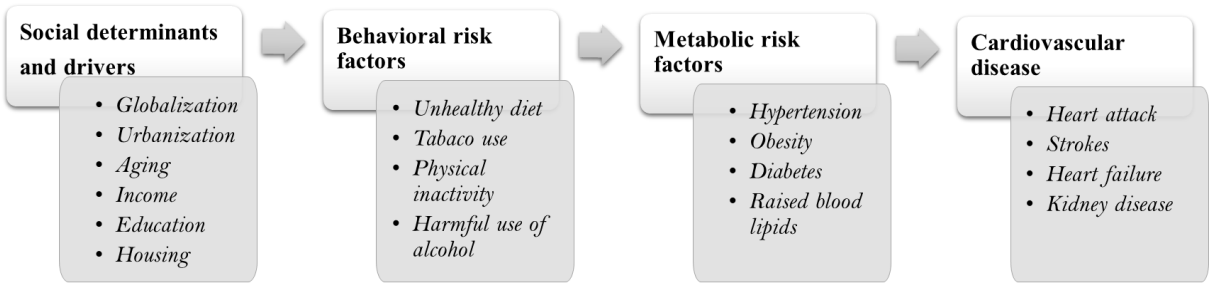


Fig. I.2. Main factors that contribute to the development of high BP, CVD, and their complications. Adapted from: [3].

Indeed, it has been estimated that hypertension along with high cholesterol, high Body Mass Index, low fruit and vegetable intake, smoking, and alcohol intake, is responsible for 83-89% of ischemic heart disease and 70-76% of stroke in the world [11]. Among all mentioned factors, it is important to highlight the impact of diet and the fruit and vegetable intake. It has been proven that an increase in fruit and vegetable intake (up to 600 g/day) reduces the burden of ischaemic heart disease and ischaemic stroke by 31% and 19%, respectively [12]. Despite the knowledge on hypertension has significantly increased, the related statistics are still discouraging. Hypertension is an important global public health challenge and its prevention, detection, and treatment should receive high priority in modern society.

I.2. The cardiovascular system at molecular level

The cardiovascular system is one of the most important systems within human body, whose main purpose is to supply nutrients to tissues and deliver wastes to the excretory system. Although the overall knowledge of the function of the cardiovascular system and its related diseases are well established, most of the molecular mechanisms behind its correct behaviour are still not completely known. This represents a fundamental piece of information in order to prevent, mitigate, and treat hypertension and cardiovascular events. Some basic aspects dealing

with biochemical processes involved in the regulation of BP at molecular level are presented in this thesis.

Every organism controls its homeostasis orchestrating complex molecular signaling events. In multicellular organisms, the molecular signaling can act over long distances and induce several physiological responses. Chemical messengers, known as hormones, trigger intercellular signaling. Upon binding to its own specific receptor on the cell surface, every hormone activates intercellular signal transduction evoking a specific effect [13]. Several different molecular pathways are involved in the regulation of BP in human organism. In this respect, renin-angiotensin system (RAS) plays a pivotal role in living organisms. RAS is not the exclusive regulator of BP since also kinin-nitric oxide system, endothelin converting enzyme system, and neutral endopeptidase system have also shown to affect BP [14]. At intracellular level, several biochemical reactions occur in which different proteins are involved. Part of this thesis will be devoted to study different proteins playing important roles in the cardiovascular function such as cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP) dependent kinases (PKA and PKG) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII).

I.2.1. The renin- angiotensin system and blood pressure regulation

Different studies have led to the discovery of RAS as the major regulator of BP, electrolyte balance, and renal, neuronal, and endocrine functions associated with cardiovascular control [14]. RAS consists of a cascade of enzymatic reactions (see Fig. I.3). Activation of RAS begins with the production of prorenin by kidneys [15]. Prorenin is, therefore, converted into renin by the action of kallikrein enzyme. Then, angiotensinogen, secreted by the liver, is exclusively converted into the decapeptide angiotensin I (Ang I, DRVYIHPFHL) by the presence of renin in bloodstream.

Angiotensinogen has exclusive functions, as it is the only precursor of Ang I and the only substrate for renin [14]. Hence, Ang I is transported into the small vessels of lungs (see Fig. I.3) where it is hydrolyzed by the action of angiotensin I converting enzyme (ACE; kinases II peptidyldipeptide hydrolase) into angiotensin II (Ang II: DRVYIHPF), an essential hormone in the organism. Additionally, ACE also inactivates the potent vasodilator bradykinin (RPPGFSPFR) by removing the dipeptide (FR) from its C-terminus [16, 17].

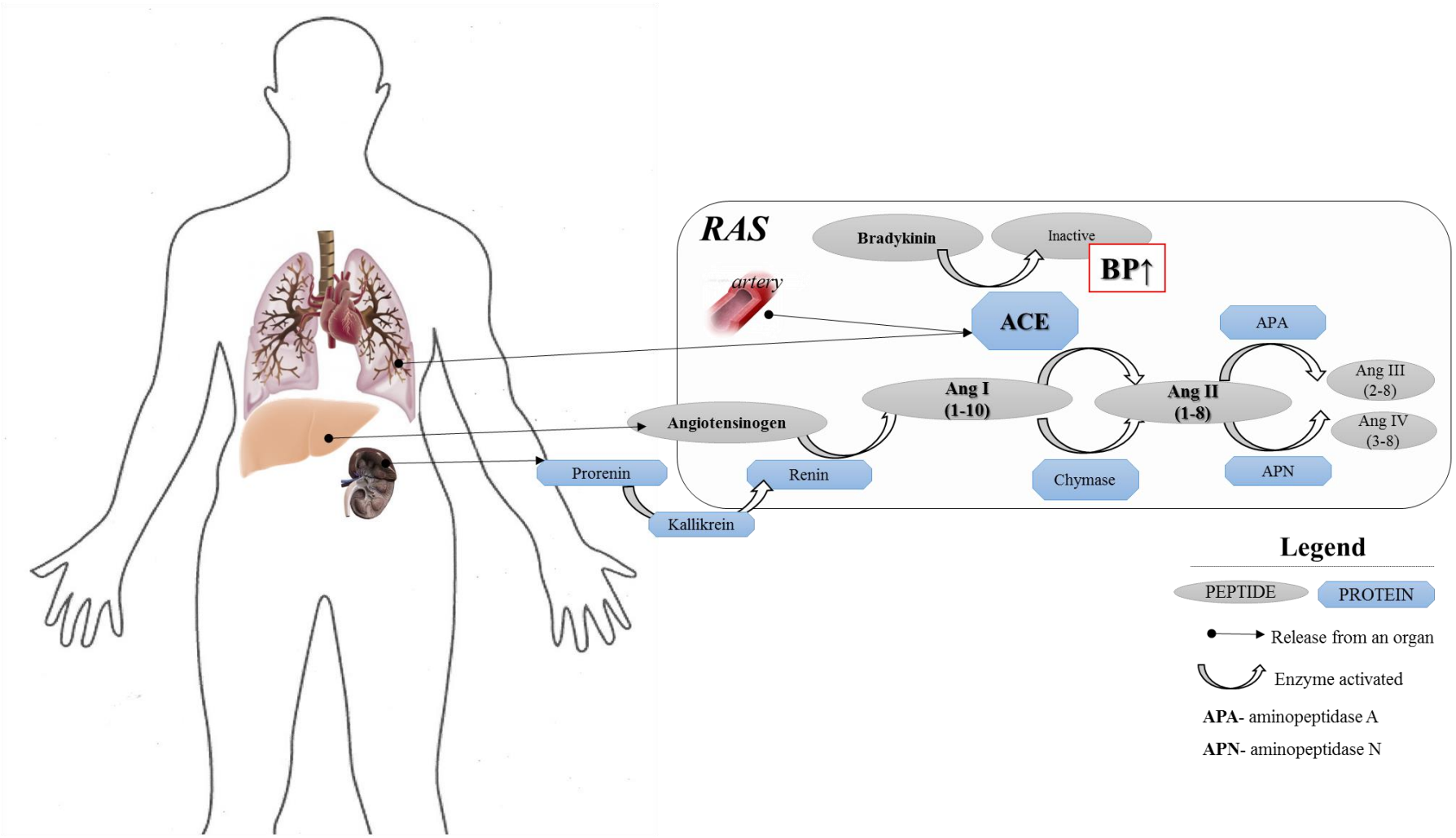


Figure I.3. Schematic view of RAS involved in the regulation of BP level in human organism.

Thus, the inactivation of this potent vasodilator by the action of ACE causes the raise of BP. The action of chymase enzyme can also catalyze the formation of Ang II from Ang I; however, this reaction dominates only in the left ventricle of the human heart [14, 17-19]. Ang II is distributed through the body and is converted by aminopeptidase A or aminopeptidase N to angiotensin III (RVYIHPF) or angiotensin IV (VYIHPF), respectively. These two peptides may play a relevant role in different tissues, though their functions are not entirely understood [17]. Moreover, Ang II is a hormone that plays a number of important regulation functions in human organism such as the regulation of perfusion pressure of a number of organs, BP regulation, salt and water volume modulation, regulation of neurotransmitter interactions, and control of the activity of gonadotropin hormone and pituitary hormone [14, 17]. Additionally, an increasing number of reports indicates the influence of Ang II on the production and release of reactive oxygen species (ROS) [17]. In fact, Ang II could amplify oxidative stress and ACE inhibitors could intensify the antioxidant defense system by the inhibition of Ang II formation in animals and humans [20].

Summarizing, the regulation of RAS by the control of Ang II and bradykinin levels can effectively modulate BP. Indeed, ACE inhibitors influencing RAS are the most prominent antihypertensive drugs and have shown to reduce CVD [17].

I.2.2. PKA, PKG, and CaMKII - regulatory proteins of cardiovascular functions

PKA and PKG are important regulatory proteins of the cardiovascular function and are both involved in BP regulation and cardiac contractility [13]. CaMKII occupies a central role in the physiology and pathology of cardiac muscle cells [21]. PKA, PKG, and CaMKII belong to the same protein family of serine/threonine kinases and they phosphorylate the hydroxyl group of serine or threonine [13, 22-24].

PKA and PKG are the major targets of secondary messengers cAMP and cGMP. Signalization through these cyclic nucleotides is at the center of a large variety of physiological responses such as memory, contraction of heart, and BP regulation. Moreover, they are also involved in many pathophysiological processes such as heart failure, hypertension, and cancer. At molecular level, the activation of PKA is associated to the production of cAMP and to the catecholamine level in blood (see Fig. I.4) (Nobel Prize in 1971). PKA is involved in several sets of functional heart pathways such as excitation-contraction coupling and calcium homeostasis. Its activation leads to the phosphorylation of other proteins that promote muscle

contraction in heart and increase heart rate. Additionally, the activation of PKA is involved in pathological remodeling of heart [25]. In this regard, it was observed that patients suffering from heart failure had increased plasma catecholamine levels [13, 26], which raises PKA level.

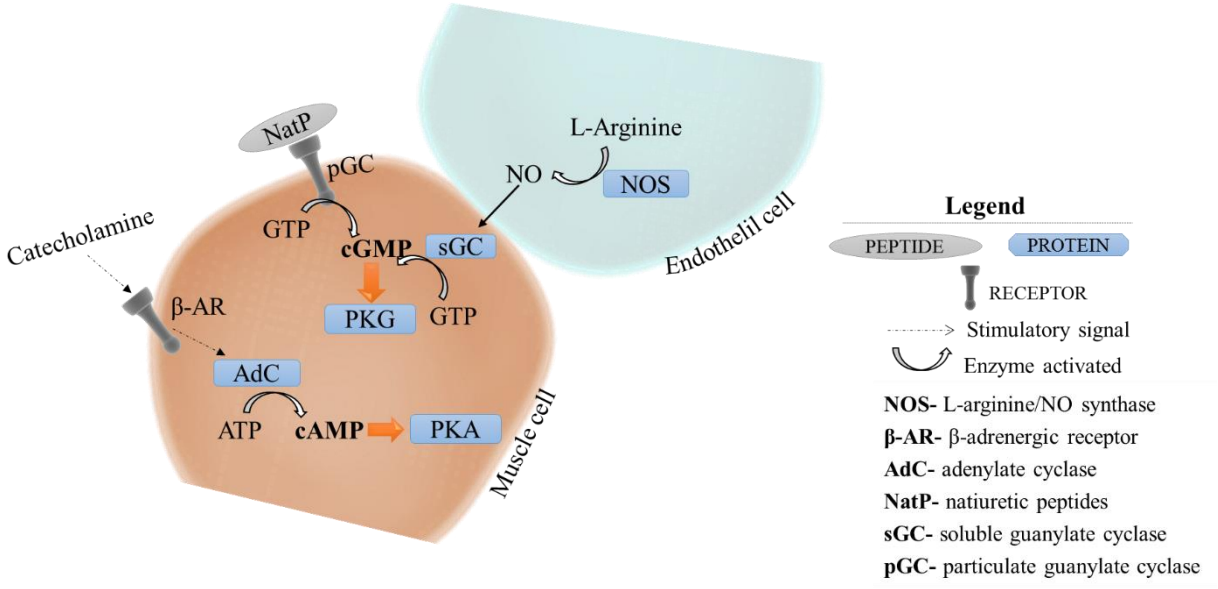


Fig. I.4. Mechanism of activation of PKA and PKG in the cell. Source: [13].

On the other hand, PKG is associated to the production of cGMP which can occur by different ways. The production of cGMP can be due to the release of highly reactive radicals (NO) from nearby endothelial cells (see Fig. I.4). These radicals, produced from L-arginine, diffuse through cells and activate soluble guanylate cyclase. Activation of soluble guanylate cyclase leads to the synthesis of cGMP from guanosine-5'- triphosphate (GTP). The formation of cGMP can also be achieved by a membrane bound particulate guanylate cyclase, a receptor of paracrine hormone peptides or natriuretic peptides that is released from heart and vascular endothelium. Increased level of cGMP causes the activation of PKG that further phosphorylates several proteins in the cell [13, 27]. PKG is mainly involved in the relaxation of muscles lining on arteries and veins and, therefore, it is involved in the control of BP. Indeed, for more than 100 years, NO generating reagents, which increase PKG level, were used in the treatment of hypertension and CVD [13] (Nobel Prize in 1998).

PKA and PKG have a similar domain architecture (see Fig. I.5). PKA is formed by 2 catalytic subunits (PKA-C) and 2 regulatory subunits (PKA-R) resulting in a heterotetrameric holoenzyme [$(PKA\text{-}R)_2$-$(PKA\text{-}C)_2$]. At low cAMP concentrations, PKA remains intact and catalytic subunits are together with regulatory ones. At high cAMP concentrations, the

holoenzyme dissociates into [$R_2(cAMP)_4$)] and the two free catalytic subunits are released to phosphorylate intracellular targets. Once the cAMP level decreases, it dissociates from the PKA-R subunits (in [$R_2(cAMP)_4$)]) which results in rebinding of PKA-C subunits. Interestingly, PKA-R subunits are encoded as four distinct isoforms (Iα, Iβ, IIα, and IIβ) and the catalytic domain (PKA-C) as three distinct splicing variants (α, β, and γ).

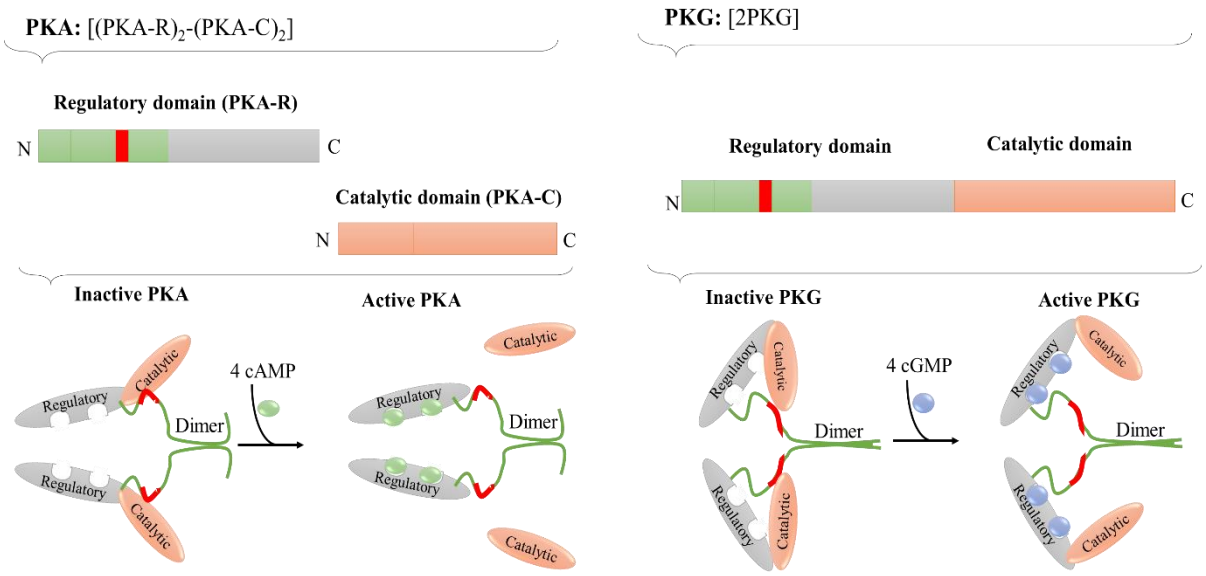


Fig. I.5. Domain organization, structural models, and activation scheme of PKA and PKG. Adapted from: [27].

PKG is activated when binding to cGMP resulting in the phosphorylation of intracellular substrates (see Fig. 1.5). Mammals have two genes encoding PKG, PKG type I and II. PKG I has two variants, Iα and I β. Little is known about the specificity and functionality of PKA and PKG isoforms, although some evidences would suggest that these isoforms are not redundant proteins [13, 25, 27].

CaMKII is an abundant protein in myocardium that is activated by increasing intracellular Ca^{2+} concentration [28]. CaMKII is a multimeric protein typically composed of 12 subunits (see Fig. I.6). Each subunit contains association, regulatory, and catalytic domains. The activation of CaMKII protein is associated to an increased intracellular calcium concentration.

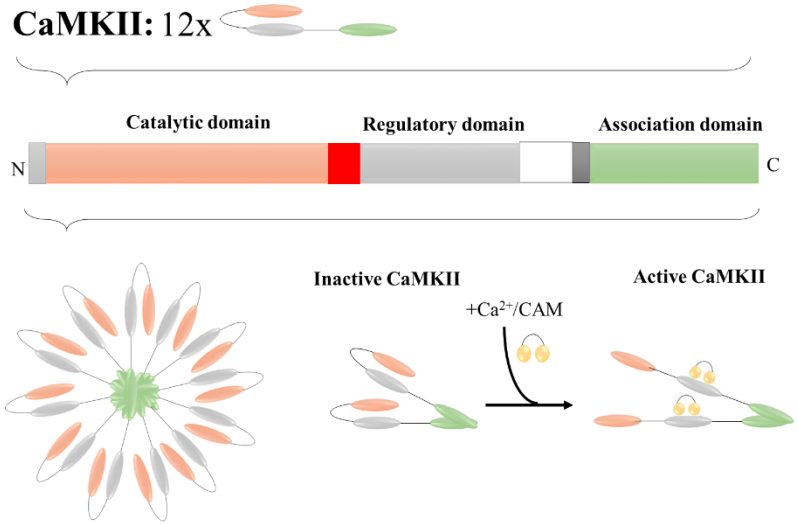


Fig. I.6. Domain organization, structural model, and activation scheme of CaMKII. Adapted from: [23].

Calcium calcifies calmodulin protein (CAM) which binds to the regulatory subunits of CaMKII and, as a consequence, it exposes its catalytic domains (active conformation of CaMKII) [23]. Once activated, CaMKII might autophosphorylate, decreasing its dependence on the Ca^{2+}/CAM interaction [21, 23]. Signaling through Ca^{2+} and CaMKII is involved in a large variety of physiological responses in heart, brain, kidney, and many other organs. CaMKII expression and activity is increased in failing human myocardium and in many animal models of cardiac hypertrophy and heart failure [29]. CaMKII is expressed as many variants: α, β, γ (A-C), and δ (1-7). Although these isoforms have different tissue distribution and may have different sensitivities to Ca^{2+}/CAM and different activation kinetics, the specific role of CaMKII isoforms is still unclear [30].

Summarizing, PKA, PKG, and CaMKII are important kinases involved mainly in the regulation of the cardiovascular system. The study of the activity, function, and structure of these proteins suggests that there are large differences among their isoforms. The straightforward quantification of these isoforms could help to understand the cardiovascular system and its performance at molecular level. Therefore, their expression in organs having cardiovascular importance like liver, kidney, or heart is of particular interest.

I.3. Treatment and prevention of hypertension

A variety of drugs such as diuretics, β-blockers, ACE inhibitors, Ang II antagonists, calcium channel blockers, AT1 receptor blockers, nitrates, renin inhibitors, vasodilators, and others [31, 32] can be used in the treatment of hypertension. Drugs that target at various levels the RAS cascade are renin inhibitors, ACE inhibitors, and receptor antagonists [33], while β-blockers,

calcium channel blockers, and nitrates, directly or indirectly, influence the level of PKA, PKG, and CaMKII proteins. Among them, calcium channel blockers (*e.g.* nifedipine) and ACE inhibitors (*e.g.* captopril) are the first choice where the last one exhibits higher effectiveness and less side effects [34]. The discovery of the first ACE inhibitor is strictly connected to the studies dealing with the venom of snake *Bothrops jararaca* performed by Mauricio Rocha e Silva. They enabled the isolation of bradykinin that decreases BP, contracts slowly intestinal smooth muscle, and enlarges vessels (from Greek word *brady* (slow) and *kinin* (to move)) [1]. Further work of Sergio Ferreira resulted in the isolation of the first ACE inhibitor in 1970 from the same snakes' venom [35]. Based on this study, in 1970 Miguel Ondetti and David Cushman designed the first synthetic antihypertensive drug called captopril [36] which is still the most commonly prescribed drug for the treatment of hypertension. Numerous potent di- and tri-peptide inhibitors of ACE or their pro-drug forms have been synthetized therefore (*e.g.* cilazapril, ramipril, enalapril, enalaprilat, ceranapril) [37]. Nevertheless, although synthetic drugs exhibit high capability to inhibit ACE, they cause various side effects [14, 38]. An excellent alternative to synthetic drugs are antihypertensive peptides naturally ocurring in some foods. Unlike synthetic drugs, food antihypertensive peptides do not yield any adverse effect although they are usually less potent [39, 40]. Despite this, even a small decrease in BP can result in a significant reduction of the risk of CVD. It was estimated that a reduction of DBP of 5 mmHg decreases CVD risk to ≈ 16% and stroke to ≈ 40%, while a reduction of SBP of 10 mmHg declines CVD up to ≈ 20-25% [6, 14, 41, 42]. Indeed, dietary therapies are acknowledged to prevent hypertension, reduce BP in hypertensive patients, and improve efficiency of antihypertensive therapies [10]. Works devoted to the study of dietary approaches to stop hypertension revealed that diets rich in proteins resulted in a greater reduction of BP, improved lipid levels, and reduced cardiovascular risk than diets rich in unsaturated fats or carbohydrates [10, 43]. Moreover, it has been proved that the maximum benefit can be obtained by the consumption of vegetable and milk proteins [10, 44]. These functional properties could be attributed to biologically active peptides encrypted in protein molecules and released by gastrointestinal digestion [45].

I.3.1. Food bioactive peptides

There is an increasing interest on improving diet and lifestyle as a basic strategy against CVD and hypertension [46]. This fact encouraged the development of food products with specific health promoting properties that are known as '*functional foods*' [47]. According to

Health Canada Federal Department '*a functional food is similar in appearance to, or may be, a conventional food that is consumed as part of an usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions*'[48]. First country recognizing the benefits of functional foods was Japan [49]. Indeed, in 1999 Japan government established the legal frame for the commercialization of these foods that were labeled as food for special health use (FOSHU) [50]. Unlike Japan, EU and USA legislation do not yet recognize functional foods as a distinct category of foods [51, 52]. On the other hand, bioactive compounds incorporated within functional foods can be defined as '*food components that can affect biological processes or substrates and hence have an impact on body function or condition and ultimately health*'. In this respect, it is important to highlight to ceveats: the component should impart a measurable effect at a realistic physiological level and the measured 'bioactivity' has to show a potential beneficial health effect [50, 53, 54].

One of the most widely studied bioactive compounds from foodstuffs are bioactive peptides. Bioactive peptides have been defined as '*specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health*' [55, 56]. Mellander *et al.* [57] described the first report on bioactive peptides in 1950. Since then, and according to the BIOPEP database, there are more than 2600 different bioactive peptides with more than 37 different types of bioactivties [58]. Nowadays, there are several commercially available functional foods and food ingredients containing bioactive peptides and claiming health benefits [59]. In majority, these products are fermented milk drinks (Ameal S, Calpis, Danaten, Evolus, Casine DP, PRODIET F200/Lactium *etc.*) or other types of hydrolysates (C12 peptide, Lowpept, PeptoPro, Tensiocontrol, Vasotensin *etc.*) [46, 55, 59-61]**.** Interestingly, most of these products claim to reduce BP level and improve cardiovascular performance [59]. Increased attention has been focused on the discovery of new bioactive peptides with specific bioactivities [46, 50]. Food bioactive peptides have been found to affect many body systems (see Fig. I.7) [55, 59, 62, 63] either directly through their presence in an undisturbed form (bioavailable peptides) or by their release from their respective host protein by hydrolysis [59].

An analysis of the data extracted from the BIOPEP database (n=2609) revealed that most bioactive peptides are below 1 kDa (51%) while peptides above 5 kDa represent just 1% of the whole database (see Fig. I.8). This observation emphasizes the inverse relationship between peptide size and peptide activity.

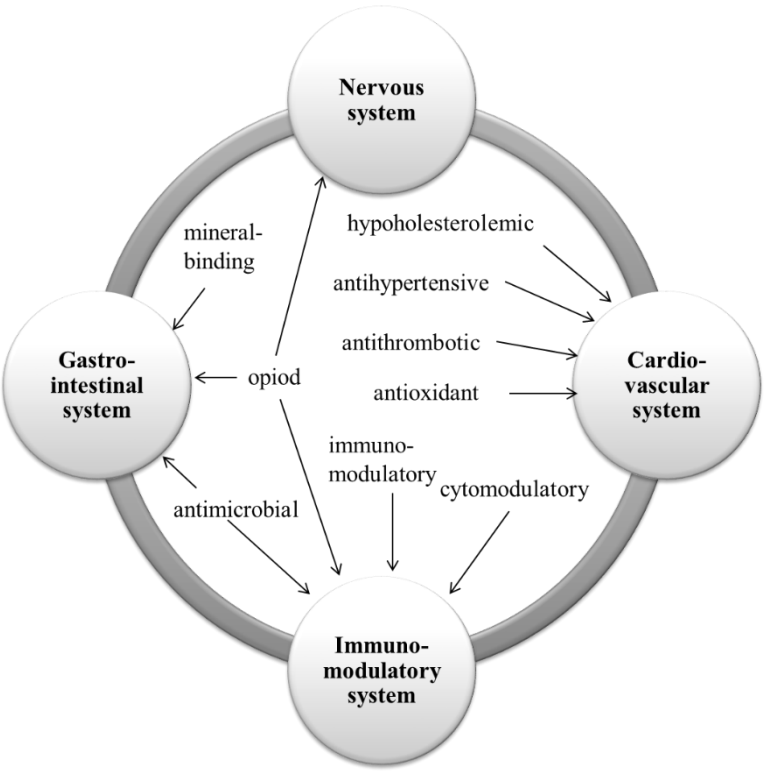


Fig. I.7. Influence of bioactive peptides on different human systems. Adapted from: [55, 59, 62].

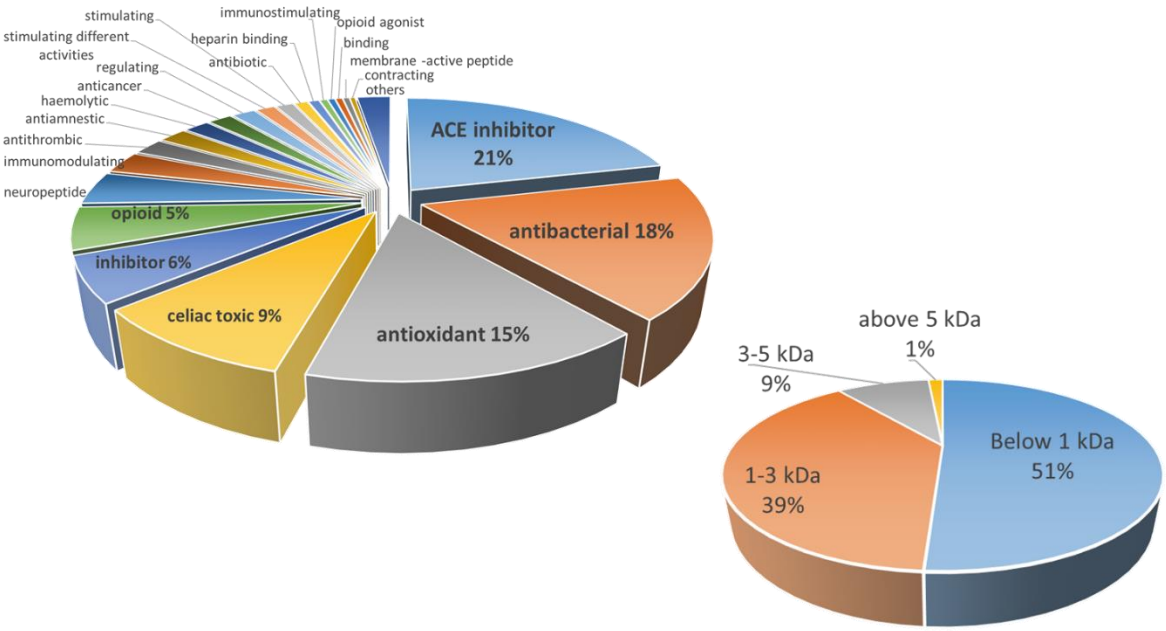


Fig. I.8. Chart representing the distribution of peptide bioactivities and peptide molecular weights. Charts prepared based on the data available in the BIOPEP database (2609 peptides in September 2013).

Bioactive peptides generally contain from 2 or 3 to 20 amino acids although sometimes this range can be extended to even 50 amino acids (*e.g.* anticancer lunasin with 43 amino acid residues) [45, 52, 54, 64, 65]. The activity of a bioactive peptide depends mainly on its amino acid composition [45, 55]. Bibliographic data demonstrate that antihypertensive (21%) peptides are the most frequently reported, followed by antibacterial (18%) and antioxidant peptides (15%) (see Fig. I.8). Additionally, some peptides can exert multifunctional activities being peptides simultaneously showing antihypertensive and antoxidant activities the most usual.

I.3.2. Antihypertensive and antioxidant peptides in foods

Antihypertensive peptides (ACE inhibitors) are the best-known class of bioactive peptides [60, 65, 66]. Antihypertensive peptides inhibit the *in vitro* activity of ACE, which results in the reduction of BP [52, 54]. The potency of an ACE inhibitor is usually expressed as IC_{50} value which is the inhibitor concentration leading to a 50% inhibition of ACE activity [56, 59]. Studies on spontaneously hypertensive rats (SHR) and some clinical trials have shown that antihypertensive peptides significantly reduce BP but they do not have effect on normotensive individuals [46]. Additionally, studies comparing antihypertensive activity of captopril and food ACE inhibitors have shown that some ACE inhibitory peptides can exhibit higher *in vivo* than *in vitro* activity [46]. It has been postulated that some peptides may influence not only the RAS system through the inhibition of ACE, but also other BP regulation systems [46, 67]. Maruyama and Suzuki described the first isolation and identification of an antihypertensive peptide in 1982. The peptide, hydrolized from bovine casein by trypsin, had the sequence FFVAPFPEVFGK [68]. Nowadays, best-known and studied ACE inhibitory peptides are VPP and IPP, identified in a Japanese sour milk drink fermented with *Lb. Helveticus* and *Saccharomyces cerevisiae* strains [50]. These peptides exert IC_{50} values of 9.13 and 5.15 μM, for VPP and IPP, respectively. These values are 1267 and 704 times higher than that for captopril [50]. ACE inhibitory peptides can be found in a large variety of foodstuffs as independent entities or can be released from foods after protein hydrolysis. Antihypertensive peptides have been discovered in marine foods (shrimps, sea cucumber, and blue mussel), fishes (Alaska Pollock, bonito, salmon, and pacific hake), meat (pork, pork loin, bullfrog, porcine, and chicken), eggs, milk and dairy products, plant foods (soybean, wheat, rice, garlic, maize, mushrooms, and amaranth) and processed products (miso paste, douchi, wakame, royal jelly, soybean sauce, and soybean paste). Some examples of peptides isolated and identified in different foodstuffs are presented in Table I.2. Despite there are some exceptions (*e.g.* VPP and IPP), most protein derived ACE inhibitory peptides have moderate potency, within 100-500

μmol/L [46]. Among vegetable foods, maize and soybean have attracted particular attention being possible the identification of peptides (LRP, LSP, LQP and VLIVP) with higher ACE inhibitory activity than VPP and IPP (see Table I.2). Although active antihypertensive peptides with up to 27 amino acids have been identified, they are generally smaller. ACE inhibitory peptides contain from 2 to 20 amino acids and a high level of hydrophobic amino acids within the sequence [52, 56, 66, 69]. The inhibition of ACE by antihypertensive peptides seems to be strongly influenced by the C-terminal sequence of the peptide. Peptides containing proline, lysine, and arginine at C-terminus are preferred residues [56, 63, 69, 70]. In fact, small peptides, with high hydrophobicity and containing proline at the C-terminal, are more likely to resist the action of gastrointestinal enzymes [65]. It was suggested that phenylalanine residue could also be essential for ACE inhibition [71]. ACE also seems to prefer substrate or competitive inhibitors containing hydrophobic amino acid residues at C-terminal positions [67, 71].

Table I.2. Examples of antihypertensive peptides, their sources, sequences, and IC_{50} values.

Source of peptide(s)	Identified peptide(s)	IC_{50}	References
Pork loin	VKKVLGNP	28.5 μM	[72]
Spanish dry-cured ham	AAATP	100 μM	[73]
Chicken bone	YYRA	33.9 μg/mL	[74]
Alaska Pollack skin	GPM, GPL	17.13, 2.65 μM	[75]
Marine shrimp	DP, GTG, ST	2.15, 5.54, 4.03 μM	[76]
Jellyfish	QPGPT, GDIGY	80.67, 32.56 μM	[77]
Duck skin by-products	WYPAAP	137 μM	[78]
Salmon by-product	VWDPPKFD, FEDYVPLSCF, FNVPLYE	9.10, 10.77, 7.72 μM	[79]
Skimmed milk	VPP, IPP	9.13, 5.15 μM	[80]
Cheese	LQP, MAP	3.4, 0.8 μM	[81]
Wheat bran	LQP, IQP, LRP, VY, IY, TF	2.2, 3.8, 0.21, 21, 3.4, 18 μM	[82]
Corn gluten meal (CGM)	AY	14.2 μM	[83]
Maize α-zein	LRP, LSP, LQP	0.29, 1.7, 2.0 μM	[84]
Glycinin from soybean	VLIVP	1.69 μM	[85]
Processed soybean milk	FFYY, WHP, FVP, LHPGDAQR, IAV, VNP, LEPP, WNPR	1.9, 4.8, 10.1, 10.3, 27.0, 32.5, 100.1, 880.0 μM	[86]

On the other hand, although some physiologically produced free radicals can exhibit beneficial functions, like signaling role or provide even defense against infections, an excess of ROS might damage cells [87]. An extensive ROS production in combination with failing of the antioxidant defense system is commonly known as oxidative stress. Oxidative stress is an important factor influencing the initiation or progression of several vascular diseases. ROS can damage most important biological macromolecules like DNA, proteins, and lipids, and their prolonged action can be a cause of the development of severe tissue injury and diseases like hypertension, cancer, and cardiovascular or inflammatory diseases [46, 63, 87]. The production of ROS, like superoxide anion and hydrogen peroxide, is increased in hypertensive individuals in which NO synthesis and bioavailability of antioxidants are reduced [60].

Antioxidant peptides contribute to the prevention of oxidative stress, a causative factor for the initiation or progression of hypertension and several vascular diseases. Antioxidant peptides prevent the formation of radicals or may scavenge radicals. Artificial antioxidants (butylated hydroxylanisole, butylated hydroxyltoluene, and *n*-propyl gallate) have shown strong activity against several oxidation systems. However, many of them are considered a health risk and, therefore, their use is restricted or even prohibited in some countries [88]. Consequently, dietary antioxidant supplements and functional foods containing antioxidants like α-tocopherols, vitamin C, or plant derived phytochemicals (lycopene, lutein, isoflavones, green tea extract, *etc.*) are highly demanded in the current marketplace [89]. Peptides derived from protein hydrolysates can exert antioxidant activity against enzymatic and non- enzymatic peroxidation of lipids and essential fatty acids, where the exact mechanism is not fully understood [46, 52, 56, 88]. Since peptidic antioxidants can exert more than one bioactivity, they are more attractive candidates than non-peptidic antioxidants as dietary ingredients [89, 90]. It is important to highlight that, in some cases, hydrolysates exhibit higher antioxidant capacity than purified peptides [87], which might be connected to the synergistic effect of antioxidants [91]. Indeed, unlike antihypertensive activity, most works devoted to antioxidant peptides do not yield specific peptide sequences and are more interested in the evaluation of antioxidant capacity of whole hydrolysates, whole extracts, *etc.* [66] (see Table I.3). Antioxidant peptides have been observed in marine foods (oyster, giant squid, blue mussel, and jumbo squid skin), fishes (loach, sardine, capelin, catfish, hoki, Alaska Pollock, bonito, salmon, pacific hake, mackerel, and much more), meat (porcine, venison, and chicken essence), eggs, milk and dairy products, and vegetable foods (soybean, rice, maize, chickpea, peanut, sunflower, and amaranth). The properties of antioxidant peptides are related to their composition, structure, and hydrophobicity

[87]. The antioxidant activity is attributed mainly to a high amount of histidine residues and the presence of hydrophobic amino acids like methionine, cysteine, tyrosine, tryptophan, and phenylalanine [87, 89]. Nevetheless, these amino acids are not effective when they are not within a peptide sequence [89].

Table I.3. Examples of antioxidant peptides, their source, sequence, and assays used to measure their capacity.

Source of peptide(s)	Identified antioxidant peptide(s)/fraction	References
Bullfrog skin (*Rana catesbeiana Shaw*)	LEELEEELEGCE	[92]
Tuna cooking juice	PSHDAHPE, SHDAHPE, VDHDHPE, PKAVHE, PAGY, PHHADS, VDYP	[93]
Pacific hake (*Merluccius productus*) fillet	Whole hydrolysate	[94]
Oyster (*Crassostrea talienwhannensis*) meat	Peptides in the fraction below 1 kDa with the highest antioxidant capacity	[95]
Camel and bovine whey proteins	Peptides in the fraction 5-10 kDa with the highest antioxidant capacity	[96]
Zein hydrolysate	Hydrolysate containing short peptides (<500 Da)	[97]
Native and heated soybean protein isolate (SPI)	Whole hydrolysate	[98]

Despite vegetable proteins have been less studied as a source of bioactive peptides, their relevance is increasing [60, 66].

I.4. Vegetable proteins

There is a trend toward to alternative sources of high quality proteins due to economical (plants are cheaper), ecological, and sustainability (production of plant proteins is less demanding than animal proteins) issues, or even consumer preferences (vegans and vegetarians) [47]. A special attention deserves widely consumed plant foods such as soybean, wheat, maize or rice. In fact, according to the Food and Agriculture Organization a major part of human diet all over the world consists of cereals and legumes. Indeed, it was estimated that around 70% of human foods comprise cereals and legumes while remaining 30% comes from animals feed on these seed meals [99]. Among plant foods, maize and soybean are of great interest since they are excellent sources of antihypertensive and antioxidant peptides (see Tables I.2 and I.3). In

fact, high hydrophobicity and high level of proline amino acids in maize and soybean proteins ensures that release peptides could exert high antihypertensive and antioxidant properties.

Seed proteins can be classified into storage and biologically active (so called housekeeping) proteins. Biologically active proteins like lectins, enzymes, and enzyme inhibitors are responsible for maintaining the normal cell metabolism and are usually minor proteins. Storage proteins are non-enzymatic and their unique function is to deliver nitrogen and sulphur required during germination and establishment of the new plant [99]. Empirical classification of seed proteins proposed by Osborne [100], distinguished four main groups of proteins based on their solubility: albumins - soluble in water, globulins- extractable in dilute salty solution, prolamins- soluble in alcohol solutions, and glutelins- soluble in dilute acids and bases or sodium dodecyl sulfate (SDS) solutions. Prolamins and glutelins are storage proteins in monocots (*e.g.* cereals) while albumins and globulins are storage proteins in dicots (*e.g.* legumes) [99, 101].

I.4.1. Maize proteins

Corn or maize *(Zea mays L.)* is a major crop for both livestock feeding and human nutrition. It was estimated that maize accounts 15-56% of the total daily calories in human diets, particularly in Africa and Latin America [102]. It is also the only indigenous cereal crop from America and the most dominant crop in United States being grown throughout the temperates, sub-tropical, and tropical zones wherever rainfall or irrigation is adequate [103, 104]. Maize kernel is composed of the pericarp (6%), the endosperm (82%), and the germ (12%). Table I.4 depicts an approximate distribution of major components in whole maize kernel and its parts.

Table I.4. Average distribution of major components in maize kernel. Source: [104].

Component	Whole kernel (%)	Endosperm	Germ	Pericarp
		(%) as dry basis		
Starch	62.0	87	8.3	7.3
Protein	7.8	8	18.4	3.7
Oil	3.8	0.8	33.2	1
Ash	1.2	0.3	10.5	0.8
Water	15.0	-	-	-
Others	10.2	3.9	29.6	87.2

Maize protein content can range from 6 to 12% (as dry basis), being mainly present in the endosperm and germ. The distribution of protein fractions in the germ (25% of proteins) and the endosperm (75% of proteins) is summarized in Table I.5. Zeins, according to Osborne nomenclature, are the prolamin fraction of maize and they are almost exclusively present in the endosperm. They also represent the most abundant fraction in maize and the most widely explored. Zeins have a high content in glutamic acid (21-26%), leucine (20%), proline (10%), and alanine (10%) and are deficient in basic and acidic amino acids. Therefore, around 59% of their amino acids are hydrophobic [104].

Table I.5. Distribution of protein fractions in maize (percentage related to total proteins). Source: [101].

Protein fraction	Endosperm	Germ
	(%) as dry basis	
Albumins	6	30
Globulins	-	30
Zeins	60	5
Glutelins	26	23
Residue	8	12

Zeins are a family of similar proteins with different molecular sizes, solubility, and charges. There are, at least, five different zeins nomenclatures being those proposed by Wilson [105] and Esen [106, 107] the most important. Based on their molecular weights and solubility in aqueous alcohols and aqueous alcohols containing β-mercaptoethanol (B-ME), Wilson classified zeins as: A-zeins (21-26 kDa), B-zeins (18-24 kDa), C-zeins (13-18 kDa), and D-zeins (9-11 kDa). Based on their molecular weights and solubility in different concentrations of isopropanol (IPA) containing B-ME and sodium acetate, Esen classified zeins as: α- zeins (21-25 kDa), β-zeins (17 kDa), γ-zeins (18 and 27 kDa), and δ- zeins (10 kDa). α-zein is the most abundant zein fraction, accounting for 75-85% of total zeins depending on genotype [106, 107]. On the other hand, isoelectrofocusing of α-zeins indicates that they consist of a mixture of at least 15 proteins [108]. Most authors distinguish two major groups of α-zeins: Z19 zein migrating at 19 kDa and Z22 zein migrating at 22 kDa. Nevertheless, the studies on zein sequences obtained from cloned cDNAs and genes, have shown that these two groups of zeins were made up of 210-245 amino acid residues and had a molecular weight (Mw) around 23-24 and 26-27 kDa, respectively. This caused a further confusion in nomenclature [108]. Moreover, two interesting works focused on the analysis of zeins by MALDI-MS (matrix assisted laser desorption mass spectrometry) have confirmed the complexity and variety of zeins [109, 110].

In order to clarify the nomenclature introduced by Wilson [105] and Esen [106, 107], the following scheme is presented in Fig. I.9.

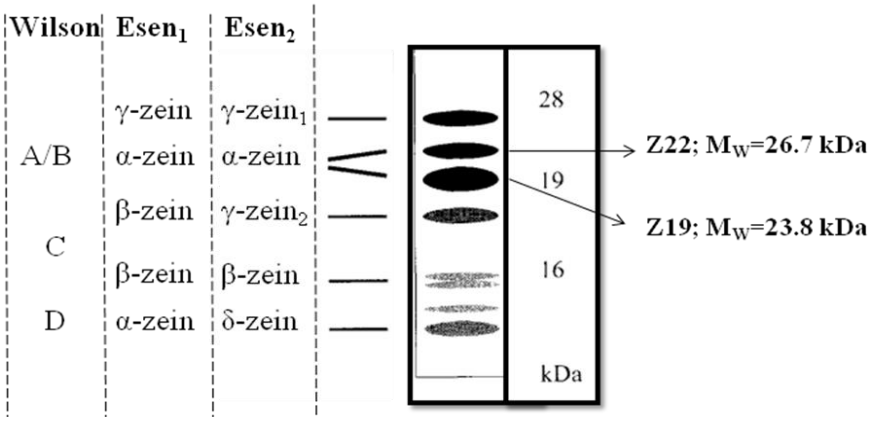


Fig. I.9. Summarized scheme of various zeins nomenclatures introduced by Wilson [105] and Esen [106, 107].

I.4.2. Soybean proteins

Soybean (*Glycine max*) is a legume with a high protein content, ranging from 20 to even 50%. Soybean, like other legumes, contains anti-nutritional compounds that can be classified into proteic compounds (termolabile) and non-proteic compounds (termostable). The first group includes biologically active proteins like protease inhibitors or amylase inhibitors, which need to be inactivated by hydrothermal treatment before consumption. The second group of anti-nutritional compounds consists of phytic acid, saponins, goitrogens, *etc.*. Therefore, it is usual the use of soybean protein isolates (SPI) and concentrates as starting materials for the production of soybean products avoiding the presence of antinutritional non-proteic compounds. Soybean proteins are present in human foods in a variety of forms, like infant formulas, sauce, miso, natto, tempeh, sufu, soybean dairy-like products, tofu, yuba, *etc.* [111, 112].

In addition to proteins, other soybean components are lipids (20%, mainly saturated fatty acids), water (≈4-10%), carbohydrates (cellulose, hemicellulose, pectins, soluble oligosaccharides), minerals (iron, zinc, magnesium, calcium, sodium, potassium, copper, magnesium), and vitamins (thiamine (B1) and riboflavin (B2)). Table I.6 summarizes an approximate distribution of major components in soybean seeds and SPI.

Table I.6. Average distribution of major components in soybean seed and SPI. Source: [113].

Component	Soybean seed (%)	SPI (%)
Protein	43.7	86.0-87.0
Oil	21.8	0.5-1.0
Ash	5.3	3.8-4.8
Water	4.0-10.0	4.0-6.0

Soybean contains three kinds of proteins: proteins involved in metabolism, structural proteins or glycoproteins, and storage proteins (globulins) which do not exert biological function. The last ones account around 80-90% of total soybean proteins, are soluble in salt solutions, and precipitate at different isoelectric points (pI). Soybean globulins have high level of glutamic (22.4%) and aspartic acid (12.6%) and are rich in hydrophobic amino acids (53%) [111]. Unlike maize zeins, soybean globulins nomenclature is quite standardized. Soybean globulins are composed of 4 major components that can be classified as 2S[2], 7S, 11S, and 15S according to their rate of sedimentation by ultracentrifugation (see Table I.7.) [111, 114]. Major storage proteins are glycinin (11S) and β and γ- conglycinin (7S) [113]. Structurally, glycinin consists of six subunits, each made up of two polypeptide chains: an (A) acidic chain (≈ 40 kDa) and a (B) basic chain (≈ 20 kDa). Both chains are joined by intra-chain disulphide bonds.

Table I.7. Components of soybean globulins. Source: [111, 114].

Soybean protein components		Content (% of total storage proteins)		Molecular weight (kDa)	pI
Ultracentrifugation	*Immuno-methods*	*Ultracentrifugation*	*Immuno-methods*		
2S globulin	α-conglycinin	15.0	13.8	21	-
7S globulin	β-conglycinin	34.0	27.9	180	4.8
	γ-conglycinin		3.0	170	
11S globulin	Glycinin	41.9	40.0	350-380	6.4
15S globulin	Polymer of glycinin	9.1	-	600	-

Five genetic variants (G1-G5) which are divided into group-I ($A_{1a}B_2$, A_2B_{1a}, $A_{1b}B_{1b}$) and group-II ($A_5A_4B_3$, A_3B_4) based on their homology [115] can be distinguished. Glycinin subunits in the Uniprot protein database can be found under the name G1 ($A_{1a}B_2$), G2 (A_2B_{1a}), G3

[2] 'S means Svedberg unit, which is a unit of sedimentation rate computed as the rate of sedimentation per unit field of centrifugation strength'. Source: [114].

($A_{1b}B_{1b}$), G4 ($A_5A_4B_3$), and G5 (A_3B_4). On the other hand, β-conglycinin is a trimer composed of three subunits: α (63 kDa), α' (67 kDa), and β (48 kDa). These subunits share a large degree of sequence homology [114].

I.5. Proteomics and peptidomics workflows for the analysis of proteins and peptides

I.5.1. Proteomics workflows for the analysis of proteins

Analysis of peptides and proteins is emerging as an important part in science and technology. Proteomics is a relatively new methodology that can analyze entire proteins (proteome) present in biological samples. Proteome is a highly dynamic system which depends on various factors and can change during the age (maturation) or impact of a disease. Its understanding can yield information about a process that takes place in a biological system and can depict how system reacts under certain conditions [116]. Proteomics can be defined as a "*large-scale analysis of proteins in a particular biological sample at a certain time*" [117]. Proteomics tools allow to characterize proteins and to study their function, nutritional and biological relevance, conformation, modifications, localization, quantification or even interactions [117-119]. Proteomics centers the identification of proteins in the analysis of protein fragments or peptides by mass spectrometry (MS). The sensitivity of MS detection in the case of peptides is much higher than in the case of proteins. Indeed, MS is more efficient to obtain sequence information from peptides up to around 20 amino acid residues than from proteins [120].

There are two main types of proteomics workflows to analyze proteins: *top-down* or *bottom-up* approaches [117, 121, 122]. In the *top-down* approach, intact proteins are both fragmented and analyzed in a mass spectrometer and information on primary protein structure is obtained. In the more frequent *bottom-up* strategy, proteins are firstly separated, digested (usually using trypsin), and finally resulting peptides are identified by MS (see Fig. I.10).

Bottom-up strategy can further be performed by two different procedures. Peptide mass fingerprint is an approach where two-dimensional gel electrophoresis is normally employed to isolate proteins of interest. Separated proteins are then enzymatically digested into peptides and subjected to MS [117]. Obtained peptide mass profile is compared against generated *in-silico* protein sequences from databases taking into account the same enzyme cleavage sites. All peptide masses that match the obtained sequence within a certain mass error tolerance are scored and ranked [123].

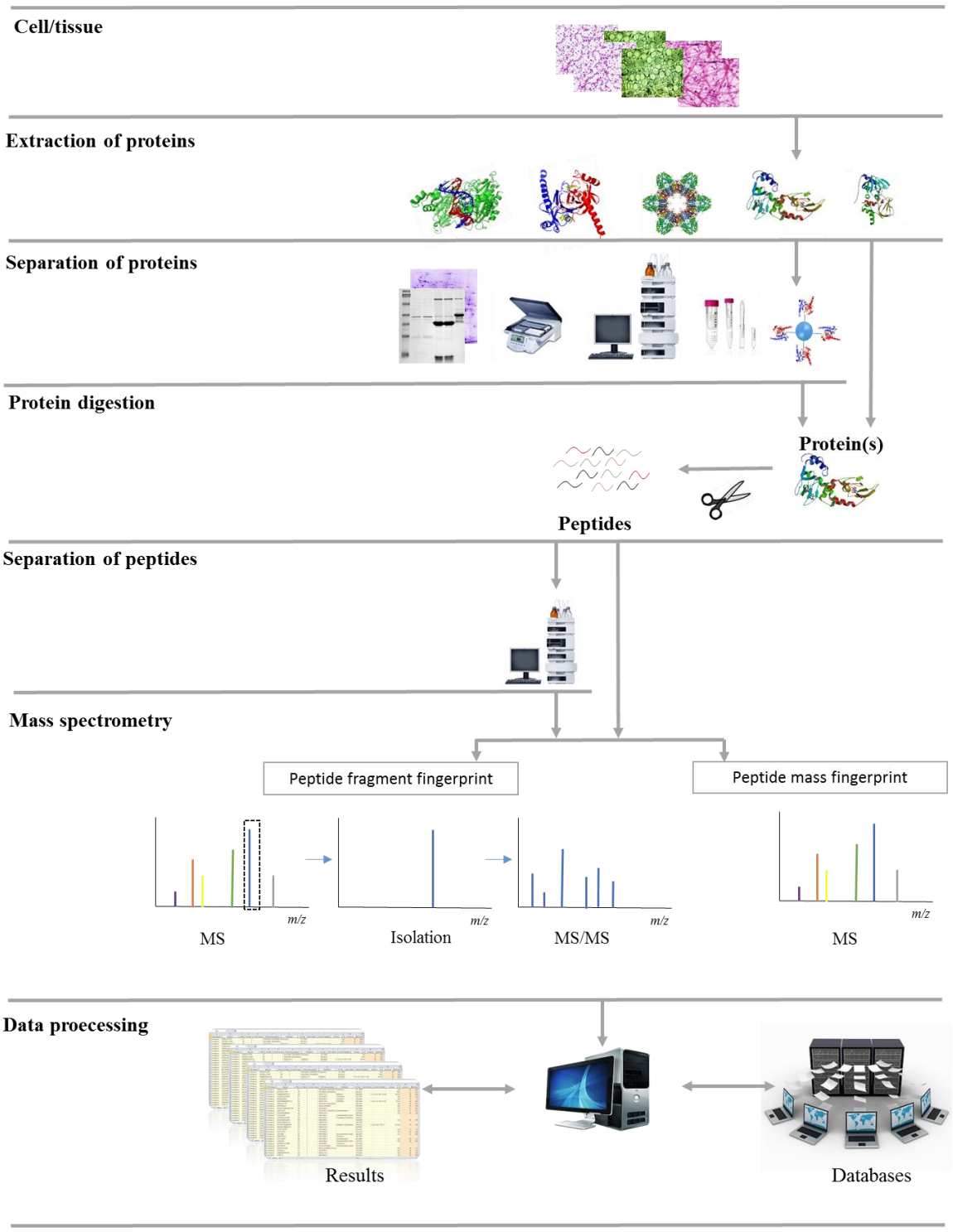


Fig. I.10. Proteomics workflow using the *bottom-up* approach. Adapted from: [122].

On the other hand, peptide fragmentation fingerprint produces fragment ion data by MS^n from one or more unique peptides to identify unambiguously parent proteins [117]. This approach provides more confident protein identification than peptide mass fingerprint [123]. In the peptide fragmentation fingerprint approach, peptides can be identified from their MS/MS spectra by searching against databases. There are several algorithms to search sequences against databases like Peptide Search, Sequest, Mascot, Sonar MS/MS or Protein Prospector [120]. Database search involves that parent proteins have previously been identified and introduced in the database. Otherwise, protein could just be tentatively identified by homology with related proteins. It must be highlighted that the proteome of several organisms is still not known or incomplete, which generates difficulties to identify proteins.

A major analytical challenge in proteomics is the dynamic range that can reach even 10^{12}. Current MS-based proteomics platforms can yield a dynamic range of just 10^4. Therefore, the study of low abundant proteomes requires partial purification, by depletion of most abundant proteins or by the selective enrichment of low-abundant proteins, before their analysis [117, 123]. There are two main modes of analysis of proteins in proteomics: data-dependent analysis and targeted proteomics. In the first one, the objective is to study the highest possible number of proteins in a sample while, in the second the aim is to identify selected proteins or peptides by focusing just specific peptide ions.

I.5.2. Peptidomics workflows for the analysis of peptides

Peptidome is defined as a "*pool of all peptides of an organism, tissue or cell*" [124]. Peptidomics is a subfield of proteomics, which focuses on the composition, interaction, and properties of peptides. Moreover, peptidomics also studies the origin and changes of peptides released from proteins during processing and storing [117, 118]. The area of food peptidomics also involves the study of product authenticity, history, functional properties, allergenicity, sensory properties, and biological activities of peptides in food products or raw materials [118, 124]. The classical workflow in the analysis of food bioactive peptides is depicted in Fig. I.11. Studied peptides can be obtained by their direct extraction from foodstuffs or by the hydrolysis of parent protein(s). The release of peptides from parent proteins can be performed by the action of proteolytic enzymes or microorganisms. In order to study whether obtained peptides exert a desired activity, bioactivity assay(s) is(are) needed. *In vitro* bioactivity assays are much more popular on this stage of research since they are faster, cheaper, easier to perform, and available in the majority of laboratories. In most cases, the purification of one or few potential peptides

is the goal of the study. However, the measurement of the activity of a whole hydrolysate or a peptide fraction of a product without isolation and identification of particular peptides is also possible.

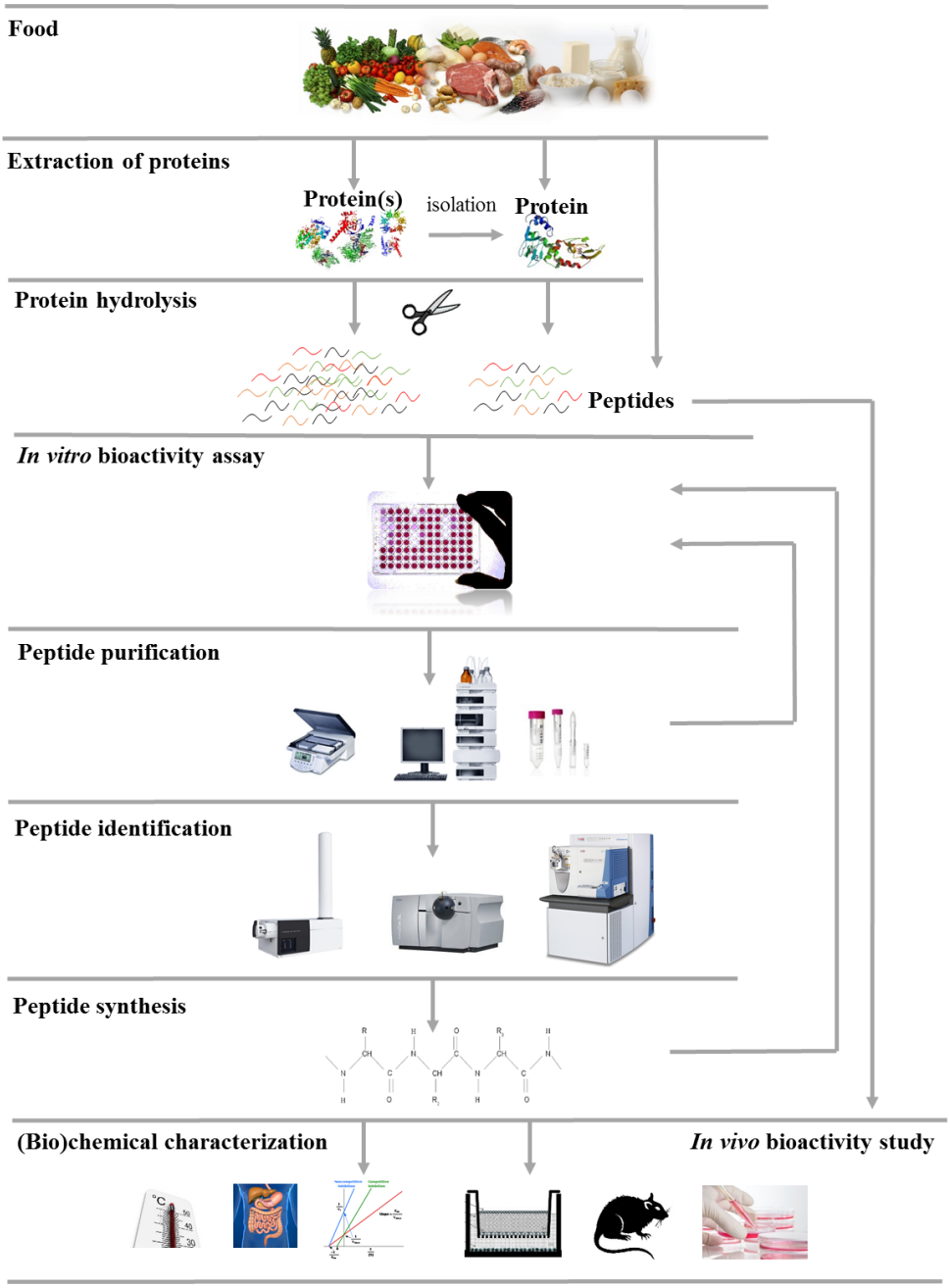


Fig. I.11. Workflow used for the analysis of bioactive peptides from foodstuffs.

Purified bioactive peptides or groups of peptides are then identified by MS. Identification of bioactive peptides from unknown proteins is not possible by database searching and requires the use of *de novo* sequencing strategy. Finally, potential peptide counterparts are synthetized and characterized using *in vitro* and/or *in vivo* assays to confirm their bioactivity.

The analysis of food bioactive peptides apparently can remind proteomics workflows since it involves an enzymatic hydrolysis, followed by peptide separation, and MS identification. However, a few basic features and differences should be highlighted (see Fig. I.12).

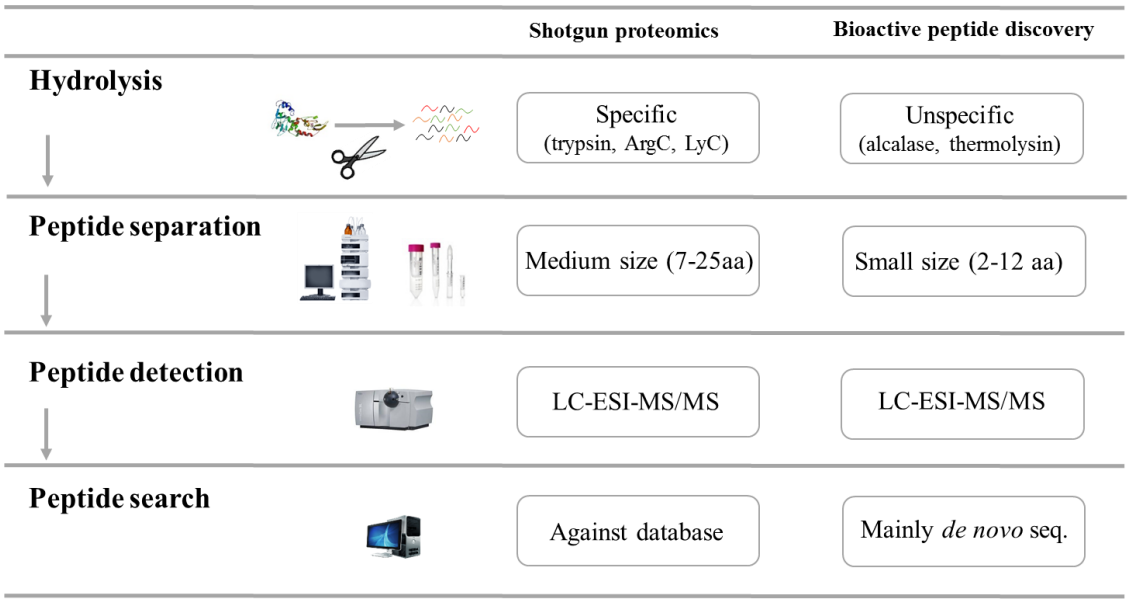


Fig. I.12. Comparison of proteomics and bioactive peptide discovery workflows. Adapted from: [125].

One of the most important differences is the kind of enzyme used to release desired peptides. In the proteomics workflow, specific enzymes like trypsin are employed. This enables to obtain peptides with similar charge (typically + 2 or +3) and length (7 to 25 amino acids) and same amino acids at C-terminal position (in trypsin digestion, basic amino acids, K and R, are at C-terminal positions in the resulting peptides) [123]. Bioactive peptides released by unspecific enzymes or microorganisms result in a much more heterogeneous mixture of small peptides (2 to 20 amino acids with charge + 1), often without any basic amino acid within the sequence, and bearing different C-terminal residues. Moreover, this peptide mixture also results much more complex in peptide length distribution and dynamic range. All these facts make necessary a higher number of fractionation steps. Furthermore, food bioactive peptide identification might be problematic since fragmentation is usually poorer and less informative when using nonspecific enzymes than when using specific trypsin. Indeed, tryptic peptides bearing many charges and basic amino acids, that maintain the charge within the sequence, can be easily identified. Moreover, there are several tools to analyze and identify tryptic peptides in an automated fashion. Additionally, the search for new bioactive peptides usually requires organisms proteomes that have not been yet sequenced. In these cases, *de novo* sequencing is the only solution to identify them. Finally, all quantitative tools in proteomics are based on the

fact that a unique peptide can identify a particular protein and that the peptide: protein ratio is equal to one. In the case of small bioactive peptides, this ratio might be different from one since very small peptide sequences can derive from more than one protein [125].

I.6. Extraction and enrichment of proteins

Suitable performance of protein extraction, isolation, and purification is critical in both previously described workflows. There is a variety of methods for the disintegration of cells (lysis) within various tissues and for the extraction of proteins. The selection of the extraction method depends on the type of studied cells/tissues (animal or plant *etc.*) and targeted protein [126]. Tissue samples are very complex matrices since they may contain many different cell types and structural material connecting tissues. This translates into a higher challenge for sample preparation procedures [127].

I.6.1. General considerations in the extraction and enrichment of proteins

Prior to protein extraction, the mechanical disruption of cells (cell lysis) is required. Manual homogenization, vortexing, grinding, or liquid nitrogen treatment are the most used methods. Cell lysis depends on the presence or absence of cell walls. Especially difficult are plant cells where walls are made up with multiple layers of cellulose, particularly strong and difficult to disrupt [128]. Proper cell lysis is significantly important when intracellular proteins are studied. They can represent a tiny fraction of total cellular proteins and, thus, they are much more difficult to extract and recover [126, 129]. Proteins in biological samples are generally in a native state associated to other proteins and often being part of large complexes or membranes. Chemical and physical techniques can be applied to disturb cell walls. They can be grouped into five major categories: mechanical homogenization, osmotic and chemical lysis, ultrasounds or pressure, and temperature treatments. The application of two or more procedures has also been reported [130]. These methods must lyse rapidly and efficiently cells to extract proteins with minimal proteolysis or oxidation [126, 128].

Mechanical homogenization implies the use of a rotor-stator homogenizer or open blade mill. In the osmotic shock strategy, cells are suspended in a gently shaken hypertonic solution. Regarding chemical lysis, most common treatment includes the use of antibiotics, chelating agents, detergents, and solvents capable of disintegrating cells. For example, organic solvents (*e.g.* acetonitrile (ACN), ethanol (EtOH), and methanol (MeOH)) are efficient for destabilizing

membrane bilayers [121]. Procedures employing detergents or strong chaotropic reagents (a substance that denatures and disrupts the structure of macromolecules, *e.g.* urea, thiourea or guanidine chloride) within the extraction buffer assure the disruption of interactions among proteins and between proteins and other compounds. Furthermore, low cost and easy to use detergents can efficiently disrupt cell membranes, break lipid-protein interactions, and solubilize proteins. Four different groups of detergents are mainly used for this puropose: bile acid salts, non-ionic, zwitterionic, and ionic. Bile acid salts (*e.g.* sodium deoxycholate) are charged soft detergents compatible with native protein extraction. Non-ionic detergents (*e.g.* Triton X series and Tween 20) are considered as mild since they disrupt protein-lipid interactions rather than inter/intra-protein interactions. Zwitterionic detergents (*e.g.* 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) show intermediate properties and they can solubilize proteins more efficiently than non-ionic detergents. Ionic detergents (*e.g.* SDS) provide the harshest conditions and cause protein denaturation. SDS is considered the best protein solubilizer but it is incompatible with MS [131]. In fact, most traditional detergents and chaotropic reagents are not compatible with common MS ionization techniques and may interfer further enzymatic digestions. Therefore, their removal must be considered [121, 128]. Basic criteria for the selection of a suitable lysis buffer for protein extraction are buffer composition, pH, ionic strength, salt concentration, temperature, and presence of detergents, chaotropes, protein reducing agents (*e.g.* B-ME, dithiothreitol (DTT)), and presence of components preventing their proteolysis (*e.g.* protease inhibitors) [128, 129]. In conclusion, the selection of a sutaible lysis buffer or method is essential to avoid possible difficulties in next steps.

High intensity focused ultrasounds (HIFU) or pressure treatment involves the application of ultrasonic waves to the solution. Ultrasounds generate a cyclic sound pressure with a frequency greater than 20 kHz [132]. This pressure can accelerate certain chemical reactions, replacing traditional techniques or accelerating them. This phenomenon is produced by the focalization of high intensity ultrasonic waves that cross the liquid media and create an effect known as cavitation. Cavitation can be defined as a physical process by which numerous tiny gas bubbles are produced. Bubbles grow, oscillate, split, and implose [132, 133]. Therefore, these bubbles can be considered as microreactors inside which there are high temperatures and pressures. Ultrasonic devices (bath or probes) are commonly applied in many analytical laboratories. The greatest difference between them is that ultrasonic probe is inserted into the solution which provides, at least, 100 times greater energy than the ultrasonic bath [133, 134]. Sonication and

high-pressure procedures have been applied to disrupt (lysis) different kinds of cells (microorganisms, plants, animal) and to extract organic and inorganic analytes from solid or liquid media [126]. Apart from protein extraction, the ultrasonic probe has also shown to reduce time in protein enzymatic digestion procedures [128].

After proteins extraction, the precipitation of proteins is very usual to separate them from interfering compounds, to change the surrounding environment if required in further steps, or to enrich proteins of interest. The precipitation of proteins can also be important when just the peptidome of a sample is studied, since it enables the removal of proteins [135]. It is a especially important step in the case of plant cell extracts, since they can contain high amounts of interfering molecules (polysaccharides, lipids, polyphenols, secondary metabolites, *etc.*). Removal of interfering compounds can be performed before or after the extraction procedure. Among various procedures, precipitation at pI, precipitation at high temperatures or precipitation using various reactants/solvents are the usual [135]. Lower protein solubility at pI can be explained by proteins zero net charge, which enables the association among protein molecules with a minimum charge repulsion. Thermal precipitation is based on the fact that proteins denature at high temperatures [135]. This method is also employed to stop enzymatic reactions. Moreover, various organic solvents such as acetone, EtOH, ACN, and their mixtures often provide effective protein removal. Additionally, protein deproteinization can be carried out by the addition of inorganic acids and salt solutions such as ammonium sulfate (salting out), trifluoroacetic acid (TFA), trichloroacetic acids, perchloric acid, or sulfosalicylic and alginic acids. In the analysis of food samples, precipitation under acidic conditions is more effective than with inorganic salts [136].

I.6.2. Special considerations in the extraction of maize and soybean proteins

In the case of seeds, the extraction of a desired group of proteins is possible by their sequential extraction with water, salt solutions, aqueous EtOH, and acid/alkali solutions containing detergents [99] or by the direct extraction of the protein group if it is the majority.

As described previously, main maize proteins are zeins while other minor proteins are globulins, albumins, and glutelins. The literature describing the extraction of zeins from maize kernels and maize co-products (CGM, corn gluten feed, *etc.*) is considerably wide. The special solubility behavior of zeins is related to a high ratio in non-polar amino acid residues and the lack of basic and acidic amino acids [104]. Three types of solvents have been used for the

extraction of zeins [137]: primary solvents at concentrations >10% (glycols, glycol-ethers, amino-alcohols or amines, and amides) that dissolve zeins alone, secondary solvents (water /organic solvent or short-chain aliphatic alcohols /organic solvent), and ternary solvents (water/short chain aliphatic alcohol /organic solvent or water/ two different organic solvents). Despite this extended list of solvents, the most frequently used are binary mixtures of water with short chain aliphatic alcohols (EtOH or IPA) since they enable an easier recovery and separation of zeins [146] (see Table I.8).

Table I.8. Procedures employed for the extraction of zeins.

Solvent	Solvent/ sample ratio	Other conditions	References
70% EtOH	4:1, v:w	40˚C, 30 min	[138]
70% EtOH	4:1, v:w	50˚C, 30 min	[139]
70% EtOH	4:1, w:w	A: 60˚C, 1 h, at 125 rpm centrifugation; filtration; overnight at -18˚C; pellet solubilization; overnight at -18˚C; pellet isolation B: 60˚C, 1.5 h, at 125 rpm; centrifugation; filtration; air and vacuum drying	[140]
30-70% ACN+ 5% B-ME 70% EtOH +5% B-ME 8 M Urea+ 5% B-ME	1:0.25, v:w	RT, 45 min	[141]
0.5% (v:v) B-ME, 0.5% (w:v) ammonium acetate, 45% ACN	0.1:3, v:w	ultrasounds, 5 min	[142]
ACN, B-ME, water, 60:5:35 (v:v:v), 120 mM ammonium hydroxide	1:0.5, v:w	RT, 5 min	[143]
55% IPA+0.2% DTT 60% t-butanol+0.2% DTT	----	----	[144]
70% EtOH	----	60˚C, 2 h	[145]

In some cases, albumins and globulins can be pre-extracted (step not included in the table). However, as reported by Adams *et al.* the previous removing of albumins and globulins did not

seem to be indispensable for the extraction of zeins [109]. Among different attempts to obtain total-zeins, extraction procedures using aqueous solutions of EtOH or IPA with or without a reducing agent are the most frequent. Parris and Dickey [145] described a method for the fractionation of total-zeins based on their different solubility. Nevertheless, this time consuming procedure was rather optimized for CGM and not for maize kernels. Yano *et al.* extracted α-zeins with 70% of EtOH from CGM [84]. Nevertheless, since all fractions of zeins (α-, β-, γ-, and potentially δ-) are soluble in 60% EtOH [101] when 70% of EtOH is used to extract α-zines, small amounts of β- and γ- proteins are also extracted [141]. Moreover, all methods using 70% EtOH to extract α-zeins are very tedious, time-consuming, and highly dependent on experimental conditions (temperature and sample-to-solvent ratio) [137]. Furthermore, these methods have never been applied to extract α-zeins from maize kernels but from maize co-products (*e.g.* CGM).

Regarding soybean, most abundant proteins are globulins. Bibliography related with the analysis of soybean proteins and their extraction from soybean seeds is quite wide. The extraction mostly starts with the defatting of grinded seeds. This step removes the high level of lipids in soybean (even ≈ 20%) which facilitates further protein extraction. Defatting step can be achieved using petroleum ether [147], hexane [148] or a mixture of them [149]. Next, a variety of protein extraction buffers can be employed. Despite organic/aqueous based media can be used, a Tris-HCl (tris (hydroxymethyl) aminomethane-hydrochloride) aqueous buffer is more frequently applied (see Table I.9) for this puropose. After obtaining a whole protein extract, further fractionation of 11S and 7S globulins is commonly performed (step not included in the table). For that purpose, the method of Thanh and Shibasaki [150] and the method of Nagano [151] are usually applied. These methods are characterized by their simplicity and ability to afford a large-scale separation of these two major proteins. Briefly, they are based on the sequential isoelectric precipitation of 11S and 7S proteins. A variety of modifications have been introduced into these methods to improve protein yield and recovery. A special attention deserves the method of Liu *et al.* [148] that was developed based on these two first methods. In the first stage of this method, a whole protein extract with a high protein yield is obtained. Further steps enable the isolation of 11S and 7S protein fractions with high purity. The only disadvantage of the mentioned method is the tedious and time-consuming protein preparation.

Table I.9. Procedures employed for the extraction of soybean proteins.

Solvent	Solvent/ sample ratio	Other conditions	References
30 mM Tris-HCl buffer, pH 8.0, 10 mM B-ME	----	agitation 1h; centrifugation	[147]
30 mM Tris-HCl buffer, pH 8.5	15:1, v:w	stirring 1h at 45°C; centrifugation; supernatant separated; pellet extracted one more time; centrifugation; two supernatants combined	[148]
A: 30 mM Tris-HCl buffer, pH 8.0, 10 mM 2 B-ME B: 50 mM Tris-HCl buffer, pH 8.8, 1.5 mM KCl, 10 mM DTT, 1 mM phenymethanesulfonyl fluoride, 0.1% SDS	----	A: 1 h vortexing; centrifugation B: 10 min in ice bath; centrifugation	[149]
25% (v/v) ACN, 0.3% (v/v) acetic acid (AA) or 0.1% (v/v), TFA	----	----	[152]
water	----	25°C for 5 min; centrifugation	[153]
10 mM Tris-HCl, pH 8.0	10:0.6, v:w	3 min sonication; centrifugation	[154, 155]

I.6.3. Special considerations in the extraction and enrichment of PKA, PKG, and CaMKII from animal tissues

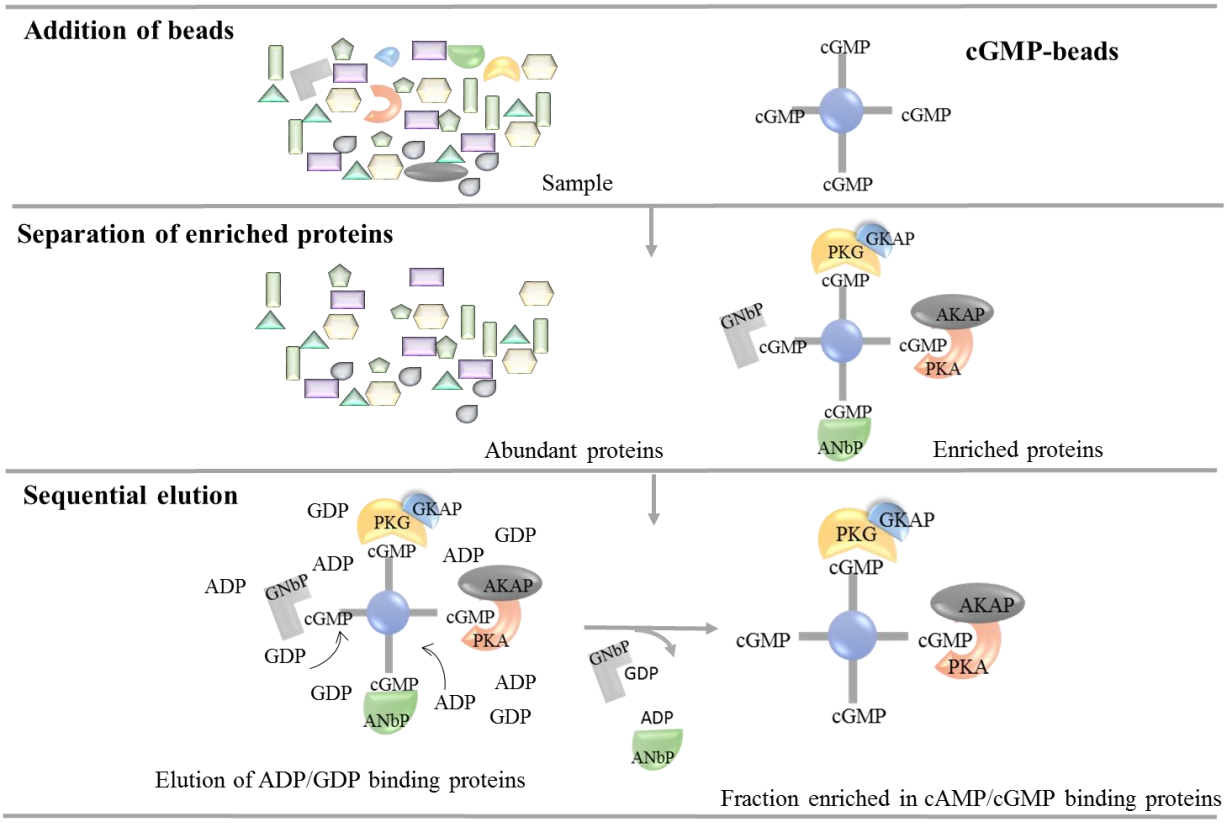
An aspect to be taken into account for the selection of a method to extract proteins from animal tissues is the further use of isolated proteins. If the aim is to extract the highest number of proteins, very harsh conditions are employed [156]. Nevertheless, if the aim is the isolation of specific proteins and their enrichment from cellular lysates, a fractionation using interactions with specific bait-proteins and antibodies are prefered. Nevertheless, protein-bait interaction requires that the whole protein assembly is maintained. Therefore, in these cases, very mild extraction conditions, as those grouped in Table I.10 are necessary.

Table I.10. Procedures employed for the extraction of PKA, PKG, and CaMKII proteins from animal tissues.

Extraction procedure	References
Whole proteome extract	
animal tissue + 500 µL lysis buffer (50 mM ammonium bicarbonate, 8 M urea, complete mini protease EDTA (ethylenediaminetetraacetic acid) free inhibitor mixture (1 tablet for 15 mL buffer), 0.1% phosphatase inhibitor mixture); room temperature (RT) for 5 min; centrifugation; supernatant 1 separated; pellet 1+ 500 µL lysis buffer; RT for 5 min; centrifugation; supernatant 2 separated; pellet 2+ 500 µL lysis buffer; sonication; centrifugation; supernatant 3 separated; three supernatant combined	[156]
Protein extract maintaining inter/intra proteins interactions	
animal tissue + lysis buffer (phosphate buffer solution (50 mM K_3PO_4, 150 mM NaCl, pH 7.0), 0.1% Tween 20, protease inhibitor cocktail); 10 min at 0°C; centrifugation	[25, 157, 158]
animal tissue + 1 mL lysis buffer (phosphate buffer solution, 0.1% Tween 20, 300 mM sucrose); RT for 5 min; 10 min at 0°C; centrifugation	[158, 159]

One of the major difficulties in the extraction of proteins from cardiac and other types of tissues is the dynamic range of protein expression. Cardiac proteome contains a set of high abundant muscle proteins that can hide less abundant but important proteins like PKA, PKG, or CaMKII [156]. In these cases, it is very usual the need for an enrichment step after protein extraction. The selective enrichment of low abundance proteins can be performed by a subproteomics approach or chemical proteomics. The subproteomics approach or chemical proteomics approach involve the use of agarose beads attached to molecules that interact with targeted proteins. Agarose beads with attached secondary messenger molecules, cAMP and cGMP, are added to the protein extract to interact with targeted kinases proteins. Next, beads with attached proteins are separated and enriched proteins are eluted (see Fig. I.13).

Secondary messengers cAMP and/or cGMP are immobilized onto agarose beads via flexible linkers in either 2- or 8- position of the nucleotide moiety. Although the principal intracellular targets of cAMP and cGMP are PKA and PKG, respectively, several other proteins can also be activated by these secondary messengers and can be pulled by their direct attachment to cAMP/cGMP.



Legend

AKAP- A- kinase anchoring proteins
GKAP- G- kinase anchoring proteins
GNbP- guanine binding proteins
ANbP- adenine binding proteins

Fig. I.13. Protocol for the enrichment of protein kinases using cAMP/cGMP agarose beads. Adapted from: [25, 157, 158].

Moreover, some proteins can also be bound through the attachment to the bonded PKA/PKG. Among these co-attached proteins are cyclic-nucleotide gated channels, phosphodiesterases, guanidine nucleotide exchange factors, A-kinase anchoring proteins, PKG anchoring proteins, and others [157]. Nevertheless, although it is difficult to 'pull-down' just selected protein kinases using these beads, the sample dynamic range obtained using this approach is very narrow. It is also possible to reduce non-specific binding by the incubation with an ADP/GDP (adenosine-5'-diphosphate/guanosine-5'-diphosphate) solution. The use of sequential elution with ADP, GDP, cAMP, and cGMP increases further specific enrichment as cross-linked nucleotide binding proteins can be pre-eluted. The use of cAMP-/cGMP agarose beads has shown to be very effective in the enrichment of these kinases in different proteomics studies [25, 27, 157, 159].

I.7. Extraction of peptides

Different procedures can be employed for the extraction of peptides depending on sample matrix and on peptide state.

I.7.1. Extraction of bioactive peptides from foodstuffs

Bioactive peptides can be naturally occurring in foods as individual entities or can be encrypted within a parent protein (see Fig. I.14).

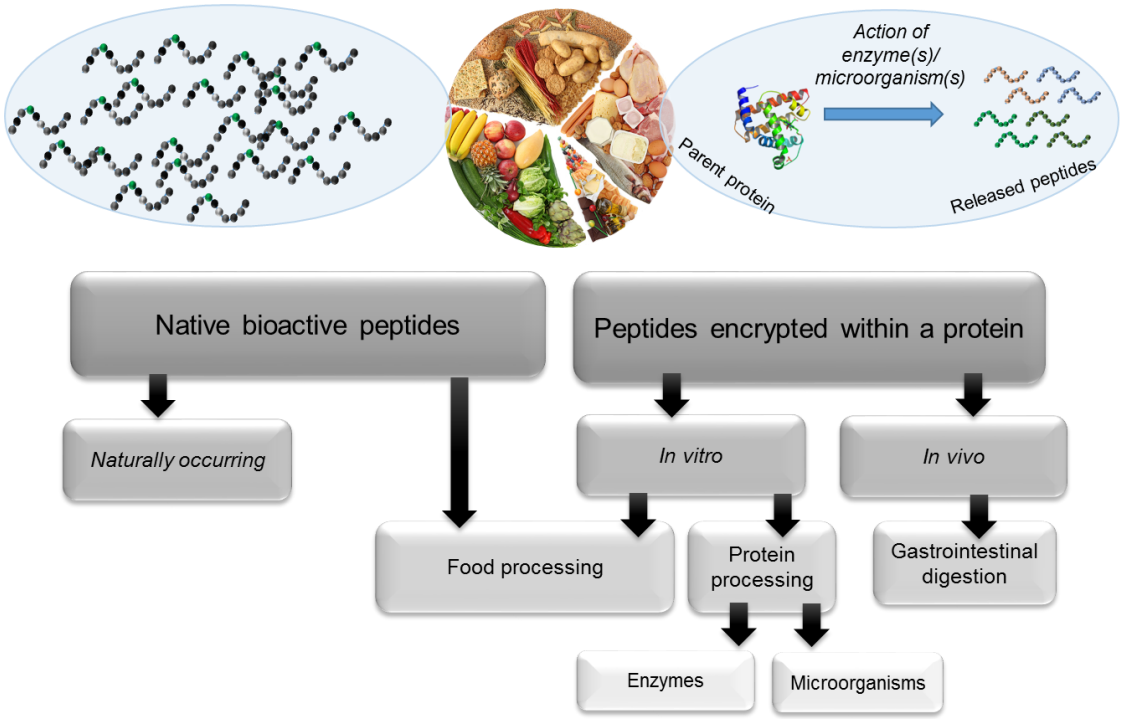


Fig. I.14. Scheme summarizing the pathways to obtain bioactive peptides from foodstuffs.

Two proteolytic pathways can be performed to release peptides from food proteins: *in vivo* and *in vitro*. The *in vivo* pathway is the result of the degradation of dietary proteins by digestive enzymes (gastrointestinal tract) in the organism. The *in vitro* manner involves protein processing by the action of microorganisms or enzymes derived from microorganisms or plants [52, 54, 55]. An interesting case are peptides provided from food processing. Although these bioactive peptides are released from food proteins, they are released just in order to obtain

desired products like yoghurt, cheese or other fermented products (*e.g.* kefir). Therefore, peptides obtained by food processing can be classified as native bioactive peptides.

Extraction of antioxidant and antihypertensive peptides naturally occurring in foodstuffs (native peptides). Native antioxidant and antihypertensive peptides have been found in a variety of unprocessed and processed foods (see Table I.11). Among unprocessed foods, the most frequently reported source of native antihypertensive peptides are mushrooms [160-162]. Regarding processed foods, the most frequently reported source of native antihypertensive peptides are dairy products [81, 163-166] and soybean based products [40, 167, 167-171] (not all works included into the table). Despite food matrix might be very different, the methods used for the extraction of antihypertensive peptides are very similar. They mostly consist of peptide extraction with water or an organic solvent like MeOH [66].

Literature concerning the extraction of native antioxidant peptides is much more narrow. In this case, the most frequently reported sources of native antioxidant peptides are milk and dairy products and soybean [172, 173]. Extraction procedures used in this case involve mainly the use of water or aqueous solutions.

Food protein hydrolysis approaches. As previously mentioned, there are two main pathways to release encrypted peptides within a parent protein, *in vivo* and *in vitro.* In the *in vitro* pathway, protein hydrolysis can be carried out by fermentation with bacterial organisms, by using proteolytic enzymes or by autolyzation. The last one is based on the proteolytic activity of natural ingredients in some foodstuffs. Despite this method is the simplest and the cheapest one, it is not the most popular since it requires long-term fermentation (even until 6 months) [179, 180]. In general, peptides produced by fermentation yield higher ACE inhibitory activities than digested peptides since bacterial strains can produce smaller peptides [181]. Nevertheless, the use of bacterial strains have some limitations like higher cost and laboriosity. This makes more common the use of proteolytic enzymes. One of the best sources of animal origin proteinases are pancreases, a by-product of meat industry. In addition, microorganisms' proteases such as thermolysin, neutrase, subtilisin, orientase, and proteases from lactic acid bacteria are relatively cheap and widely used sources of proteases [182]. The advantage of using commercially available microbial proteinases is their low cost, safety, and very high product yield [64].

Table I.11. Procedures employed for the extraction of native bioactive peptides from foodstuffs.

Product	Extraction procedure	Peptide	References
Antihypertensive peptides			
Pleurotus cornucopiae mushroom	Extraction with MeOH-water	RLPSEFDLSAFLRA, RLSGQTIEVTSEYLFRH	[161]
Garlic	Homogenization in water, precipitation with MeOH	SY, GY, FY, NY, SF, QF, DF	[174]
Wakame	Extraction with hot water, centrifugation	10 dipeptides	[175]
Buckwheat	Extraction with water at pH 9.0, centrifugation	GPP	[176]
Probiotic yogurt	Adjusting pH 4.5, centrifugation	VPP, IPP, and other 6 peptides	[177]
Different Spanish cheeses	Extraction with water, ultrafiltration Mwco 1 kDa	A total of 41 peptides	[164]
Commercial fermented soybean paste	Extraction with water	HHL	[168]
Douchi, douche qu, natto	Extraction with water, centrifugation	---	[167]
Antioxidant peptides			
Apium graveolens (celery)	Extraction using a phosphate buffer (pH 7.4), with KCl, EDTA, and polyvinylpyrrolidone, centrifugation	5 kDa peptide with partial sequence ADNAARPVRETDAVP	[178]
Cheddar cheese	Extraction with water, centrifugation, protein precipitation at pH 4.6	---	[173]
Douchi	Extraction with water, boiling, centrifugation	---	[172]

A variety of enzymes can be employed for the release of antihypertensive peptides. Enzymes such as subtilisin, chymotrypsin, pepsin [39, 183], thermolysin [184, 185], and alcalase [83, 183, 186] have been used for this purpose. These enzymes cleave peptide bonds near to hydrophobic amino acid residues leading to peptides favorable residues for antihypertensive activity at C-terminal position [183, 187]. In the case of antioxidant peptides, enzymatic

hydrolysis has been the main process for the production of bioactive peptides [89]. Enzymes such as trypsin, α-chymotrypsin and pepsin [188-190], or alcalase [191-193] were employed for this purpose. When the aim is the production of peptides with both antioxidant and antihypertensive activities, enzymes with low specificity are preferred. Enzymatic digestion can be achieved using a selected enzyme or by sequential digestion with different kinds of enzymes. There are also many kinds of commercially available proteinases which can contain a mixture of enzymes exerting both exopeptidases[3] and endopeptidases[4] activities [194]. Final composition of hydrolysates depends on the protein substrate, proteolytic enzyme(s) employed, enzyme to substrate ratio, physicochemical conditions (pH, preheat treatment, digestion buffer, hydrolysis time, and temperature), degree of hydrolysis, post-hydrolysis modifications, *etc.* [90].

I.7.2. Release of proteotypic peptides from tissue proteins

Enzymatic digestion of proteins is also used to obtain peptides that are further used for the identification and quantification of parent proteins. To achieve that, enzymatic digestion mainly using trypsin enzyme is employed. Trypsin is an aggressive, stable, specific protease that cleaves at the carboxy-terminal side of arginine and lysine residues. Obtained peptides are suitable for MS/MS analysis due to their appropriate mass range for sequencing (7-25 amino acid residues) and the presence of basic residues (R or K) at peptide C-terminal position. Lys-C endoprotease is another frequently used specific enzyme that cleaves at the C-terminal of K. Due to its stability in harsh solubilizing protein conditions (*e.g.* 8 M urea), it is commonly employed just before trypsin digestion (so called 'two stage digestion'). Other frequently used highly specific but less active proteases are Asp-N or Glu-C, which generate peptides complementary to tryptic peptides. Less specific proteases are usually avoided since they cut proteins into many small peptides overlapping protein coverage and generating highly complex matrices [120, 121]. There are three main strategies for this enzymatic digestion: *in-solution*, *in-gel*, and *in-column* [128], being the first two the most frequently applied. Additionally, a new approach known as FASP (filter aided sample preparation) introduced by Wiśniewski *et al.* in 2009 [195] is recently gaining a lot of interest.

In-solution digestion. The easiest and most widely applied approach to digest proteins is directly in the lysis buffer itself (in-solution). The digestion rate from sample to sample can be

[3] enzymes that break peptide bonds at the end of the chain.
[4] enzymes that break peptide bonds in non-terminal amino acids (within the chain).

influenced by the heterogeneity of the sample matrix and by the physicochemical properties of proteins. In order to disrupt intra molecular complexes, the protein mixture is subjected to its denaturation (*e.g.* using 8 M urea). Before trypsin digestion, the reduction and alkylation of protein disulphide bridges is commonly performed with DTT and iodoacetic acid or iodoacetamide. To allow digestion by trypsin action, sample is diluted to milder conditions (around 1 M urea) and the digestion is performed for 12-24 h. In order to reduce digestion time, several approaches have been developed. Among them, the application of microwaves, infrared radiation, ultraviolet radiation, use of a modified trypsin, or the use of HIFU are usual. Next, the addition of strong acids (formic acid (FA), AA, and TFA) or heating is mostly used to stop enzymatic digestion. The main drawback of this approach is the complexity of the peptide sample and the need for further fractionation of peptides [128].

In-gel digestion. In this strategy, proteins in the sample are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional electrophoresis before enzymatic digestion. Afterwards, selected gel bands or spots from mono or bi-dimensional electrophoresis are cut off and gel pieces are destained using suitable procedures. For the most common Coomassie Brillant Blue destaining, several washes with 50% ACN in ammonium bicarbonate at neutral pH is the most appropriate. In order to reach an efficient protein digestion within the gel, gel pieces must be dehydrated and dried. Therefore, gel piece can easily swell the trypsin solution. Enzymatic digestion with trypsin is carried out using standard reduction and alkylation. Two-stage digestion with Lys-C and trypsin is also a common practice. After digestion, peptides are extracted in a stepwise fashion. The main advantage of in-gel digestion is the reduced complexity of the sample and the lack of interferences. In addition, the apparent molecular weight of proteins is already known. Nevertheless, in-gel digestion suffers some limitations. First, some peptide bonds could not be accessible to the enzyme and, second, the extraction of peptides from the gel could be limited [128, 130].

FASP. In-solution digestion is an easily automated method that minimizes sample handling but may be hampered by interfering compounds. Moreover, obtained proteome might be incomplete. On the other hand, in-gel digestion is extremely robust against impurities but hard to automate. In addition, gel may prevent appropriate recovery of some important peptides. Therefore, a new approach combining the advantages of in-solution and in-gel digestion, has recently been introduced. FASP enables to combine strong detergents for universal protein solubilization and sample clean up before digestion while avoiding the disadvantages of using

a gel [195]. The basic steps involved in the FASP approach have been depicted in Fig. I.15 and include the use of ultrafiltration Mwco filters as a basic tool. Critical steps in FASP are: 1) separation of proteins from interfering compounds (*e.g.* SDS) by ultrafiltration through Mwco filters by centrifugation; 2) reduction and alkylation of proteins (when trypsin is used); 3) protein digestion; 4) elution of pure peptides. The key feature in FASP is the ability to retain high molecular weight substances on the Mwco filter and the ability to elute interferences. The major advantage of this protocol, over the two previously mentioned, is its ability to accommodate a wide range of digestion conditions [195].

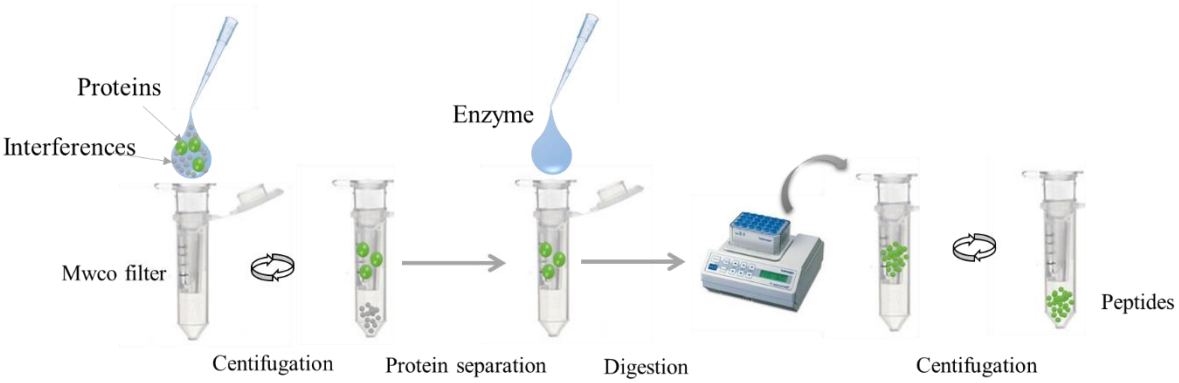


Fig. I.15. FASP protocol workflow.

I.8. Techniques used for the separation, purification, and determination of peptides and proteins

I.8.1. Separation and purification techniques

The complexity of biological samples usually requires the use of techniques enabling the separation of peptides and proteins. Separation techniques mostly used in the analysis of proteins and peptides are grouped in Table I.12.

Table I.12. Separation and purification techniques used in the analysis of proteins and peptides. Adapted from: [196].

Separation technique	Physical/chemical property
Electrophoretic techniques	
Gel electrophoresis	Stroke's radius
Isoelectrofocusing (IEF)	Isoelectric point
Chromatographic techniques	
Size exclusion chromatography	Stroke's radius
Ion exchange chromatography	Charge
Reversed phase chromatography (RP-LC)	Hydrophobicity
Affinity chromatography	Specific biomolecular interaction

I.8.1.1. Electrophoretic techniques

Electrophoresis is based on the movement of charged compounds under the application of an electric field. The direction and velocity of substances is determined by their charge and ion mobility [197]. This phenomenon offers a powerful tool to separate proteins, peptides, and other molecules. The velocity of migration (υ) of charged compounds within an electric field depends on the electric field strength (*Es*), the net charge of molecules (*z*), and the frictional coefficient (*f*). The frictional coefficient depends on both mass and shape (for a sphere of radius *r*) of separating molecules and the viscosity of the medium (η). Therefore, the velocity of migration can be expressed by the following equation:

$$\upsilon = \frac{Es\ z}{6\pi r\eta} \qquad (\textit{Equation 1})$$

For an established electric field and medium viscosity, the velocity of migration of an ion in an electrolyte depends directly on the net charge and on the molecule radius. Consequently, small molecules with high net charge have high electrophoretic mobility and move faster in an electric field than large molecules with low net charge [198]. Based on this principle, various electrophoretic techniques have been developed for the separation of proteins and peptides being SDS-PAGE and IEF the most commonly applied.

SDS-PAGE. Simple SDS-PAGE enables to resolve around 100 proteins in one run [116]. SDS-PAGE remains a gold standard technique to separate complex protein mixtures according to their molecular masses on a gel surface [131]. Electrophoretic separation is carried out in gels (solid support), which act as molecular sieves. Inert and readily formed polyacrylamide gels obtained by the polymerization of acrylamide and cross-linkers (methylenebisacrylamide) are the most commonly used [198]. Each gel is characterized by the total percentage concentration (%T) of both monomers (acrylamide and cross-linker). This value limits the size range of molecules that can be separated on the gel: the higher the %T, the smaller the molecules that can be separated [122]. Separation of proteins by SDS-PAGE is carried out in a device similar to that shown in Fig. I.16 A.

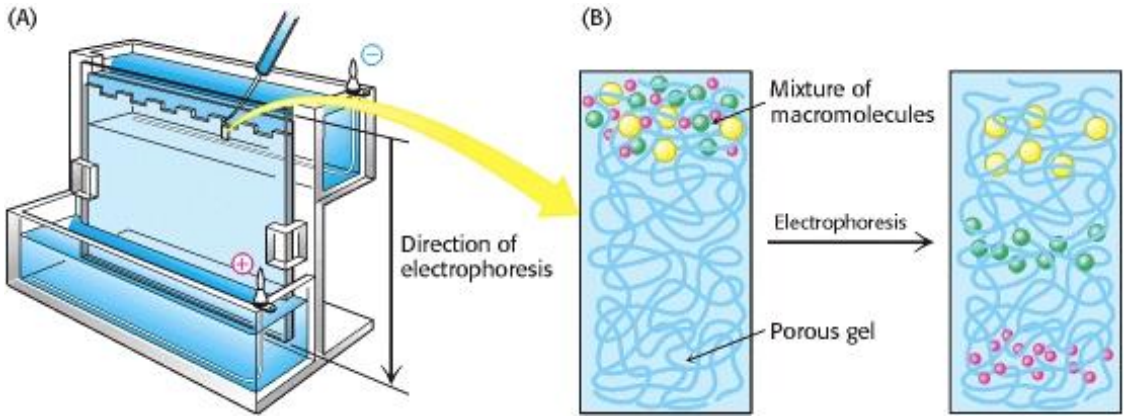


Fig. I.16. Polyacrylamide gel electrophoresis device (A) and scheme of the electrophoretic separation procedure (B). Source: [198].

Several samples can be separated by electrophoresis on one gel. Proteins are normally separated in polyacrylamide gels under denaturing conditions using SDS detergent and B-ME or DTT. SDS anions bind to protein chains creating a complex with a large negative charge which masks the real protein charge. In this way, the net charge per unit mass is approximately constant in all separating molecules and their separation is based just on their molecular masses.

SDS-protein complexes migrate towards the positively charged electrode (anode) during the electrophoretic separation. Small molecules pass through the gel pores rapidly, larger molecules remain immobilized, and intermediate molecules move with different degrees of difficulty (see Fig. I.16 B). The mobility of most proteins under these conditions is linearly proportional to the logarithm of their mass [198].

Separated proteins can be detected using various stains like Coomassie Brilliant Blue, silver dyes, fluorescence dyes or radiolabeling. Despite silver staining has a great sensitivity, it does not show signal linearity and is less compatible with MS detection. Therefore, Coomassie Brilliant Blue is the most common staining technique in SDS-PAGE separation [131].

Isoelectrofocusing. IEF is an electrophoretic technique enabling the separation of amphoteric molecules based on their characteristic pI. The pI of any amphoteric molecule is defined as the pH at which its net charge is equal to zero. Based on equation 1, the electrophoretic mobility of amphoteric molecules at their pI is null. pI values of proteins and peptides depend on the relative content of acidic and basic residues within their sequence. IEF is performed normally by the stablishment of a pH gradient of polymeric ampholytes exerting small differences in their pI values. The pH gradient of ampholytes can be created in a gel, in a capillary or can be immobilized on a strip. In the OFFGEL equipment, a protein or peptide liquid sample is distributed into different wells that are positioned on the top of an immobilized pH gradient (IPG) gel strip (see Fig. I.17).

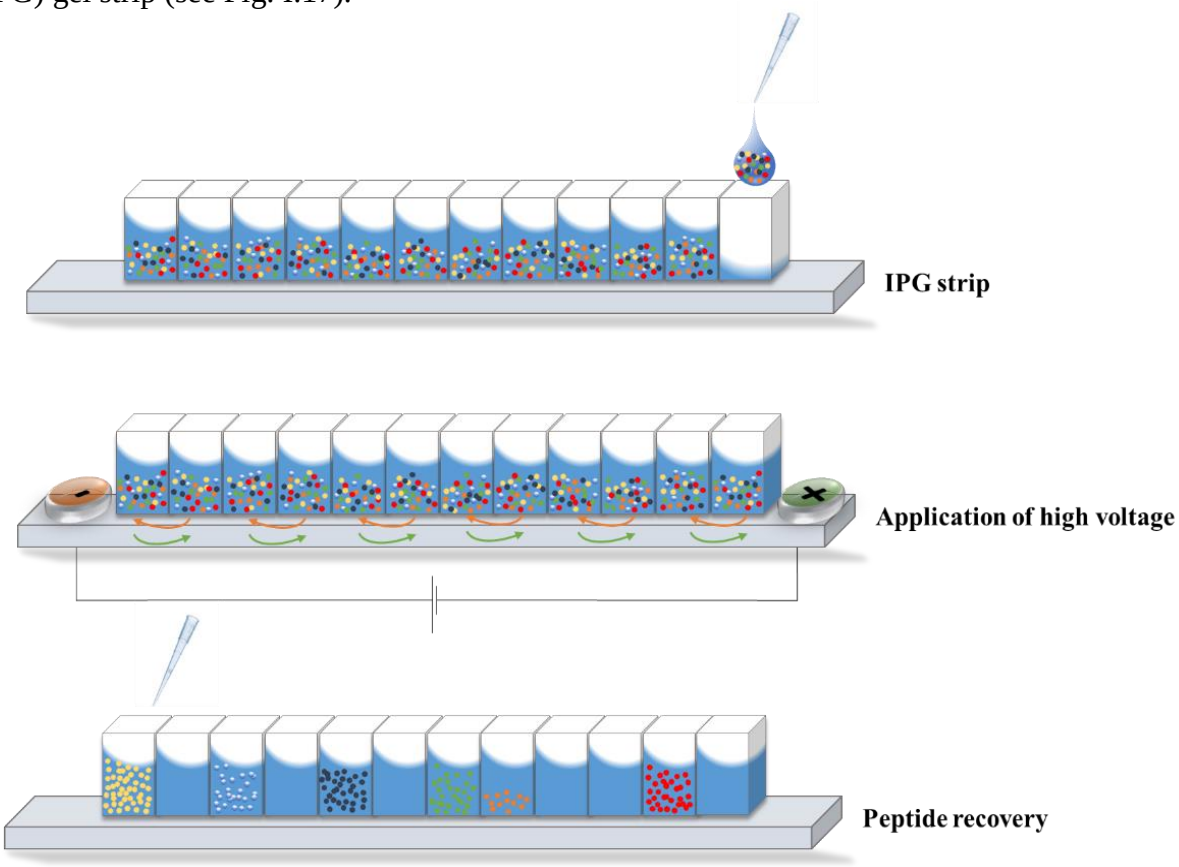


Fig. I.17. Scheme of fractionation using OFFGEL IEF with IPG strips.

The application of an electric field to the extremes of the IPG strips makes peptides and proteins to migrate with ampholytes through wells until they reach a pH equal to their pI. At this point, molecules lose their charge and focus in wells [116, 122, 198]. OFFGEL equipment enables a free flow IEF where proteins or peptides are separated in a multi-well device (12 or 24 wells) from which they can be directly recovered after separation for their further analysis. Dehydrated IPG strips are commercially available with different pH gradients [131]. Separation resolution depends on the number of used wells and the range of pH gradient.

I.8.1.2. High performance liquid chromatography (HPLC)

HPLC plays a central role in the separation of both proteins and peptides. The physicochemical diversity of these molecules (see Table I.12) makes them suitable for nearly every kind of chromatographic mode [199]. Size exclusion or ion exchange chromatography (including both anion and cation exchange modes) are frequently applied at the beginning of a peptide/protein fractionation, while RP-LC is usually employed as last step due to its high resolving power and compatibility with MS [200].

The separation by RP-LC is based on the hydrophobic interaction between molecules and packing material. Mobile phases used in the separation of peptides/proteins by RP-LC usually consist of water with an organic modifier being ACN followed by alcohols such as MeOH, EtOH or IPA, the most popular options. ACN is the most frequently selected due to its volatility, low viscosity, and transparence to ultraviolet (UV) light. Separation under gradient conditions is the most usual in the case of peptides and proteins [199]. In order to increase the hydrophobicity of charged peptides or proteins, the addition of acidic ion-pairing reagents to the mobile phase is also frequent. Ion-pairing reagents like AA, FA, phosphoric acid, heptafluorobutyric acid or quaternary ammonium salts are very common, although most widely employed ion-pairing reagent is TFA. TFA is transparent to UV light, it does not block amino groups, it is highly volatile, and it is easily miscible in most organic mobile phases. Nevertheless, the use of TFA is not advisable when using MS detection since it suppresses electrospray (ESI) ionization causing significant signal decrease [201]. Alternatively, UV online detection can be performed. Wavelengths at which peptides are detected are 210-220 nm (corresponding to the absorption of peptide bonds) and 254 and 280 nm (specific absorption of aromatic amino acids as tryptophan, phenylalanine, and tyrosine) [202].

Silica-based supports used to be the first choice in RP-LC since they enable a wide range of selectivities, depending on the bonded phase. Hydrophobic molecules like large peptides and proteins are usually separated on less retentive stationary phases (C_4-C_{12} phases). On the other hand, most typical choice for peptides is the use of C_{18} phases. C_{18} stationary phases offer retention and selectivity for a wide range of compounds that contain both polar and non-polar groups. Columns with phases as cyano, hexyl, phenyl, hexyl/phenyl, and perfluorinated are also available [203, 204].

In conventional columns, the separation speed is limited by the rate of analyte transference between mobile and stationary phase [199]. This step is particulary critical in the case of macromolecules like proteins due to their slow diffusion [205]. There are two main approaches in HPLC to reduce separation time without compromising resolution and separation efficiency: 1) use of supports with enhanced permeability and 2) use of stationary phases with smaller particle sizes [205-207]. Perfusion columns facilitate the permeability of proteins through the stationary phase by the use of particles containing large through- pores (6000-8000 Å) that are connected by small diffusive pores (800-1500 Å). These through-pores permit small percentage of convective flow through particles accelerating large molecules pass through them. This approach reduces the distance over which diffusion to the particle-binding surface occurs and, consequently, the retention time (see Fig. I.18 A and B). This strategy allows to rise the flow rate without loss of resolution. Another strategy to increase the permeability is to use columns made of one single piece (monolithic columns), prepared by compression or by polymerization of monomers inside the column [208]. These single rigid or semi-rigid rods contain both flow-through channels and a system of conventional diffusive pores (see Fig. I.18 C).

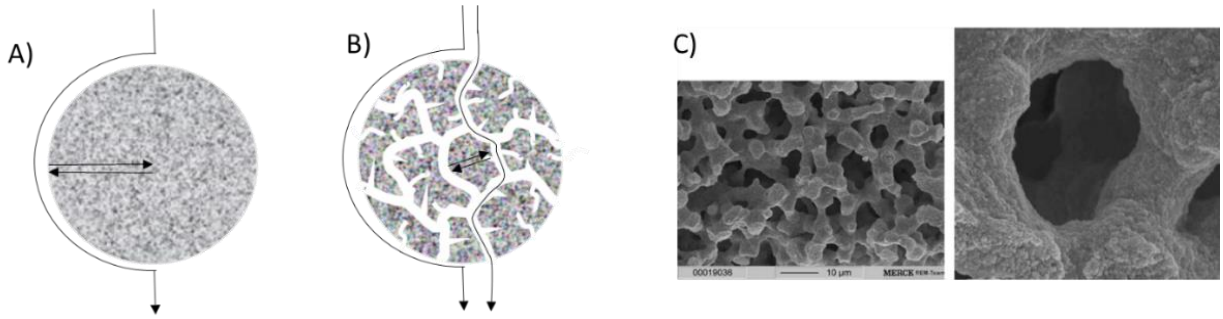


Fig. I.18. Comparison of stationary phases. A) Particle with normal porosity, B) particle with flow through pores, and C) scanning electron microscope image of the porous structure of a typical monolithic silica column (left) and enlarged view of the entrance to a through-pore (right). Pictures A) and B) adapted from: [209]. Picture C) from: [210].

Monolithic columns can be divided in two groups: organic monoliths (polymer based, *e.g.* polystyrene-divinylbenzene and polymethacrylate), suitable for the separation of macromolecules, and inorganic monoliths (silica-based), suitable for the separation of small molecules [199, 200].

The easiest strategy to reduce analysis time is to increase flow-rate. However, in conventional HPLC, particles present an optimum flow-rate at which column performance is maximized. Below and above this flow-rate, efficiency is lost due to different kinetic parameters described in the van Deemter equation:

$$H = A + \frac{B}{u} + Cu \qquad (Equation\ 2)$$

where H is the theoretical plate height, u is the linear velocity, and A, B, and C are constants that account for contributions to band broadening by Eddy diffusion, longitudinal diffusion, and mass transfer resistance, respectively [207]. The plot of the Van Deemter equation shows the variation of H versus the linear velocity (Fig. I. 19.). Smaller particles provide, at any practical flow-rate, higher efficiency and faster analysis without loss of efficiency. As a consequence, the development of chromatographic supports with smaller particles have been of great interest during the last decades (see Fig. I.19). Therefore, flatter Van Deemter plots are obtained and, higher flow-rates and maximum efficiencies are allowed [207].

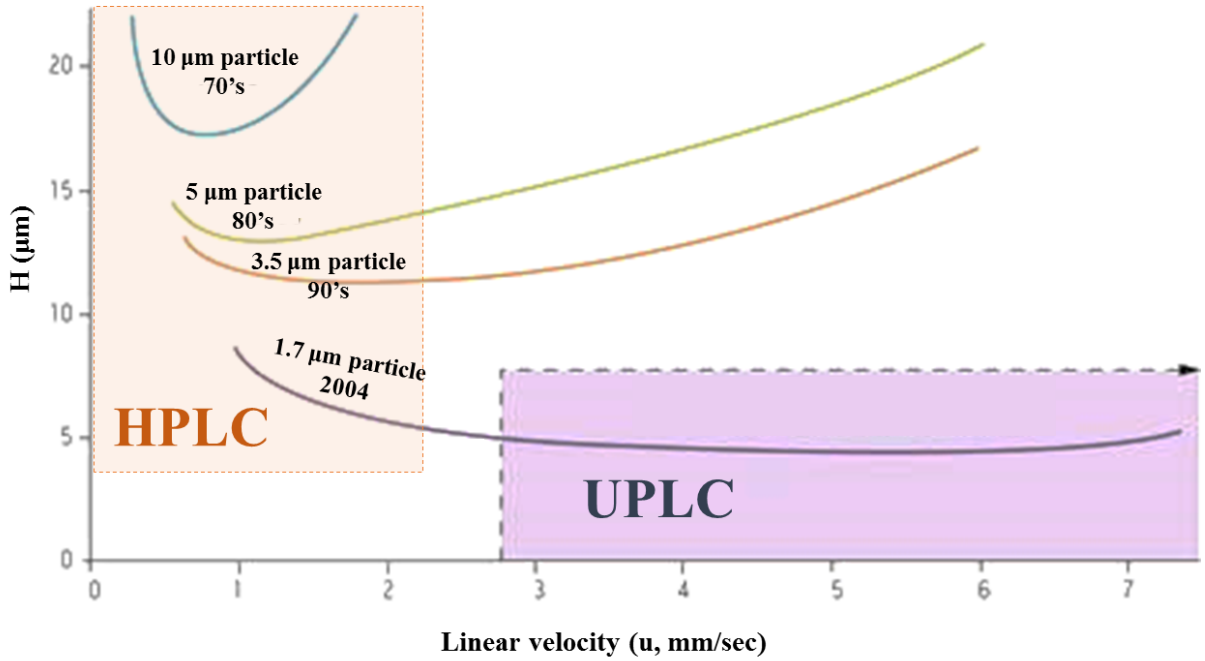


Fig. I.19. Van Deemter plot and evolution of particle sizes over the last three decades. Adapted from: [211].

Nevertheless, the backpressure varies with the inverse square of the average particle diameter, according to the following equation:

$$\Delta P = \frac{(uL\eta\Phi)}{d_p^2} \qquad (Equation\ 3)$$

where *P* is pressure, *L* is column length, Φ is the column resistance factor, and d_p is particle size [199]. Therefore, columns packed with particles smaller than 2 µm require suitable equipments supporting high backpressures (commonly named: UPLC). An alternative to sub-2 µm particles is the use of pellicular packings also known as 'fused core', 'core-shell', or 'superficially porous' silica particles. Unlike sub-2 µm particles, fused-core silica particles enable the reduction of analysis times and maintain column efficiency with relatively low backpressure.

Fused-core particles consist of a 0.35-0.5 µm porous silica layer fused onto a 1.7-1.9 µm solid inner core (see Fig. I.20). Small diffusion paths (0.5 µm), in comparison with traditional particles (1.5 µm), ensure a faster mass transfer and a better performance at high mobile phase velocities. Moreover, a robust packed bed and a homogeneous path length net minimize analyte diffusion through the column. Columns with 2.7 µm fused-core particles produce, approximately, half of the backpressure observed with 1.8 µm particles (UPLC) being possible the use of traditional HPLC systems [212]. Columns with fused-core particles play an important role in chromatography and their use in bio-analytical methods has been reviewed [213].

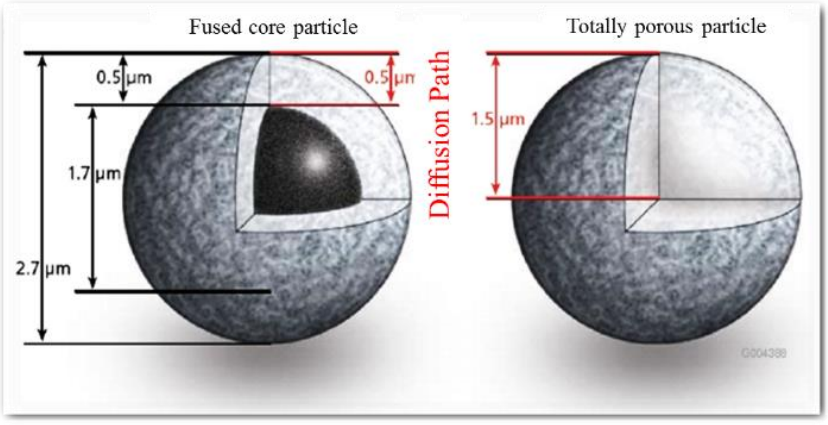


Fig. I.20. Scheme of a fused-core particle (on left) and comparison of the diffusion path with that of a totally porous particle.

Besides the reduction of analysis time, another topic that has attracted much attention in HPLC is the improvement of sensitivity. The use of HPLC columns with miniaturized dimensions provides an increase in sensitivity. For that purpose, flow rates, connecting tubes, and injection volumes should also be downscaled [214]. LC techniques can be classified based on column internal diameter (I.D.) in: micro (0.5-1 mm), capillary (0.1-0.5 mm), and nano (0.01-0.1 mm) HPLC [134]. The reduction of column internal diameter offers several advantages, such as: 1) lower requirement of all types of chemicals (mobile and stationary phases) and samples; 2) less wastes; 3) higher sensitivity due to a lower dilution of the sample during its separation; 4) higher capacity of thermostatization of the column; 5) increased ESI - MS detector response, since ionization is easier; and 6) higher efficiency [122, 134, 215, 216]. An important issue in miniaturized LC is the injection volume that should not exceed 30% of the column volume. To overcome this problem, it is especially common in nano-LC the use of switching systems. In these systems, a short trapping column with a highly retaining stationary phase keeps compounds, which are then eluted in a longer analytical column. This approach also ensures an online sample cleaning from unwanted or clogging interferences [214-216].

I.8.2. Mass spectrometry

MS involves the ionization of analytes in the ion source and their separation depending on their mass per charge (*m/z*) ratio in one or more mass analyzers. Subsequently, a detector registers the ion current from the analyzers, yielding the corresponding mass spectrum. During several years, a fundamental issue in the analysis of biological samples was the transference of polar and non volatile molecules into the gas phase without destroying them. The proposed solution commonly known as soft ionization techniques (MALDI and ESI) had a high impact on the peptide and protein research field. Nobel Prize awarded the latter technique in 2002 in the field of chemistry [120]. One of the most important advantages of ESI over MALDI is the possibility of on-line connection with the chromatographic eluent. In addition, while MALDI is used for relatively simple peptide mixtures, ESI-MS system is preferred for complex samples [217].

Most popular analyzers in proteomics are ion trap (IT), quadrupole (Q), time of flight (ToF) tubes, and Orbitrap cells [120, 123]. Hybrid mass spectrometers can yield additional information on analyte structure. Different fragmentation mechanisms (collision induced dissociation (CID), also known as collision activated dissociation, electron capture and electron transfer dissociation (ECD and ETD) *etc.*) are available [117].

Principles of ESI. ESI is a technique of ionization at atmospheric pressure where a sample is nebulized and ionized at the end of a capillary due to the action of a strong electric field (several kV) (see Fig. I.21). After ionization and nebulization, charged droplets move forward due to the established electric field gradient and the pressure gradient (ionization takes place at atmospheric pressure while mass analyzers are at very low pressure). Moreover, the control of chamber temperature enables the evaporation of charged droplets during this transition. As a consequence of solvent evaporation, the size of droplets decreases and the charge density increases, which results in a repetitive droplet instability and explosion into finer droplets. Finally, electrostatic repulsion is sufficiently high to cause desorption of analyte ions which then pass to the MS. Ions generated by ESI usually bear multiple charges [119, 120, 122, 128].

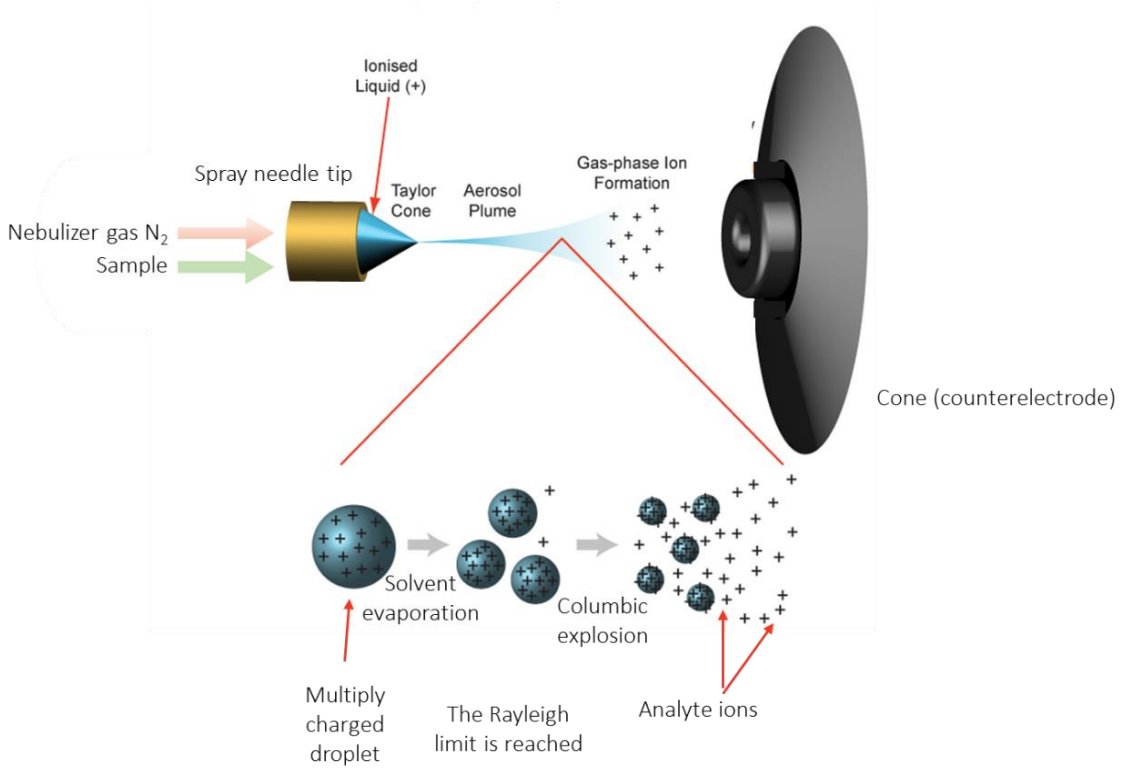


Fig. I.21. ESI ionization overview.

In ESI, analytes compete for charge as they are extruded from spray droplets. Consequently, the main drawback of ESI is its sensitivity to easily chargeable salts and detergents [218, 219]. Recently, an additional feature in ESI named "Jet Stream" has been introduced. This technology consists of an additional sheath gas heated at high temperature that focuses the nebulizer spray and desolvates ions more efficiently, thus improving sensitivity at high LC flow rates [205].

Mass analyzers and hybrids. Key parameters of any mass analyzer are sensitivity, resolution, mass accuracy, and ability to generate MS/MS spectra. There are four main analyzers:

quadrupole, ion trap, time of flight, and Orbitrap [217]. The schemes of all four mass analyzers are depicted on Fig. I.22. Moreover, different hybrid instruments have been developed by fusing various mass analyzers, ions optic, and fragmentation tools [131].

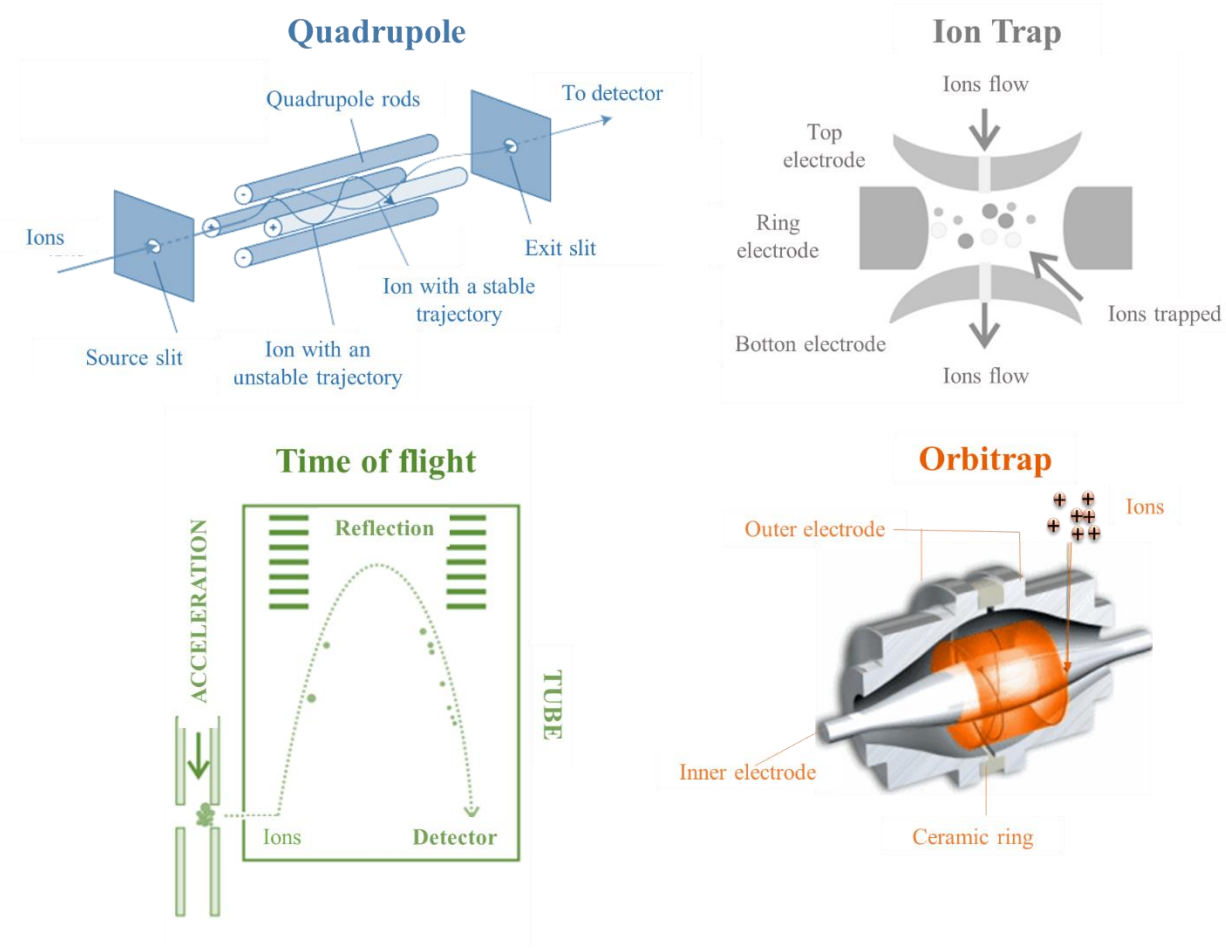


Fig. I.22. Schemes of Q, IT, ToF, and Orbitrap mass analyzers.

First mass analyzer introduced in the market was the quadrupole (Q). It consists of four parallel rods to which a fixed and direct current (DC) and an alternating radio frequency (RF) are applied [121] (see Fig. I.22). By applying appropriate RF/DC voltages, only a narrow *m/z* range can reach the detector [219]. Q is limited in mass range (until 4000 *m/z*) and provides low resolution. This mass analyzer can operate in single ion monitoring (SIM) or in scan modes. The SIM mode provides a significantly higher sensitivity. In the SIM mode, Q parameters are adjusted to select only one specific *m/z*. The time required to collect data of a particular mass is the transmission efficiency. When various *m/z* values are detected, the instrument works sequentially (from low to high *m/z*) [205]. When a high number of ions is selected, the transmission efficiency for every ion is reduced which directly decreases sensitivity. Three consecutive Q configured together (QqQ) can enhance significantly selectivity. The first and

third Q are used for scanning/filtrating ions, while the middle one is used as a collision cell. Ions are fragmented using CID, which is a low energy 'beam type collision' with a ground gas (*e.g.* nitrogen) [218]. Many MS/MS scan modes are possible in QqQ, like product ion, precursor ion, neutral loss or selected reaction monitoring (SRM or multiple reactions monitoring (MRM)). The two first modes are especially useful to identify closely related molecules or functional groups, and are out of the scope of this thesis. In the SRM mode (see Fig. I.23), Q1 and Q3 are programmed to filter just selected precursor and fragment ions, thereby increasing selectivity. When the number of monitored compounds during an analysis is too high and the transmission efficiency is very low, different time-scheduled windows with different SRM transitions and time intervals can be employed [206].

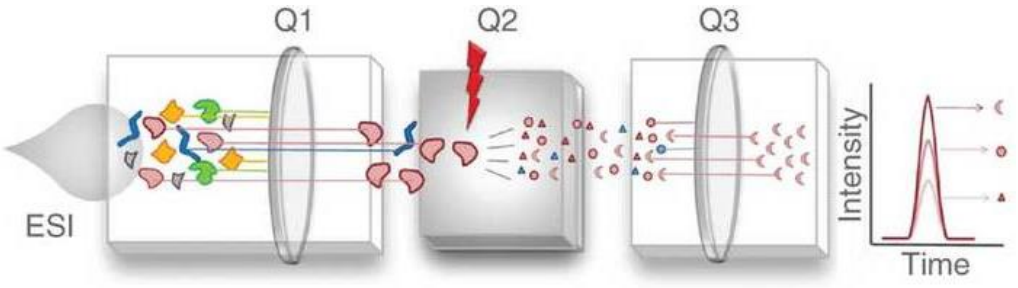


Fig. I.23. Overview of the selected reaction monitoring mode in QqQ. Source: [220].

Quadrupole IT consists of two parallel oval rods enabling the application of variable RF and one circular ring enabling the application of a fixed RF (see Fig. I.22). IT permits to isolate and fragmentate ions in the same space. Ions are trapped into a small volume by an oscillating electric field (RF/DC) and scanned by increasing the RF applied to the trap. IT is filled with helium gas that takes the excess of ions kinetic energy and focus them in the center of the trap. Further isolation of selected precursor ions is performed by ejecting all ions except that selected as precursor. Isolated ions are translationally excited before the collisions with helium gas. The translational energy is converted to internal energy during the collisions with helium, which leads to the ion fragmentation (so called resonance CID). Obtained daughter ions are then scanned out. The main advantage of IT is its quick shift between scanning for masses of analytes and generating fragmentation spectra of these ions [131]. IT is very sensitive since it can concentrate ions in the trapping field for different amounts of time. Additionally, IT is the only mass analyzer that can provide multiple MS^n fragmentation and, alone, can be used to identify peptides. 'Pseudo- SRM' mode is used with IT analyzer when upon fragmentation of a precursor ion, MS/MS data are acquired on a partial mass range, which is centered on a

fragment ion [221]. The main disadvantage of IT is its low mass accuracy, partly due to the limited number of ions that can be accumulated at its center. Linear ion traps (LIT) are a recent improvement of IT, where ions are stored in a cylindric volume that is larger than a conventional IT. This feature improves IT sensitivity, resolution, and mass accuracy [131, 217, 219]. Dual pressure LIT improves sampling speed and sensitivity. In this hybrid, the first IT efficiently captures and fragments ions at relatively high pressure, whereas the second IT performs extremely fast scan at reduced pressure [131, 222].

ToF-MS is a mass analyzer where ionized molecules are accelerated by a fixed amount of kinetic energy and travel down to a flight tube (see Fig. I.22). Due to the differences in masses, ions have different velocities and reach the detector, at the far end flight tube, at different times. ToF instruments provide high mass resolution and accuracy over a broad mass range. Typically, ToF instruments can achieve very high resolutions [218, 219]. The combination of ToF with Q is other commonly applied hybrid. Q-ToF consists of one Q, one hexapole collision cell, and a ToF mass analyzer (see Fig. I.24). This configuration provides additional advantages since it is possible to select parent ions for their fragmentation and to separate fragments using high resolving power ToF [121].

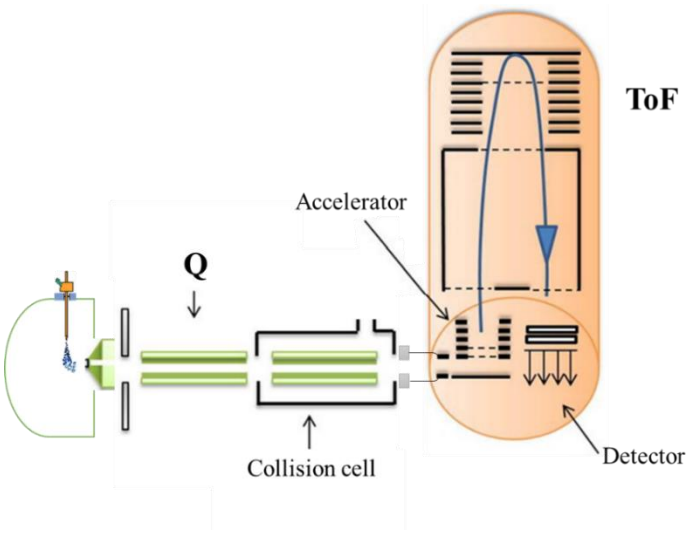


Fig. I.24. Schematic diagram of a Q-ToF. Adapted from: [131].

Orbitrap is one of the newest mass analyzers. It consists of an outer electrode enclosing a central inner electrode and a ceramic ring (see Fig. I.22). In Orbitrap, moving ions are trapped into an electrostatic field. The attraction toward the central electrode is compensated by the centrifugal force that comes from the initial tangential velocity of ions (similar to satellites on orbit). This electrostatic field forces ions to move in complex spiral patterns. The axial component of ions oscillations (independent from initial energy, angles, and position) are

detected as a current in two parts of the electrode that encapsulate the core. Fourier transform turns those currents into oscillation frequencies of ions at different masses, which allows obtaining their accurate *m/z* values. Although it may be possible to fragment ions in the Orbitrap, it is more practical and much faster to hybrid Orbitrap with other systems. In this regards, a hybrid with sensitive and very fast LIT has been developed (LTQ-Orbitrap). LTQ-Orbitrap contains three main parts: a LIT analyzer to obtain MS and MS^n spectra with very high sensitivity and mass accuracy, a C-Trap system (simplified Q) to accumulate and store ions, and an Orbitrap to analyze ions accumulated in the C-Trap (see Fig. I.25). Depending on requirements, two analyzers can be used independently or in concert. When both analyzers work simultaneously, high resolution/mass accuracy spectra are acquired by Orbitrap while fast fragmentation and MS/MS detection is carried out by LIT [223]. The next generation of LTQ Orbitrap system, termed Velos, provides even more improved sensitivity and scan speed. The most important implemented changes are: the use of a dual LIT instead of a simple LIT which accelerates the acquisition speed, improves and makes more efficient the fragmentation by a higher energy collision dissociation cell (HCD) system, *etc.* [222].

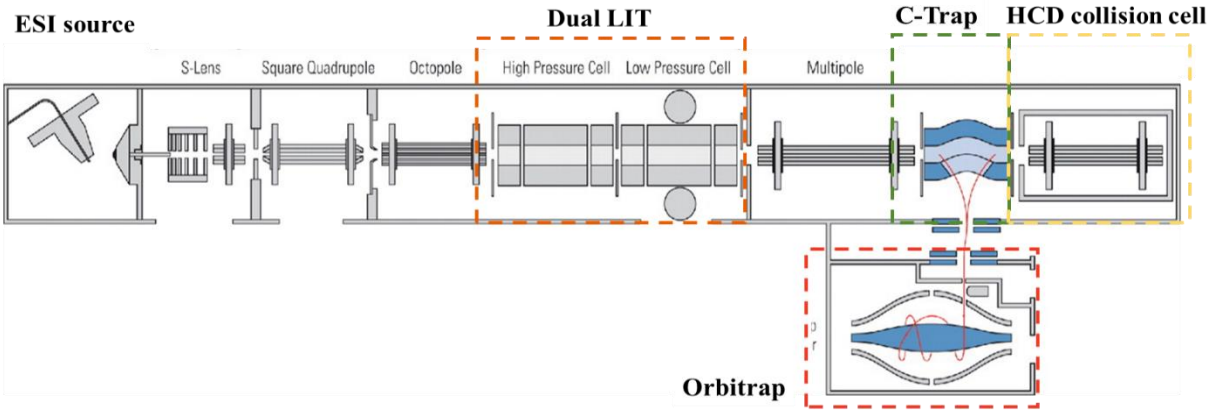


Fig. I.25. Schematic diagram of the LTQ Orbitrap Velos MS. Adapted from: [222].

Finally, Table I.13 groups some characterictics of the mass analyzers and hybrids described. Q-ToF and LTQ-Orbitraps are the MS equipments with the highest resolving power and mass accuracy. Regarding *m/z* range, Q-ToF is the system enabling to work at higher *m/z* values and, thus, the most suitable to work with large molecules. Last extremely important parameter is acquisition speed, being IT and LIT the systems showing the highest values [224].

Table I.13. Overview of some selected commercially available mass spectrometers with their technical specifications provided. Source: [224].

Mass analyzer type	Resolving Power[5] (defined at *m/z*)	Resolution[6] (Δ*m/z*)	Mass accuracy (ppm) using internal calibration[7]	*m/z* range[8]	Acquisition speed (Hz)
QqQ	7,500 (*m/z* 508)	0.07	5	10-3,000	5
IT	-	0.1	-	50-6,000	52
LIT	-	0.05	-	15-4,000	66
Q-ToF	42,000 (*m/z* 922)	0.02	<1	50-10,000	50
LTQ-Orbitrap	240,000 (*m/z* 400)	0.002	<1	50-4,000	8 (at RP= 15,000)

The selection of a suitable MS system obviously depends on the requirements of the analysis in terms of resolution, mass accuracy, and acquisition speed. QqQ provides good linear dynamic range, high precision, and less matrix interferences for product ion measurements. QqQ is perfectly adapted for targeted analysis and quantitative applications. In fact, SRM using QqQ is a golden standard for LC-MS quantification. IT and LIT are fast, sensitive, and able to perform multi-stage fragmentation. They are perfect for both targeted and non-targeted analysis coupled to fast and highly efficient LC systems. Q-ToF instrument can acquire data over a wide mass range with high mass accuracy, resolving power, and speed. Therefore, it is particularly well suited for non-targeted analysis and, in some cases, for targeted [224]. On the other hand, Orbitrap instruments offer extremely high resolving power. However, such high quality measurements sacrifice the time of analysis proportionally with requested resolution [223]. LTQ-Orbitrap shows low sensitivity and, due to the slower data acquisition rate, it requires slower chromatography. Latest generation of this instrument has improved significantly this inconvenient by the introduction of the dual-LIT. Therefore, LTQ-Orbitrap instruments are especially important in non-targeted analysis of complex samples.

Application of tandem MS to the sequencing of peptides. Tandem MS enables to obtain the primary structure of peptides. In the first stage, the peptide ion is isolated and fragmented and the MS/MS spectrum of peptide fragments is generated. Fragment ions produced by tandem MS can be separated into two classes. One class retains the charge on the N-terminal while the

[5] Defined as *m/z* value of particular peak divided by the peak full width at half maximum. Resolving Power is defined for a particular *m/z* value.
[6] Defined as the inverse of Resolving Power expressed as Δ*m/z* for a given *m/z* value.
[7] Defined as the relative difference between the experimental *m/z* value related to its theoretical value including the sign (+ or -) and expressed in ppm.
[8] Defined as the limits of *m/z* over which the mass analyzer can measure ions.

cleavage is observed in the C-terminal. This fragmentation can occur at three different positions, each of which is sequence designated as types a_n, b_n, and c_n (see Fig. I.26.). The second class of fragment ions generated from the N-terminal retains the charge on the C-terminal, while cleavage is observed from the N-terminal. Like the first class, this fragmentation occurs at three different positions, types x_n, y_n, and z_n (see Fig. I. 26).

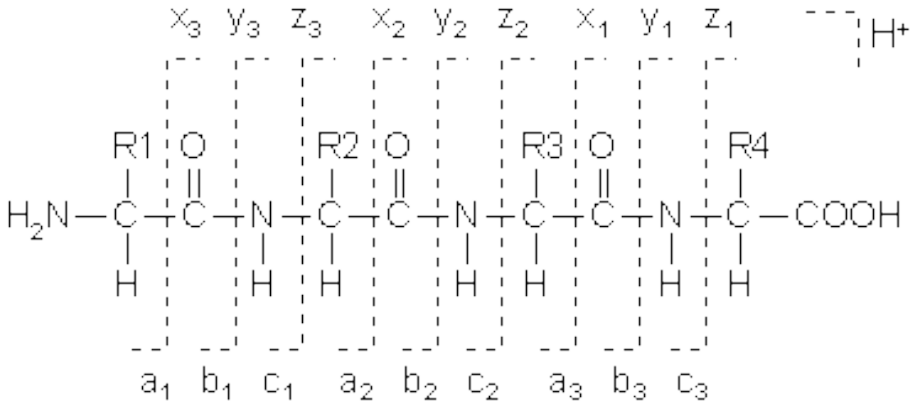


Fig. I.26. Peptide fragmentation nomenclature.

MS/MS fragmentation can be performed by various fragmentation methods. CID is the most widely applied where peptide ions undergo dissociation at amide bonds generating *b*- and *y*-type fragment ions. In contrast, ECD and ETD lead to the cleavage of N-C_α backbone bonds generating *c*- and *z*- type fragment ions [225]. While the differences among spectra obtained with CID and ECD/ETD are obvious, interestingly differences have also been observed among CID types. Indeed, the energy applied to a peptide in IT (resonance CID) and Q (beam type CID) analyzers is different. Obtained spectra differences have been associated mainly to gas conditions and kinetic energy. Lately, introduced HCD fragmentation type is other beam CID that has shown to generate spectra similar to Q fragmentation [226].

On the other hand, peptide fragmentation information can sometimes be incomplete or some peaks can belong to other peptide series, which result in complex MS/MS spectra analysis. Indeed, peptides obtained by enzymes, which do not cut at basic residues, do not possess charge at C- or N-terminal of peptides. In this case, the abundance of *b*- and *y*- ions series can be reduced and some abundant internal ions are generated. This fact complicates spectrum interpretation and peptide identification. Peptides with basic residues at C- or N-terminus, like tryptic peptides, cleave easily in MS/MS obtaining fragments that deliver richer sequence

information on *b*- and *y*- ion series and, consequently, are much easier to interpret. Obtained data can be treated using database search or *de novo* sequencing. *De novo sequencing* is influenced by the quality of data, in terms of mass accuracy and resolution, as well as the information obtained from the MS/MS spectrum. Database search is easier since the number of possible peptide amino acids sequences that occur in nature is limited [120].

I.9. Quantification of targeted proteins/peptides

Quantification methods can be divided into classical (*e.g.* Bradford assay, o-phtaldialdehyde assay, *etc.*), where total protein/peptide content is estimated, and more specific, where just targeted peptides or proteins are quantified.

I.9.1. Quantification of targeted bioactive peptides in foods

To date, studies in the area of bioactive peptides in foodstuffs have been mainly focused on the discovery of novel peptides with antihypertensive or antioxidant activity, their isolation, purification, identification, and *in vitro* or/and *in vivo* characterization. However, quick development of functional foods with bioactive peptides requires new methodologies for the quantification of peptides along with stability studies in complex matrices. This is especially true in the case of antihypertensive peptides since they have shown to be highly dosage dependent and they do not exert synergistic effects. In this sense, the quantification of antioxidant peptides in food hydrolysates could be pointless. Quantification methodologies to assess the content of antihypertensive peptides in foods are necessary in order to evaluate safety of functional foods, to establish healthy claims, and to expand policy and regulations controlling the addition of peptides to foodstuffs [227, 228]. Table I.14 summarizes the methodologies used to determine antihypertensive peptides in foodstuffs. LC has been the most frequently employed technique, using UV or MS detection. MS is the dominant technique for the reliable detection and quantification of antihypertensive peptides. A special attention should be directed to matrix effects since they can suppress peptide ionization in MS detection [227]. Label free approach using MS or MS/MS detection has been the unique strategy employed for bioactive peptides. In addition to the scarce number of existing methodologies for the determination of antihypertensive peptides in foodstuffs, most of them are focused on foods from animal origin.

Table I.14. Summary of methodologies used for the determination of antihypertensive peptides in foodstuffs.

Source of peptide(s)	Quantified peptide(s)	Methodology	References
Yoghurt like products	YP	LC-UV	[229]
Royal jelly	IY, VY, IVY	LC-UV	[230]
Wakame	7 dipeptides	LC-MS	[231]
Salmon muscle	8 dipeptides	LC-MS	[232]
Goat milk	TGPIPN, SLPQ, SQPK	LC-MS	[233]
Miso paste	IPP, VPP	LC-MS	[234]
Fermented milk	LHLPLP	LC-MS and LC-MS/MS	[235]
Bonito muscle	LKPNM	LC-MS and LC-MS/MS	[236]
Fermented soybean seasoning and soybean sauce	9 dipeptides	LC-MS/MS	[40]
Swiss and non-Swiss cheeses	VPP, IPP	$LC\text{-}MS^3$	[237]
Cheeses with different time of ripening	VPP, IPP	$LC\text{-}MS^3$	[238]
Soybean	Soymetide	LC-UV and LC-MS	[154, 239]
Rye malt sourdoughs	LQP, LLP, VPP, IPP	LC-MS/MS	[240]

I.9.2. Quantification of targeted proteins in living organism tissues

The quantification of protein populations in samples with different states or obtained under different conditions (control vs. case) is an essential topic in proteomics. To achieve that, different approaches for the quantification of proteins in proteomics have been developed (see Fig. I.27).

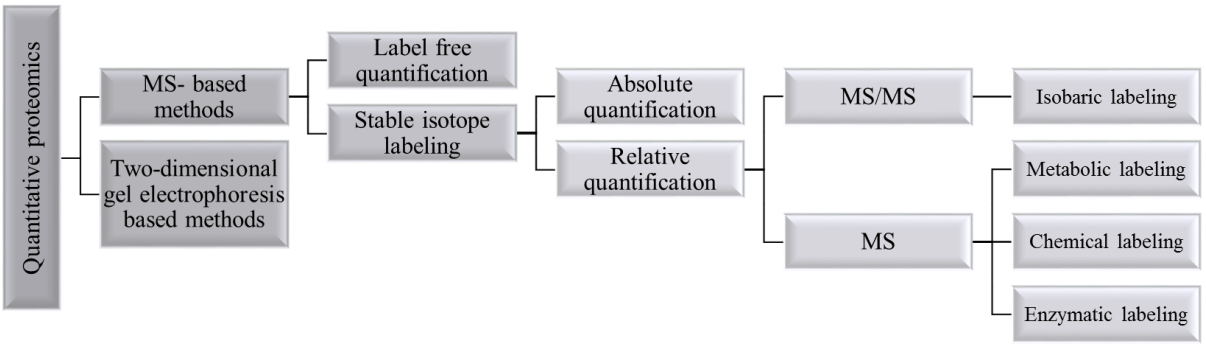


Fig. I.27. Classification of strategies applied in quantitative proteomics. Adapted from: [131].

Classical two-dimensional gel electrophoresis methods are not suitable to analyze low abundant proteins in complex samples. MS-based approaches can be divided into label- free and label-based methods. Label free methods determine protein content based on the MS- ion current signal of a peptide/protein or the MS/MS spectra (spectral counts) of a protein [116, 123, 128, 200]. Moreover, these methods provide comparable results within biological samples [131]. In label-based methods, proteins/peptides are labeled at different stages using mass tags. This strategy provides both relative and absolute quantification although this is less precise than label-free strategies. In absolute quantification, isotopically labeled synthetic peptides are needed as internal standards for each targeted protein [117, 123, 123]. Absolute quantification can be obtained by spiking the sample with stable isotopically labeled peptides or proteins. Briefly, it is based on unique peptides (proteotypic peptide- *PTP*) which can be related just to one certain protein. A peptide with identical sequence to this PTP is synthetized and isotopically labelled. Both types of PTPs (labeled and non-labeled) are monitored, identified, and quantified by MS/MS using SRM [123]. This approach has recently emerged as a targeted proteomics technique for accurate quantification of specific sets of proteins in very complex backgrounds. Although it is highly sensitive and selective, it still has not been broadly used since it is only useful if working with a limited number of proteins [241]. The typical workflow to establish SRM experiments to quantify targeted proteins is presented in Fig. I.28.

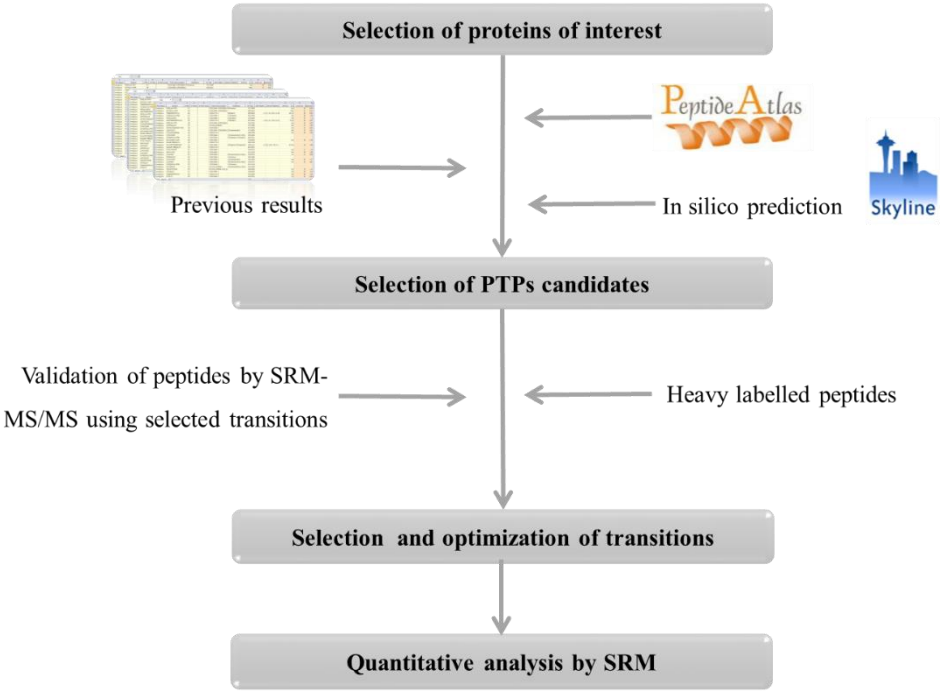


Fig. I.28. Workflow for SRM-based proteomics experiments. Adapted from: [221].

The most important part is the appropriate selection of PTPs and the selection of specific *m/z* settings for precursor and fragments (transitions). Firstly, PTP selection is based on *in-silico* targeted protein digestion together with mining in previously acquired results to predict most promising candidates and to confirm their uniqueness within PeptideAtlas database. Next, the selection of transitions is based on data from discovery experiments or it is experimentally determined in a QqQ instrument. While the first approach can result in biased information due to differences in fragmentation patterns, the second one is much more reliable but might be time consuming. Despite the high selectivity obtained by this approach, the appropriate selection of transitions must be validated for every peptide using heavily labelled peptides counterparts. The optimization of collision energy and time scheduling is normally performed to increase detection sensitivity [220, 221, 241].

I.10. Characterization of bioactive peptides

Once protein hydrolysate or bioactive peptide is obtained, its bioactivity must be confirmed by the use of one or more *in vivo* or *in vitro* assays. In addition, several characteristics can be assessed such as the kind of inhibition, resistance to high temperatures and other processing factors, and bioavailability. In the case of hydrolysates, the amino acid composition, molecular weight distribution, and peptide content are some additional characteristics that could be studied.

I.10.1. Bioactivity assays

Antihypertensive activity assays. Antihypertensive assays monitor the conversion of an appropriate substrate by ACE in the presence and absence of peptide inhibitors. Commonly used substrates are hippuryl-histidyl-leucine (HHL) and 2-furanacryloyl-phenylalanyl-glycyl-glycine [64]. Original method developed by Cushman and Cheung [242] is still the most widely adopted assay, where HHL is hydrolysated by ACE to hippuric acid (HA) and HL peptide [71]. The increase of HA level can be monitored at 228 nm. Recently, this assay has been carried out by RP-LC which ensures a suitable separation between HHL and HA [66]. Lately, highly sensitive, selective, and quick UPLC-MS methodology has also been proposed [243].

Antioxidant activity assays. In order to study the antioxidant capacity of peptides, several *in vitro* methods have been developed (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH), 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) assay (ABTS), oxygen radical absorbing capacity assay, ferric reducing antioxidant power, hydroxyl radical

scavenging, *etc.*). Nevertheless, there is no official standardized method due to the complex nature of antioxidants. Consequently, it is suggested the use of different methods to evaluate antioxidant capacity of potential antioxidants [90, 118]. *In vitro* antioxidant assays can be divided into two groups: assays based on hydrogen atom transfer (HAT) and those based on electron transfer (ET). HAT assays are based on a competitive reaction scheme, where antioxidant and substrate compete for thermally generated radicals. ET assays are non-competitive and are based on the measurement of the capacity of reduction of oxidants by antioxidants. While oxygen radical absorbing capacity assay and hydroxyl radical scavenging assays can be classified as HAT, DPPH, ferric reducing antioxidant power, and ABTS assays belong to the ET assay group [90, 244].

DPPH, ABTS, and hydroxyl radical scavenging assays have been commonly used for the evaluation of antioxidant activity in proteins and peptides from vegetable foodstuffs. In the DPPH assay, the scavenging capacity of peptides is tested on DPPH free radicals. DPPH dissolved in EtOH is incubated under light and temperature protection with the mixture of peptides. Upon reduction, the solution color fades proportionally to the antioxidant capacity of investigated peptides and the absorbance at 515-517 nm is measured. On the other hand, in the ABTS assay, ABTS radicals are used as probes and oxidants. ABTS radicals are produced after the incubation of ABTS with potassium persulfate. This radical is able to scavenge electrons from antioxidant peptides and change its color. Decoloring process is monitored at 734, 690 or 420 nm in the presence and absence of tested peptides. The hydroxyl radical scavenging assay can be performed in various systems. The most popular is the $FeSO_4$-phenanthroline-H_2O_2. Both, $FeSO_4$ and H_2O_2 generate hydroxyl ($OH^{\cdot}$) radicals which hydroxylate 1,10-phenantroline-Fe^{2+} to 1,10-phenantroline-Fe^{3+}, causing the reduction of the absorbance at 536 nm corresponding to 1,10-phenantroline-Fe^{2+} complex. Antioxidant peptides inhibit this oxidation reaction [66, 90, 244].

I.10.2. Bioavailability study

In order to exert *in vivo* effects, bioactive peptides have to be bioavailable [46]. This means that a bioactive peptide has to demonstrate its capacity to be absorbed and available for use. Consequently, bioavailability studies are essential. They involve to study the resistance of peptides to gastrointestinal track conditions, to study the absorption of peptides through the intestinal barrier, and to study the resistance of peptides against brush border aminopeptidases, serum, or blood enzymes. In order to study the intestinal absorption, *in vitro* tests with

monolayers of intestinal cell lines (commonly Caco-2 cultures) simulating epithelium are usually applied. Additionally, the incubation with serum or blood enzymes, including ACE in case of antihypertensive peptides, can also be checked. A simulated gastrointestinal digestion enables to evaluate the resistance of peptides to gastrointestinal conditions [60, 90]. Various methodologies have been developed for this *in vitro* digestion from which that of Garrett *et al.* [245] is the most commonly used. It consists of a pepsin HCl digestion followed by a pancreatin (commercial protease mixture isolated from pancreas and made up of trypsin, chymotrypsin, elastase, and carboxypeptidases A and B) digestion with bile salts which simulates the conditions in the small intestine.

Part of the information presented in this chapter is included in detail in the following book chapter and review article:

- **Book Chapter:** *"Peptides"*
  P. Puchalska, C. Esteve, M. L. Marina, M. C. García
  IN: "Handbook of Food Analysis", 3rd Edition, Chapter 17. CRC Press, Taylor& Francis, USA, 2014, ISBN: 978-1-46655-654-6.

- **Article 1:** *Isolation and characterization of peptides with antihypertensive activity in foodstuffs*
  P. Puchalska, M. L. Marina, M. C. García
  Crit. Rev. Food Sci. Nutr., in press (DOI: 10.1080/10408398.2012.664829)

Book chapter

Peptides

P. Puchalska, C. Esteve, M. L. Marina, M. C. García

IN: "Handbook of Food Analysis", 3rd Edition, Chapter 17. CRC Press, Taylor & Francis, USA, 2014, ISBN: 978-1-46655-654-6.

Content

1. Introduction

The word "peptide" (πεπτός) originally comes from Greek and means "small digestible". Structurally, peptides are constituted by amino acids linked by amide bonds. Commonly, it is assumed that peptides contain up to 100 amino acids, more amino acids residues is referred normally to protein. Peptides play important physiological and biochemical functions in human body being involved in numerous biochemical processes within the nervous, immunological, and cardiovascular systems or intestine [1]. Among them, peptides working as neurotransmitters, neuromodulators or hormones (receptor-mediated signal transduction) are the best known.

The main source of peptides in living organisms is food. Peptides can occur naturally in foods as independent entities (*e.g.* garlic [2] or mushrooms [3, 4]) or can be as part of proteins [5, 6]. Moreover, peptides can also be released from proteins during food processing (*e.g.* yoghurt [7-9], cheese [10-12], or soybean products like douchi [13], natto and tempeh [14] *etc.*). Food processing can be performed by the action of proteolytic enzymes (with various low specificity enzymes: alcalase, thermolysin, papain, *etc.* [15]), microorganism fermentation (mainly *Lactic acid bacteria*) or autolysis. Although it has been proven that fermentation, in general, produces peptides with higher activity, the procedure is expensive, laborious, and requires special conditions. Animal origin proteases, which are the by-product of meat industry (*e.g.* pancreases) or are isolated from microorganism proteases (alcalase from *Bacillus Licheniformis* or thermolysin from *Bacillus thermo-proteolyticus rokko*), are relatively cheap sources of enzymes [16]. Moreover, the fact that proteolytic enzymes are already ingredients of foodstuffs is also exploited to obtain protein hydrolyzates. The main advantages of autolysis are simplicity of process and low cost [17]. On the other hand, gastrointestinal digestion can also release peptides and simulated gastrointestinal digestion is employed in bioavailability studies to evaluate peptide resistance to gastrointestinal digestion. The most commonly used method was developed by Garrett [18] and it involved the application of sequential digestion with pepsin and pancreatin enzymes. Additionally, the use of other enzyme mixtures containing pepsin, trypsin, chymotrypsin, pancreatin or corolase P ® has also been reported [5, 19]. In addition to simulated gastrointestinal digestion, bioavailability also involves a digestion with brush border peptidases, absorption through the intestinal barrier, as well as digestion with blood enzymes, once peptides reached the circulation

[20]. Enzymatic release becomes a very complex and unspecific mechanism in which several enzymes, from food processing or the digestive system, with various activities are involved [21].

Depending on the amino acid sequence, peptides ingested or released from proteins in organism may affect the major body systems. These effects may be attributed to the numerous activities possessing food peptides, commonly named "bioactive peptides" (see *section 4.2.)*. In general, bioactive peptides contain 2-20 amino acids, but in some cases this range can be extended [22].

Food products can also be supplemented with peptides coming from other sources in order to obtain foodstuffs with certain properties. This type of foods is commonly called "functional food". Food products enriched with peptides are already commercially available (see Table 1.) [5, 20, 23-25].

Table 1. Commercial food products supplemented with peptides with desired properties. Adapted from: [5, 20, 23-25]**.**

Commercial product name	Product type	Peptide sequence	Health/ function claims	Company
Ameal S	Fermented milk	VPPIPP	Blood pressure reduction	Calpis Co, Japan
BioPURE-GMP	Whey protein isolate	κ-casein *f*(106-109) (Glycomacropeptide)	Anticariogenic, antimicrobial, antithrombotic	Davisco, USA
Biozate	Whey protein hydrolysate	β-lactoglobulin fragments	Blood pressure reduction	Davisco, USA
Calpis	Fermented milk	VPP, IPP	Blood pressure reduction	Calpis Co, Japan
Capolac	Ingredient	Caseinophosphopeptide	Helps mineral absorption	Arla Foods Ingredients, Sweden
Casine DP	Casein protein hydrolysate	FFVAPFEVFGK	Blood pressure reduction	Kanebo Ltd., Japan
CE90CPP	Ingredient	Caseinophosphopeptide (20%)	Helps mineral absorption	DMV International, The Netherlands
CholesteBlock	Drink powder	Soybean peptides bound to phospholipids	Hypocholesterolemic	Kyowa Hakko, Japan

Cystein peptide	Ingredient/ hydrolysate	Milk derived peptides	Helps to raise energy level and sleep	DMV International, The Netherlands
C12 peptide	Casein protein hydrolysate	FFVAPFEVFGK	Blood pressure reduction	DMV International, The Netherlands
Danaten	Fermented milk	Not described	Blood pressure reduction	Danone, France
Evolus	Calcium enriched fermented milk	VPP, IPP	Blood pressure reduction	Valio Ltd., Finland
Festivo	Fermented low fat hard cheese	α_{s1}-casein *f*(1-9), α_{s1}-casein *f*(1-7), α_{s1}-casein *f*(1-6),	No health claim as yet	MTT Agrifood Research, Finland
Glutamin peptide	Dry milk protein hydrolysate	Glutamin- rich peptide	Immunomodulatory	DMV International, The Netherlands
Kotsu Kotsu calcium	Soft drink	Caseinophosphopeptide	Helps mineral absorption	Asahi, Japan
Lowpept	Casein hydrolysate	RYLGY, AYFYPEL	Blood pressure reduction	InnavesBiorech SA, Spain
PeptoPro	Ingredient/ hydrolysate	Casein derived peptides	Improves athletic performance and muscle recovery	DSM Food Specialities, The Netherlands
Peptide Soup	Soup	Bonito derived peptides	Blood pressure reduction	NIPPON, Japan
PRODIET F200/Lactium	Milk drink, confectionery, capsules	YLGYLEQLLR	Reduction of stress effects	Ingredia, France
Tekkotsu Inryou	Soft drink	Caseinophosphopeptide	Helps mineral absorption	Suntory, Japan
Tensiocontrol	Egg protein hydrolysate	RADHPFL, YAEERYPIL, IVF	Blood pressure reduction	Bioactor, The Netherlands
Vasotensin	Bonito protein hydrolysate	LKPNM, LKP	Blood pressure reduction	Metagenics, Australia
Vivinal Alpha	Ingredient/ hydrolysate	Whey derived peptides	Helps relaxation and sleep	Borculo Domo Ingredients (BDI), The Netherlands

Nevertheless, there is not a consensus in the definition of health claims in functional foods among different countries [26]. EU legislation does not yet recognize functional foods as a distinct category of foods, and European Food Safety Authority has the role to decide which nutritional claims are valid based on the scientific evidence. Unlike the EU, Japan licensed in 1991 the legal system in relation to allowable health claims on functional foods (FOSHU, Foods for Specified Health Use) [27]. According to Japanese legislation, before FOSHU is granted, health claims must be substantiated through scientific evidences [28]. Regarding USA, although the Institute of Medicine of US National Academy of Sciences describes functional foods, there is no legal position which defines functional or health enhancing foods [26]. Additionally, peptides released from foodstuffs with characteristic masses and sequences (unique peptides) are frequently employed in food analysis for the unequivocal identification of a protein source exhibiting a certain activity [29]. In fact, 'peptide centric' approach is one of the most powerful aspects in proteomics studies [30]. In this case, peptides serve as a precise molecular indication (biomarker) of the presence of specific proteins in foodstuffs (see *section 4.1.)*. These peptides can unambiguously be inferred back to one parent protein sequence accounting for its key biological activity. In order to obtain a unique peptide (proteotypic peptide), *in vitro* enzymatic digestion with a highly specific enzyme (commonly trypsin) must be performed. Thus, generated peptides commonly contain 7-25 amino acids.

As well as the biological activity, food peptides are also important for their influence on functional food properties and product taste. Functional properties of peptides include solubilization, foaming, emulsifying, and gelling. Peptide length can change solubility and gelling properties. Overall, peptides act like surfactants contributing to the formation of droplets by lowering the interfacial tension and by preventing recoalescence [31]. Emulsion forming behavior and stability of protein hydrolyzates are often related to the degree of hydrolysis and the apparent molecular weight distribution [32]. An extensive protein hydrolysis results in an increase in turbidity and viscosity finally leading to the formation of a gel [33]. However, as proved by Ferreira *et al.* [34], smallest peptides are unable to form gels and, thus, gelation time and gel strength depend on the degree of hydrolysis. Since gels confer structure, texture, and stability to food products and allow the retention of water and other small molecules, gelation capacity of peptides is highly appreciated by food manufacturers. Despite bitter-tasting peptides are described most extensively, peptides may also reveal other tastes like sweet, sour, salty, or umami [35].

Bitter taste comes from small peptides (< 6 kDa) containing hydrophobic amino acids (L, P, F, Y, I, and W). Internally sited hydrophobic amino acids exhibit higher bitterness than terminal ones [36]. Debittering of protein hydrolyzates can be performed by the adsorption of bitter peptides on activated carbon, by their chromatographic removal using different matrices, or by their selective extraction with alcohols. Bitter taste can also be masked by the addition of polyphosphates, amino acids (D and E), α-cyclodextrins, hydrolysates with intact proteins, or by transpeptidation reaction [36]. Other methods include further hydrolysis of bitter peptides with enzymes such as aminopeptidase, alkaline/neutral protease, and carboxypeptidase or the use of *Lactobacillus* as a starter adjunct [37]. Encapsulation also enables to mask bitter taste of peptides as well as reduce the metabolic activity of some bacteria or improve peptide stability. In the case of bioactive peptides, masking methods involve the use of monosodium glutamate or glutamylglutamic acid, encapsulation, addition of cyclodextrins, phospholipids, and lysophospholipids [38].

Despite food processing may improve its safety and may preserve food, it can also have harmful influence on peptides. Indeed, evaluation of dehydratation, thermal heating, and fermentation on peptides are important issues. Dehydratation preserves food, decreases the action of microorganisms, and facilitates its storage and transport. However, food dehydration can also change peptide composition, reduce amino acid content, and produce non-enzymatic browning [38]. On the other hand, thermal processing favors racemization, decomposition of residues (*e.g.* R converts to ornithine), glycation (browning, Maillard reaction), and cross-linking [38]. It is important to highlight that peptides can be altered or degraded without intervention of any reactive species by temperature (*e.g.* degradation of D [39]), pH (*e.g.* loss of ammonia from N and Q [40]), moisture (high water content results in an enhanced mobility and chemical reactivity [41]), or high pressures [42]. Peptide susceptibility to degradation or modification increases with molecular mass. Peptide modifications include intramolecular cyclization (N at N-terminus [43]), backbone modifications (intramolecular cyclization of C, S, T or β-aminoalanine [35]), side chain modifications (formation of dehydro amino acids from S, C, T), cross-linking (mainly from the dehydro amino acids or formation of disulfide bridge), or peptide breakdown (formation of carcinogenic acrylamide [44, 45]). Additionally, peptides can react with food components such as carbohydrates, lipids or their degradation products or with minor compounds like vitamins, additives, carbonyl

compounds from other reactions, or even inorganic additives. These interactions must be taken into consideration for novel functional foods formulation since they can affect peptide functionality. Some strategies for the enhancement of peptide stability and bioavailability such as lipidization, glycosilation, cationization, and microencaplsulation are extensively discussed by Witt *et al.* [46].

2. Sample preparation

Sample preparation is the first critical step affecting the outcome of the entire peptide analysis. Isolation of peptides from food is a difficult task since foods constitute complex and heterogeneous matrices. It is complicated to analyze food peptides with good accuracy without a suitable sample preparation combining, in many cases, fractionation, purification, and pre-concentration steps [47].

2.1. Peptide standard solubilization

Appropriate selection of peptide media is essential since improper peptide solubilization can lead to its loss or even experiment failure. Peptide solubility is sequence dependent. In fact, in order to facilitate peptide solubility more than 20% residues should be charged. Short peptides (till 5 residues) or hydrophilic peptides containing > 25% charged residues (E, D, K, R) and < 25% hydrophobic residues are water soluble. Hydrophobic peptides containing 50-75% hydrophobic residues may be just partly soluble in water solutions. In this case, the addition of an organic solvent like acetonitrile (ACN), alcohols, acetic acid (AA), or dimethyl sulfoxide (DMSO) (if there is no C, W or M; in case of C, dimethylformamide (DMF) is recommended instead of DMSO) might be necessary. For peptides that tend to aggregate, the addition of guanidine hydrochloride or urea is recommended. Very hydrophobic peptides (>75% hydrophobic residues) require the addition of strong solvents (trifluoroacetic acid (TFA), formic acid (FA)) or high concentration of organic solvents or denaturant reagents [29]. Peptides are more stable at solid state than in aqueous solutions. The stability of peptides is influenced by solvent, concentration, pH, and temperature [35]. Instability of peptides in solution can be caused by adsorption onto vial walls, inactivation, racemization, oxidation (C or M), deamination (N or Q), chain cleavage, diketopeperazine formation or rearrangements [29].

2.2. Peptide release from foodstuffs

Extraction of peptides from foodstuffs depends on how the peptide is present. Peptides present as individual entities are usually extracted with different solvents. Hydrophilic peptides are usually extracted using water or short-chain alcohols (*e.g.* ethanol or methanol). The use of alcohols also enables the precipitation of proteins, and thus, their removal. In most cases, protein removal (deproteinization) is a key clean-up step [48] which can include precipitation (with acetone, TFA, TCA, ethanol, methanol, ammonium sulfate or salting out), centrifugation, filtration (also ultrafiltration) or simply boiling [47, 49]. Extraction of peptides encrypted in high structural protein/s to obtain biomarker peptides requires a previous hydrolysis with enzyme(s). Chemical hydrolysis, involving the use of acids or alkalis, is simple and less expensive than any available approach. However, it is not commonly applied due to the difficulties to control it, showing low selectivity and specificity and giving rise to the damage of some amino acids [48]. For these reasons, enzymes of high purity and specificity are usually applied, being trypsin the most common one [50] (see Table 2.). Generated peptides share similar properties, having lengths between 7-25 amino acids (molecular masses between 700 and 2500 Da), which reduces the analytical range to be covered, being suitable for mass spectrometry (MS) analysis. Different strategies can be used for trypsin enzymatic protein digestion such as in-gel digestion [51] (proteins are previously separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)), in-solution digestion (proteins are dissolved in an aqueous solution) [52] or filter aided sample preparation (FASP) digestion. In-gel trypsin digestion of proteins is a standard procedure in the field of proteomics. Briefly, excised pieces of gels (from 1D or 2D gels) are destained, digested with trypsin, and obtained peptides are extracted. Although a large number of protocols have been published for in-gel digestion, they only differ on their sensitivity and throughput [53]. In-solution digestion is the easiest and the most commonly applied approach. However, typical digestion protocols usually take long time. In order to speed up and simplify digestion, different strategies have been reported: heat, microwaves, high pressure, and infrared and ultrasonic energy [54]. FASP digestion, recently introduced by Wiśniewski *et al.* [55], is still not commonly employed in peptide food analysis. It consists of digesting proteins previously retained on a membrane filter and recovering of peptides by centrifugation. FASP digestion allows the use of more aggressive conditions which ensures better protein solubilization.

Table 2. Recent works using biomarker peptides for food composition analysis, product authentication, and food allergens detection.

Foodstuffs	Brief Description	Peptide Isolation	Peptide Analysis	References
Food composition analysis				
Milk	Determination of peptide profile of milk and its changes during thermal treatment and storage	IMAC	MALDI-ToF	[56]
Gouda cheese	Identification of key kukumi peptides enhancing mouthfulness in mature Gouda cheese	SEC	LC-MS/MS	[57]
Ovine milk cheese	Study of the role of hydrophobic peptides size in bitterness	SEC	RP-LC-UV	[58]
Soybean	Isolation and identification of bitter peptides in tryptic hydrolysates of soybean 11S glycinin	2-LC: SEC RP-LC	Edman degradation	[59]
Puffer fish	Isolation and identification of flavor peptides from Puffer fish muscle	UF SEC RP-LC	Electronic tongue MALDI-ToF/ToF	[60]
Dry-cured ham	Prediction of curing time using peptide profiles	Precipitation with acetone	CE-UV	[61]
Dry-cured ham	Identification of small peptides generated during curing	SEC RP-LC cIEC	Edman degradation	[62]
Serrano dry-cured ham	Identification of naturally generated small peptides from myofibrillar proteins during curing	2-LC: RP-LC RP-LC	MALDI-ToF-MS nLC-nESI-QToF-MS MS/MS	[63]
Dry-cured ham	Identification of peptides released from muscle glycolytic enzymes during curing	SEC RP-LC	nLC-nESI-QToF-MS/MS	[64]
Dry-cured ham	Identification of small myoglobin peptides generated during curing	RP-LC	nLC-QToF-MS/MS	[65]
Product authentication				
Mussel species	Identification of species-specific peptides for the characterization of European marine mussels	2-DE Trypsin digestion	MALDI-ToF-MS nESI-MS/MS μLC-MS/MS	[66]
Merlucciidae species	Characterization of species-specific peptides for the correct classification of commercial fish species belonging to the *Merlucciidae* family	2-DE Trypsin digestion	MALDI-ToF-MS LC-MS/MS nESI-MS/MS	[67]
Shrimp species	Characterization of species-specific peptides for the identification of commercially *Decapoda* shrimp species	2-DE Trypsin digestion	MALDI-ToF nESI-MS/MS	[68]

Table 2. Continuation.

Foodstuffs	Brief Description	Peptide Isolation	Peptide Analysis	References
Shrimp species	Characterization of species-specific peptides obtained by ultrasonic-assisted digestion for the classification of seven commercial species of *Decapoda* shrimps	HIFU	LC-MS/MS	[69]
Merlucciidae species	Characterization of species-specific peptides obtained by ultrasonic-assisted digestion for the classification of commercial species belonging to the *Merlucciidae* family	Heating at 70ºC HIFU	LC-MS/MS	[70]
Meat and fish samples	Determination of marker peptides for screening food products for the addition of bovine blood-based binding agents	Acid precipitation SPE	MALDI-ToF-MS LC-MS/MS	[71]
Chicken and meat mix	Detection of adulterations due to the addition of chicken in meat mixtures by the detection of chicken-specific peptides	OFFGEL SDS-PAGE in-gel or in-solution Trypsin digestion	MALDI-ToF-MS LC-MS/MS	[72]
Gelatin	Detection of marker peptides to differentiate between bovine and porcine gelatin	SEC Trypsin digestion	MALDI-ToF LC-MS/MS	[73]
	Food allergens detection			
Milk in food	Identification of marker casein peptides for the detection of milk allergens in food samples	IEC UF SDS-PAGE Trypsin digestion	ELISA µLC-MS/MS	[74]
Milk in food	Determination of marker peptides for the detection of ruminant milk allergens in dairy and chocolate samples	Trypsin digestion	LC-MS/MS	[75]
Milk in food	Identification of marker peptides that resist protein glycation for the detection of cow's milk allergens in food samples	SDS-PAGE Trypsin digestion	MALDI-ToF-MS MS/MS	[76]
Peanut	Identification of marker peptides for raw and roasted peanut allergens	Trypsin digestion	µLC-MS/MS nESI-MS/MS	[77]
Peanut in chocolate	Detection of peptide markers for the presence of peanut allergen Ara h 1 in foods	Trypsin digestion SPE	LC-MS/MS	[78]
Peanut in foods	Detection of peptide markers for the presence of peanut allergens Ara h 2 and Ara h 3/4 in foods	UF LLE SPE Trypsin digestion	LC-MS MS/MS	[79]

Table 2. Continuation.

Foodstuffs	Brief Description	Peptide Isolation	Peptide Analysis	References
Peanut in cereal-chocolate-based snacks	Determination of peanut allergens Ara h 2 and Ara h 3/4 in cereal-chocolate-based snacks	UF Trypsin digestion	ELISA-ICP-MS LC-ESI-MS/MS	[80]
Peanut in foods	Determination of peanut allergen Ara h 3/4 in breakfast cereals	Immunomagnetic beads extraction Microwave-assisted Trypsin digestion	LC-MS/MS	[81]
Peanut in baked cookies	Selection of marker peptides for the detection of peanut allergens in baked cookies	ProteoMiner protein enrichment SDS-PAGE Trypsin digestion	nUPLC-MS/MS	[82]
Hazelnut	Identification and selection of marker peptides for the detection of hazelnut and occurrence in other nuts	SDS-PAGE Western blot Trypsin digestion	LC-MS/MS	[83]
Hazelnut	Identification of stable marker peptides of hazelnut	Trypsin digestion	MALDI-ToF-MS MS/MS	[84]
Nuts in foods	Identification of marker peptides for the detection of cashew-nut, hazelnut, almond, peanut, and walnut allergens in breakfast foodstuffs	Trypsin digestion	LC-MS/MS MS^3	[85]
Fish	Detection of parvalbumin peptide biomarkers in fish food products	Heating at 70ºC HIFU	LC-MS/MS	[86]

2.3. Purification of peptides

The objective of purification is the separation of target molecules from others. Purification is essential when studying peptide structure or properties. Peptides can be purified by the application of several approaches based on different types of chromatography, electrophoresis, and membrane separation techniques. Purification and fractionation of peptides can be performed based on their hydrophobic/hydrophilic properties (reversed phase (RP) or normal phase (NP) liquid chromatography (LC), hydrophobic interaction liquid chromatography (HILIC)), charge properties (ion exchange chromatography (IEC), isoelectrofocusing electrophoresis), molecular size (size exclusion chromatography (SEC), ultrafiltration (UF)), or affinity properties (affinity chromatography (AC)) [48]. Purification of peptides from complex matrices normally requires several purification steps using orthogonal separation techniques (multidimensional purification).

2.3.1. Membrane separation

Ultrafiltration (UF) is a common membrane separation technique which can be used for fractionation or for the removal of interfering macromolecules [47]. Additionally, it enables concentration and enrichment of peptides. Peptide solution is applied on a semipermeable membrane (mostly polysulfone or cellulose) working as molecular sieve and fractionating peptides according to molecular size [49]. Cellulose membranes are less resistant but have reduced fouling while polysulfone membranes are more rigid but are more susceptible to fouling [47]. UF is a pressure driven separation technique although centrifugation can also be applied instead. Poor selectivity of UF can be a big disadvantage. Wide range of molecular weight cut off (Mwco) membranes is commercially available (500 Da-100 kDa). Frequently, sequential fractionation of bioactive peptides on small Mwco membranes is performed (1-10 kDa). UF membranes can also be used as a surface for the tryptic digestion of proteins (FASP, see *section 2.2.*).

2.3.2. Liquid chromatography

Chromatographic modes mostly employed for the separation of peptides are size exclusion, ion exchange, normal phase, and reversed-phase [29]. Complex mixtures can be resolved by multi-dimensional approaches where two or more chromatographic modes are combined.

Size-Exclusion Chromatography (SEC). Since SEC separates molecules over a wide mass range (0.1–100 kDa) and it is a low resolution technique, it is just useful when peptide raw material requires a first purification from main interferences (such as protein or salts) [49]. Moreover, SEC is also used as first dimension in multidimensional separation strategies. SEC is commonly performed on dextran (Sephadex) resins, agarose/dextran (Superdex), polyacrylamide (BioGel P), and divinylbenzene polymers [49]. The elution on SEC depends on resin composition and can be carried out using water, organic acids, salts or alcohols [47].

Ion-Exchange Chromatography (IEC). Porous and nonporous matrices with hydrophilic materials like cellulose, cross-linked dextrans, polystyrene polymers or Bio-Rex membranes are the most used for peptide separation [47]. Cation exchange (cIEC) matrices are attached to negatively charged groups (sulfopropyl, methyl sulfonate or carboxymethyl) while anion exchange (AEC) matrices have positively charged groups (quaternary ammonium, quaternary aminoethyl or diethylaminoethyl). Peptides are mainly eluted from IEC columns by increasing mobile phase ion strength while maintaining pH (to break up the ionic interaction) being less common the elution by pH change [49]. Although both IEC modes can be applied to peptide separation, strong cation exchange chromatography (cIEC) is more common. IEC resists harsh cleaning conditions and allows the purification of peptides with low cost and relatively high resolution.

Affinity Chromatography (AC) and Immobilized Metal Ion Affinity Chromatography (IMAC). The few works devoted to the separation of peptides from foodstuffs via AC mostly employed Immobilized Metal Ion Affinity Chromatography (IMAC). Separation is based on the interaction of metals like Cu^{2+} [56, 87], Fe^{3+} [88, 89], Zn^{2+} [90] or Ti^{4+} [91] immobilized on a solid support with peptides in solution. The separation depends on the coordination between chelating metal ions and electron donor groups of peptides [92]. Although some commercial IMAC supports (beds, spin column) are available, immobilization of metals on solid supports is frequently performed in-house. Typically, metal ions are immobilized on chelating ligands like iminodiacetic acid (IDA) which is attached to a sepharose [89, 93] or a chitosan [90] matrix. As an alternative, Shen *et al.* presented the efficient IMAC-Ti^{4+} chelated on phosphorylated cellulose [91]. Metal-chelated peptides usually contain H, S, C, E, and D residues. Metal ions prefer binding carboxyl groups (E, D) or oxygen (phosphate group) or nitrogen (H) rich groups [87].

IMAC-Cu^{2+} binds peptides via carboxylic groups, phosphate groups, and amino acid side chains with electron donor groups (H, C, W). The wide specificity of interactions allows high recovery of peptide fractions [56]. Moreover, phosphopeptides enrichment, based on electrostatic interactions between positively charged metal ions and negatively charged phosphate groups of peptides, is usually carried out with metal ions like Fe^{3+}, Ga^{3+}, Zr^{4+}, and Ti^{4+}. Although this method is relatively specific, acidic peptides can also bind [91]. In fact, IMAC-Fe^{3+} proved to also attach peptides with H being necessary a previous separation by cIEC [93]. Liu *et al.* compared metal ion chelating capacity, adsorption, and separation efficiency of peptides in IMAC with different metal ions (Fe^{3+}, Cu^{2+}, Zn^{2+}, Ca^{2+}) immobilized on an IDA-Sepharose matrix [94]. They observed that binding ability of soybean protein peptide on the column was Fe^{3+}> Cu^{2+}> Zn^{2+}> Ca^{2+}, which was associated to different adsorption behaviors of metal ions. IMAC enables removal of majority of low molecular weight interferences, which led to better signal-to-noise ratio. High throughput, reproducible, easy handling, and short analysis times are additional advantages [56].

Reversed-Phase Chromatography (RP). There is a wide selection of chromatographic materials to separate peptides by RP-LC [95]. Columns packed with silica-based reversed-phase particles are the most widely used material for the purification of peptides. The C_{18} bound phase is the most popular for separation of peptides. When the alkyl chain is large, smaller or more hydrophilic peptides are recovered in high yield but larger or more hydrophobic peptides are lost. When alkyl chain is shorter the situation is opposite. Mobile phase in RP-LC usually is a mixture of water with ACN due to its high volatility, low viscosity, and relatively transparency to UV detection. Alcohols such as methanol, ethanol or isopropanol can also be employed instead of ACN. Elution of peptides in RP-LC is normally performed in the gradient mode. The addition of an ion-pairing reagent aids to maintain low pH, create complexes with positively charged peptides, and minimize their ionic interactions with stationary phase. TFA, AA or FA are the most common ion-pairing reagents (see more in *section 3.1.1*).

Hydrophilic interaction chromatography (HILIC). It is a powerful tool for the separation of polar compounds. The separation of analytes on HILIC is based on the interaction with a hydrophilic stationary phase like in normal phase (NP) chromatography. However, NP is performed just with non-aqueous, non-water-miscible

solvent buffers, whereas HILIC is performed with water-miscible solvents (*e.g.* ACN or methanol) and elution is achieved by a water gradient [96]. Columns used for NP can also be successfully applied in HILIC conditions [97]. HILIC stationary phases can be divided into three different groups: neutral (*e.g.* diol and amide phases, without electrostatic interactions with the analyte), charged (*e.g.* plain silica and aminopropyl phases which have strong electrostatic interactions with the analyte), and zwitterionic phases (*e.g.* sulfobetaine silica phases with weak electrostatic interactions with the analyte) [98]. The most commonly used HILIC columns for the separation of peptides from food matrices are TSK-gel Amide-80 (neutral) [99, 100], Atlantis HILIC Silica (charged) [101, 102], and, in some cases, ZIC-HILIC (zwitterionic) [103]. For HILIC, the best and most common organic solvent is ACN. Methanol yields wider peaks in approximately the same retention time while tetrahydrofuran may change the elution order. Aqueous solutions typically contain salts of ammonium, formate or acetate. The type of organic solvent and the type, concentration, and pH of buffer can affect the selectivity and retention time in HILIC [98]. A detailed review about HILIC [104] and an excellent review dealing with the application of HILIC to food matrices have recently been published [98] (see more in *section 3.1.1*).

Solid-phase extraction (SPE). SPE is primary used to retain peptides enabling the removal of sample matrix interferences and/or peptide concentration. Basically, all types of SPE sorbents are commercially available (C_2-C_{18}, phenyl, cyanopropyl, IEC *etc.*) [47]. The separation principles and the mode of use depend on the selected sorbents. Micro-device counterparts designed for small sample amounts like spin-columns and zip-tips are gaining popularity.

2.3.3. Electrophoretic isoelectrofocusing

Peptides can also be fractionated by electrophoretic isoelectrofocusing (IEF). Commercially available 'OFFGEL' fractionator systems are excellent devices for that purpose. Separation of peptide mixtures is performed on strips containing immobilized carrier-ampholytes required to establish a pH gradient. The application of an electric field enables the separation of peptides according to their isoelectric point (pI). 'OFFGEL' have proved to be an efficient and reproducible separation technique. Its micropreparative scale provides fraction volumes large enough to perform subsequent analyses [105].

Although, this relatively new technique has shown to be a complementary tool to obtain peptide fractions, its application in the area of food peptides is still scarce.

3. Analytical methods

3.1. Separation of peptides

Chromatographic and electrophoretic techniques are usually employed for the separation of peptides [29] due to their high-resolving power and compatibility with MS detection.

3.1.1. Liquid chromatography

RP-LC is the most common chromatographic mode used for the separation of peptides [95]. The choice of the packing material has the greatest impact on the separation and resolution of peptides. A significant progress has been the replacement of conventional silica-based columns by new stationary phases enabling higher resolutions and reduced analysis times. Monolithic columns consist of a single, rigid or semi-rigid, porous rod that can be organic-based (polymeric) or silica-based [29]. Monolithic supports contain two kinds of pores that are interconnected enhancing permeability and mass transfer of molecules. Consequently, overall performance and efficiency are improved and analysis times are significantly reduced. Another strategy enabling to improve efficiency and to increase linear velocity and mass transfer are columns filled with sub-2 μm particles. However, in order to withstand resulting high back pressures, these columns require the use of special instrumentation (ultra-performance liquid chromatography systems (UPLC)). Fused-core particle technology has been introduced as an alternative to obtain high separation efficiencies with low backpressure [106]. In the last decades, conventional flow columns have been replaced by micro and nanocolumns [107]. The reduction in column's internal diameter permits decreasing the flow-rate, which results in a higher sensitivity. Additional advantages are a lower sample volume required for the analysis, a reduced consumption of solvents, and a lower waste.

Highly polar di- and tri-peptides are often poorly or not separated due to their weak retention on RP supports. HILIC constitutes an increasingly used alternative for the separation of these peptides [108]. The recent increase in popularity of HILIC could be due to the widespread use of LC-MS. The use of partly aqueous eluents with high ratios

in ACN ideally matches ESI ionization [108]. Moreover, HILIC provides reduced back pressure and, as a consequence, the separation can be performed with smaller particles and higher flow rates [97].

The separation of a large number of peptides from very complex matrices is not frequently possible using only one chromatographic or electrophoretic dimension. In these cases, a multidimensional approach is often needed. The number and kind of separation steps depends on the complexity of sample, the dynamic range of peptides, and the aim of the study [109]. Probably, the widest combination used for two-dimensional separation of peptide mixtures is IEC (usually cIEC) in the first dimension and RP-LC in the second dimension. One of the reasons of this combination is that IEC uses high concentration of salts, being necessary the introduction of a middle RP-LC separation for its connection to MS. Another approach more recently introduced for two-dimension chromatography is HILIC-RP [100, 102, 110, 111].

3.1.2. Electrophoresis

Capillary electrophoresis (CE). The use of CE-MS in food analysis provides important advantages due to the combination of the great separation capabilities of CE and the power of MS as an identification and confirmation technique [112]. Among CE modes, capillary zone electrophoresis (CZE) is the most widely used. The separation is usually performed in a fused silica capillary (inner diameter 25-100 μm, and length 30-100 cm) filled with background electrolyte at a selected pH. Analytes are separated into the capillary due to their electrophoretic mobility which depends on peptide charge and size. CE is used in the separation of food peptides but in much less extent than HPLC. The collection of separated fractions from CE is difficult to achieve and not efficient. Additionally, adsorption of peptides to the inner surface of fused silica capillaries causes reduction in separation efficiency and peptide recovery [113]. There are three main strategies to avoid peptide adsorption: separation at pHs < 2.0 (at which silanol groups are protonated), the use of high ionic strength background electrolyte or the use of coated capillaries. Dynamic (reversible) coating by small ions or polymers (cellulose derivates, synthetic polysaccharides) or static (permanent) coating by the binding or adsorption of polymers (polyacrylamide, PVA, PEG, cellulose) are commonly applied [113].

3.2. Detection and identification of peptides

Sensitive and selective detection of peptides, particularly small peptides, has been cumbersome due to the lack of suitable chromophores, fluorophores or electrophores. Peptides were commonly detected using UV/Vis spectroscopy. Absorption at λ 185-220 nm results from peptide bonds while absorption at 254 and 280 nm is due to W, F or Y. In some cases, peptide derivatization has been required especially when using (laser-induced) fluorescence (LIF) detection [113].

3.2.1. Detection and identification of peptides by MS

MS offers the ability to detect, identify, and quantify peptides with a wide dynamic range. Early ionization techniques were not amenable for the analysis of proteins and peptides [114]. MS application to the analysis of peptides was possible by the development of 'soft' ionization techniques (Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption/Ionization (MALDI)) [115]. Soft ionization allows the transfer of polypeptide ions into the gas phase without their in-source fragmentation.

MALDI source results mainly in singly charged ions and it is considered as a robust method of ionization in presence of salts and detergents, much less prone to ionization suppression effects than ESI [116]. However, since MALDI requires off-line sample deposition onto a target plate, it is less convenient to couple with HPLC and to perform quantitative analysis. In ESI, charged droplets are produced by passing a solubilized sample through a high voltage needle at atmospheric pressure. This ionization technique is often coupled with a chromatographic system, typically RP-LC or HILIC. ESI peptide ionization is typically carried out in the positive mode. Higher voltages favor lower charged peptides forms while lower voltages are better for smaller analytes. In the last decade, the introduction of nanoelectrospray (nanoESI) has enabled to increase sensitivity. Indeed, the reduction of the internal diameter of the MS capillary from 150 to 15 μm results in a decreased flow rate and a 100 fold increase in ionization efficiency. In the ESI positive mode, protonated peptides are better produced in acid mixtures of water and organic solvents, such as ACN or MeOH. On increasing the percentage of organic solvent, the surface tension decreases making easier solvent evaporation. TFA, widely used as counter-ion in peptide chromatographic separations, is avoided for acidification since it results in strong signal suppression [117]. Instead, both AA and FA are used for

acidification resulting in reduced separation efficiency in RP-LC. One of the limitations of ESI is its high sensitivity to contaminants such as salts, chaotropes, and detergents, which may form clusters and adducts with the analytes or simply spoil the spray [50]. In fact, the addition of additives, such as urea at concentrations of 1 M and non-volatile buffers in mM range, is not recommended. Detergents usually cause deleterious effects, often type and concentration dependent. They may form adducts with peptides and frequently the signal is suppressed. In most cases, detergent concentration must be maintained below 0.01%. Therefore, in order to prevent adduct formation with salts or detergents, a previous purification of sample is needed. Nowadays, solid phase extraction (SPE) cartridges, tips (zip-tips) or spin columns with different chemistries are available from several manufacturers for this purpose. The selection of the appropriate approach depends on the chemistry of peptides (*e.g.* hydrophobic peptides), the volume of sample (zip-tips, and spin columns require μL), and its cost (see *section 2.3.2*).

Four types of mass analyzers are commonly used for peptide analysis: quadrupole (Q), time of flight (ToF), ion trap (IT) (quadrupole ion trap: QIT and linear ion trap: LIT or LTQ), and Fourier transform ion cyclotron resonance (FTICR) [118]. Additionally, multi-stage and 'hybrid' instruments such as QqQ, QqLIT, QToF, ToF/ToF, and LTQ-FTICR have also been employed. New instrument referable to the LTQ-Fourier-transform technology is commercially available as LTQ-Orbitrap system [115]. Most hybrid instruments are used in research laboratories [114] all with their different pros and cons. IT is rapid, robust, sensitive, and inexpensive but it provides low accuracy and resolution [118]. QToF, on the other hand, yields high accuracy and resolution but shows relatively low duty cycles. FT-ICR and LIT-Orbitrap show ultrahigh mass accuracy, mass range, and resolution, but their cost of acquisition and maintenance is high [114]. An excellent overview of commercial MS analyzers, their updated technical specifications, parameters, and applications has been published [116].

Identification and quantification of peptides. MS is the alternative to classical Edman degradation, used for the identification of peptides. Edman degradation is based on the labeling of N-terminal amino acid residues and cleaving from the protein in a sequential manner [119]. MS can directly provide information on the mass of a particular peptide but can also generate amino acid sequence information from tandem mass spectra (MS/MS). Peptide fragmentation is achieved by various fragmentation modes,

increasingly complementary and even competitive. Collision induced dissociation (CID) is, by far, the most common fragmentation method. Higher energy collision dissociation CID (HCD), electron capture dissociation (ECD), and electron- transfer dissociation (ETD) are other options. Peptides fragment in certain ways and a peptide fragmentation nomenclature (Biemann notation) has been introduced (see Fig.1) [114, 120, 121]. The sequence can be read from the distance between peaks of peptide ion fragments. Different dissociation techniques can yield different peptide fragments. Low energy fragmentation, such as CID, breaks the weakest bonds in the peptide (peptide bonds) yielding primary *'b'* and *'y'* ions [114] (see Fig. 1). HCD provides highly similar spectra to CID [122]. Much less used but more efficient ECD and ETD fragmentations show MS spectra dominated by *'c'* and *'z'* ions [114]. Fragmentation patterns of peptides depend on the number of peptide charges. Peptides can be singly or multiply protonated. In case of singly protonated peptides, signal intensity decreases during fragmentation since it results in just one charge peptide fragment. The amino acid residues hosting this charge are R, K, and H amino acids. Thus, MS/MS fragmentation spectra of tryptic peptides with R or K at the end of the sequence (biomarker peptides) contain strong *'y'* ion series and poor *'b'* ion series, especially at high *m/z* [114]. Small peptides with little or no R, K, and H amino acids (such as bioactive peptides) result in charge spread, which will make their identification more difficult. Peptide identification is based on precise measurement of peptide precursor mass, on product ions, and on the use of established spectra interpretation rules. For example, it is well known that linear *'b'* ions are unstable and can form stable cyclic structures or can decompose to an ion losing the CO (-28 uma) residue ('*a*' ions). Loss of ammonia (-17 uma) from peptide fragments indicates the presence of R, K, Q, and N amino acids, while loss of water (-18 uma) indicates S, T, E, and D residues [123]. Some amino acid residues tend to form immonium ions which provide information about peptide composition but not about its sequence [114]. Highly similar molecular masses of I/L, and Q/K residues make their differentiation hard or impossible to obtain in low accuracy MS.

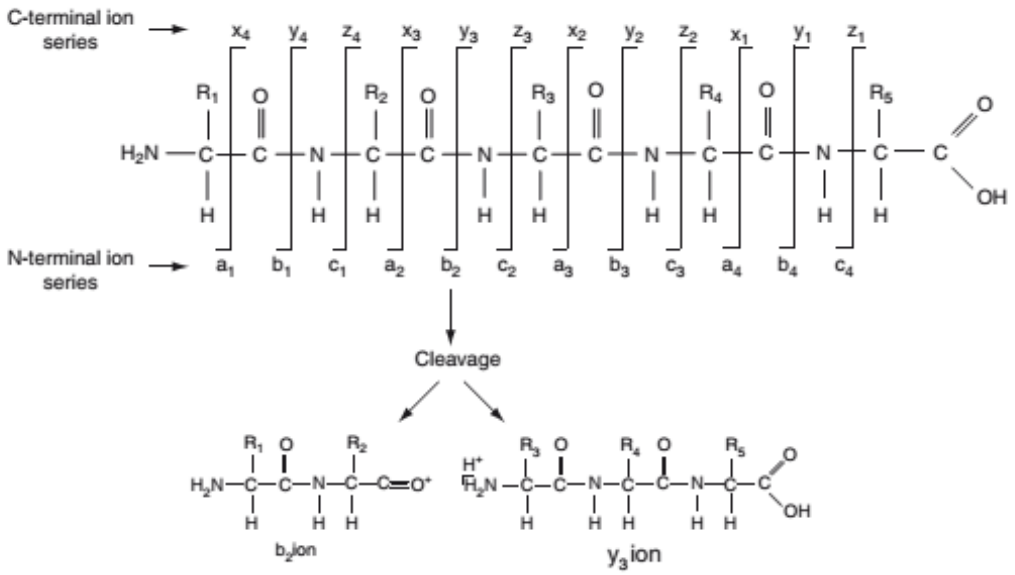


Fig. 1. Biemann notation of peptide fragmentation ions. Source: [114].

Two main approaches have been developed to identify peptide MS/MS spectra in an automated fashion: database dependent methods and database independent methods. Database dependent methods were firstly adopted while database independent methods are relatively new [114]. Database dependent methods are based on the comparison of theoretical peptide spectra from *in-silico* digestion of proteins and on the classification of the best candidates using cross-correlation or probabilistic scores [29]. This is performed just when the genome of studied peptides is known and common enzymes (like trypsin) are used. This is the case of studies with biomarker peptides. There are different algorithms that use tandem MS spectra data to search sequences against known databases: Sequest [124], Mascot [125], PeptideSearch, Sonar, ProteinProspector [120], X!Tandem, ProbID, Phenyx [114]. Although, the basis of these engines is the same, they use different scoring schemes to rank peptide matches. Most used protein databases are: Entrez Protein Database (redundant but large) of National Center for Biotechnology (NCBI), Unigene (compact but minimal), International Protein Index (deposit protein and translated cDNA sequences and predicted genes) [120], UniProt, SwissProt, and Ensembl. This approach is efficiently and widely applied for peptide identification. In order to automatically assess and validate database search results, false positive rates at a determined error rate are calculated. False discovery identification (when noise is mistaken as signal), scoring imbalance (long low quality peptides has higher score than higher quality short peptides), false identification (for post-transitional modifications or sequence polymorphisms), and

requirement of studied organism proteome database are weak points of this approach [114]. Another database dependent method includes correlation of acquired fragment ion spectra with previously obtained experimental MS/MS libraries [126]. This method ensures good performance in terms of speed and accuracy. Main disadvantage of this strategy is the non possible identification of peptides when their MS/MS spectra are never reliably identified [114]. Moreover, this strategy is not suitable in studies with bioactive peptides since they require the use of unspecific proteases and there is no much information on databases on food proteomes. In this case, database independent methods, also known as *de novo* sequencing, are more suitable. It allows identification of unknown peptides (no genome sequence information) and post-translation modifications [126]. In the *de novo* sequencing approach the amino acid sequence of a peptide is explicitly read from the fragment ion spectrum. Initially, this was accomplished manually, but recently, an array of tools has been developed [124]. In this case, the ability of software to read peptide sequence depends on MS mass accuracy, resolution, and MS/MS spectra quality. Currently, available programs are: PEAKS, Lutefisk, PepNovo, SHERENGA [114], PARSEK II [66] or DeNovoX [67]. PEAKS software, from Bioinformatics Solutions Inc., is the most popular software for *de novo* sequencing. Indeed, it has shown the best accuracy among all currently available *de novo* sequencing software packages [114]. When information about peptides' parental protein is required, extracted MS/MS spectra of peptide can be matched against already known proteins using BLAST.

Peptide quantification in foodstuffs is generally carried out by UV and MS detection. First approach is less usual since it requires that peptide was separated as pure signal from the complex sample. In case of non-pure peptides, laborious standard addition method must be applied. A MS-driven detection provides a valuable tool in bioactive peptide quantification. MS quantification approach uses commonly a label free strategy. This method is based on the relationship between peptide abundance and sampling statistics, such as peptide count and spectral count. To be statistically significant, chromatographic separation reproducibility must be very high. Methodologies using stable isotopes for differential labeling of peptides can also be used but they are not very usual in the quantification of food peptides. A common approach to quantify peptides is targeted MS. Monitoring of transitions (suitable pairs of precursor and fragment ions) in selective reaction monitoring (SRM) or multiple reaction monitoring (MRM) can be used. This setup provides high analytical reproducibility, good signal-to-noise ratio (SNR), and

increased dynamic range [70]. The optimization of an SRM assay is a time-consuming procedure and, unfortunately, its use for the quantitation of very small peptides present in complex matrices might show insufficient selectivity.

4. Recent examples

The possibility to develop standardized peptide extraction protocols together with the considerably higher resistance of peptide sequences to food processing in comparison to DNA sequences, makes the analysis of peptides an interesting and promising alternative for the analysis of food safety, for food authentication, food composition analysis, *etc.* In this case, recent advances in the field of proteomics have allowed the search of exact peptide biomarkers as potential indicators of product quality and traceability. On the other hand, bioactive peptides are gaining much attention and works devoted to the discovery of new potential bioactive peptides are being released. Despite the huge diversity of bioactivities that can exert bioactive peptides, most efforts have been aimed to antihypertensive and antioxidant peptides, especially in animal food origin foods.

4.1. Examples dealing with biomarker peptides

Food safety is a concept which encompasses different areas: food quality (food composition), traceability (food origin), and food safety *per se* (absence of allergens, pathogens or other contaminants) [127]. Two strategies have been mainly followed to solve food safety problems: the comparison of peptide profiles and the search for biomarker peptides. A peptide profile is a graphic representation of a set of peptides reflecting an exact situation, which permits to study how specific changes can affect peptides content. In some works, the study of the profile of a complex group of peptides allowed to obtain more information than the study of a target peptide. On the other hand, the identification of specific peptide biomarkers for a particular specie, tissue or ingredient by proteomics technologies is an interesting and promising strategy. This approach has shown high discriminating power, robustness, and sensitivity. For that purpose, the *bottom-up* approach is the most popular strategy. Target peptides are released, in this case, by the digestion with an enzyme such as trypsin. Next, peptides are analyzed by MS/MS for their accurate sequence analysis. Final peptide or protein identification is obtained by comparing MS/MS spectra with database using a suitable algorithm for database searching.

Food composition analysis. The first step in any food analysis or control is the knowledge of food composition. Since food proceeds from living organisms, peptide composition is affected by changes during agricultural production, industrial processing, and storing. Partial hydrolysis of proteins is quite often, desired and undesired, in the food industry. For example, hydrolytic activity of several milk proteases during milk treatment results in peptide formation [128]. Nevertheless, a high content of hydrolytic fragments from milk proteins have a negative impact on the gelation behavior or on the clotting properties in cheese production. Therefore, peptide profiles in raw milk and their changes during heat treatment have been investigated by Meltretter *et al.* [56]. In this case, milk clean-up was performed by IMAC-Cu and it was possible to observe by MALDI-ToF/ToF-MS five new peptides from the α_{S1}-casein that appeared when heating.

As mentioned in the introduction, peptides can also participate in the formation of odor and taste in some foods. Flavor peptides are a group of oligopeptides with molecular masses bellow 3 kDa that possess unique taste properties including sweet, bitter, umami, sour, and salty. Toelstede *et al.* [57] characterized peptides imparting typical mouthfulness and complex body in Gouda cheese. In order to isolate those molecules underlying the so-called kukumi sensation of the 44-week-matured Gouda cheese, SEC in combination with analytical sensory tools were applied on a water-soluble extract. HPLC-MS/MS analysis of isolated SEC fractions enabled to identify 10 γ-L-glutamyl dipeptides as responsible for the characteristic kukumi sensation of matured Gouda cheese. Bitter taste is also one of the most common quality defects of cheese. It is caused by the formation of bitter peptides from casein hydrolysis during cheese ripening. Hydrophobic peptides of cheese have shown the major contribution to this bitter flavor. Moreover, it was observed that hydrophobic peptides size also affected bitterness in ovine milk cheese [58]. Bitterness intensity of cheeses also depended on the coagulant used in their manufacture. In fact, cheeses made with microbial coagulants were perceived as the most bitter and also contained the highest concentration of bitter peptides. Also, soybean proteins have demonstrated to produce strong bitter peptides. 21 peptides from the bitter fraction of tryptic hydrolysates of soybean 11S glycinin were purified using a 2D chromatographic system consisting of sequential SEC and RP-LC separations [129]. The amino acid sequence of bitter peptides was determined using Edman degradation. When compared these peptides with those released from proglycinin, many bitter peptides were basic mimics of the common structure. This indicates the significance of the primary

structure of a peptide in the bitter taste perception. Flavor is also a very important component in meat quality. At this regard, there is interest to determine the factors influencing flavor quality during the production and processing of meat and fish. Key flavor peptides in cooked puffer fish were identified by a previous purification of peptides using UF, Sephadex G-15 SEC, and RP-LC [60]. Peptide contributing to sweet taste was isolated and identified by MALDI-ToF/ToF MS/MS as YGGTPPFV. The relation between hydrophilic amino acids residues (Y, G, G, T, and F) and umami and sweet tastes was also demonstrated. Moreover, this peptide was suggested as an important contributor to the mellowness and tenderness taste of puffer fish. The generation of peptides during curing of dry-cured products has also attracted much attention for their influence in product taste. At this regard, Sentandreu *et al.* studied peptide development during ham curing [130]. Many biochemical mechanisms such as intense proteolysis of sarcoplasmic and myofibrillar proteins by endogenous muscle enzymes take place during the curing period. It is well known that long ripening periods ensure better ham quality. Analyzing peptide extracts from hams having 6, 8, and 12 months of curing time by capillary zone electrophoresis (CZE) showed changes in protein profiles [61]. These changes enabled to develop a mathematical model using multiple lineal regression (MLR) to predict suitable ripening times. The prediction of ham curing time was possible with an error below 2.5%. The identification of peptides in dry-cured ham was also performed [62-64]. Peptide purification was possible by the combination of SEC, RP-LC, and cIEC. Isolated peptides were identified by Edman degradation [62] and different MS and MS/MS devices [63, 64]. Small peptides (especially dipeptides) were identified in the water-soluble extracts of dry-cured ham demonstrating a high proteolysis level during ham ripening [62]. In a second study, a total of 14 peptides fragments derived from myosin light chain I and titin, probably released by the action of dipeptidyl peptidases, were identified using MALDI-ToF-MS, nLC-MS, and MS/MS [63]. The intense proteolysis of the sarcoplasmic fraction of dry-cured ham was determined by nLC-MS/MS [64]. Obtained sequences suggested the contribution of both endopeptidases and exopeptidases during ripening. Finally, 11 fragments of myoglobin, a sarcoplastic protein responsible for the color of meat and meat products, were identified by nLC-MS/MS [65]. In these studies, the comparison of Paragon and Mascot search engines, together with UniProt and NCBI databases was performed. It permitted the selection of the most adequate tool in the identification of naturally generated peptides. It also demonstrated the utility of these common searching tools for the analysis of complex samples [61, 65].

Food authentication. The assessment of food authentication and origin is a major concern not only for the prevention of commercial frauds but also to avoid safety risks derived from the inadvertent introduction of any food ingredient that might be harmful for human health. It is of high importance to develop analytical methods to distinct closely related species and prevents inadvertent or deliberate mislabeling and adulteration of food products.

The identification of marine species is an issue of primary relevance for the seafood industry due to global commercial requirements concerning labeling and traceability. For that purpose, species-specific biomarkers capable to provide information about the composition of food are required. A pioneer work in the identification of species-specific peptides from marine species was performed by López *et al.* [66]. Three European marine mussel species were characterized by MALDI-ToF-MS. Peptide maps generated from 6 random selected spots of two-dimensional gel electrophoresis analysis were compared and species-specific differences corresponding to the protein tropomyosin were detected. The analysis of peptide extracts from tropomyosin by μ-HPLC-ESI-MS/MS in SIM mode demonstrated unambiguous identification of species. A similar strategy was used for the characterization of different protein nucleoside diphosphate kinase B (NDK B) from commercial hakes and grenadiers (*Merlucciidae* family) [67]. Species-specific peptides used for fish authentication were characterized by MALDI-ToF and LC-MS/MS. Shrimp species have also been identified based on the characterization of species-specific peptides from arginine kinase [68]. Since genomes from these fish species were poorly explored, *de novo* sequencing of peptides was required. It involved the manual interpretation of ion series in the spectra with the aid of the software package PARSEK II [66] or DeNovoX (Thermo-Finnigan) [67] and the BLAST program which use homology searches between the given sequences and those available in the NCBI database [68]. In a later study, the long time digestion protocol was reduced using a high-intensity focused ultrasound (HIFU)-assisted in-solution digestion to just 2 min [69, 70]. The use of this ultrasonic probe allowed the differentiation of 7 shrimp species within just 90 min [69]. Another example of the use of peptides for food authentication was the monitoring of 11 parvalbumins (PRVB) peptide biomarkers for the unequivocal identification of closely related *Merlucciidae* species in processed and precooked seafood products [70].

Another aspect in food authentication is the presence of adulterations. Adulteration is usually carried out to increase the profit of a product by its partial replacement with a cheaper counterpart. The development of meat binders to incorporate meat and fish cuts into consumer products demands accurate portion control in commercial meat and fish products. To solve this problem, a method designed to target specific peptides markers of the presence of fibrinogen has been described [71]. Fibrinopeptide A, released from blood protein fibrinogen during thrombin gelling, was isolated from food matrices by acid precipitation and SPE. Isolated fibrinopeptides were analyzed by MALDI-ToF and LC-QqQ. Fibrinopeptide A was found to be an effective marker in fresh, processed, and cooked food matrices with 5% (v/m) of bovine binding agent. A proteomics-based method was also developed for the detection of chicken meat within mixed meat preparations [72]. The first step of the procedure consisted of an enrichment of myofibrillar proteins by OFFGEL IEF. Next, myosin light chain 3 was submitted to in-solution trypsin digestion or SDS-PAGE separation and in-gel trypsin digestion. Generated peptides were analyzed both by MALDI-ToF and LC-MS/MS. The use of AQUA stable isotope peptides permitted to carry out a quantitative detection of selected species-specific peptide biomarkers. The method enabled the detection of adulterations of 0.5% of chicken meat in both raw and cooked pork meats. The use of peptide biomarkers to detect food adulterations constitute an interesting alternative to currently used immunoassay, hampered when the tertiary protein structure is affected by food processing. Marker peptides have also been used to differentiate bovine from porcine gelatins [73]. The similarity between gelatins makes difficult to trace their species. Gelatin proteins were subjected to in-gel trypsin digestion and SEC analysis. Thanks to the differences in bovine and porcine type I collagen sequences, marker peptides specific for bovine and porcine gelatins were successfully detected.

Food allergen detection. Food allergy is a significant worldwide public health issue estimated to affect up to 4% of infants and adults in developed countries [131]. Proteins termed allergens are mostly responsible for food allergenic reactions, being capable of triggering severe adverse reactions in sensitized individuals. To guarantee the security of consumers, a number of regulations in terms of food allergy have been implemented [132]. In the European Union, these regulations compel the producers to label 14 established food allergens when these have been intentionally introduced in foodstuffs. However, some products on the market could contain traces of allergens due to cross-

contaminations during food manufacturing. In order to protect consumer, reliable detection methods are needed to ensure meticulous labeling and to control allergen-free products. Immunoassay methods can be employed to detect the presence of allergens [74]. Nevertheless, these antibody-based assays usually exhibit cross-reactivities to other related species. As demonstrated by hazelnut allergens, this problem is solved thanks to the specificity of MS experiments [83]. In fact, different MS-based methods have been developed to identify set of peptides that could work as allergen markers.

Milk allergy is one of the most common food allergies in young children from birth to 1 year old. This together with severe and prevalent tree nuts (peanuts and hazelnuts) and fish allergens are the most studied. Emphasis was given to the identification of major allergens in casein [74, 75] and whey [76] fractions from milk, Ara h 1, Ara h 2, and Ara h 3/4 allergens from peanut [80, 81], and parvalbumins beta (β-PRFBs) from fish [86]. Sample treatment is especially critical when working with allergenic proteins. In fact, protein enrichment methods like ProteoMiner^{TM} (based on a large and highly diverse bead-based library of combinatorial peptide ligands) [82, 133, 134], selective immunomagnetic beads (based on the use of monoclonal antibodies) [81] or a combination of AEC with UF (Centripep filters) [74] are usually required to detect allergens at a trace level in complex food matrices. In order to obtain the best sensitivity and to improve peptide detection limits, sample treatment was usually optimized. The comparison of UF, LLE, and SPE for the isolation of peanut allergens showed the best peptide recovery when UF was applied [79]. Food processing affects the stability of proteins, which influences the detection of allergen sequence tags. Therefore, different works have been aimed to study the influence of food processing on biomarker peptides of food allergens. An example is the study of the effect of Maillard reaction on peptides from whey [76] and hazelnut [84] allergens. Also roasting can affect protein stability. Consequently, raw and roasted peanuts have been analyzed to identify peanut-specific sequence tags that could work as markers of specific allergens in processed foodstuffs [77].

Peanut allergen Ara h 1 was analyzed by its previous extraction and enzymatic digestion. Better LODs in comparison to post-extraction trypsin digestion was obtained [78]. On the other hand, chocolate is a difficult matrix since proteins can interact with chocolate tannins making more difficult protein extraction. In this case, pre-extraction

digestion greatly reduces these interactions. To speed up the protocol, a microwave-assisted [81] and a HIFU-assisted enzyme digestions [86] were implemented.

Once the hydrolyzates are obtained, the identification and selection of marker peptides is usually performed using MS-based methodologies. A fundamental role in the outcome of MS analysis in complex mixtures is a previous chromatographic separation. For the simultaneous determination of selective biomarkers from five nut allergens, two different chromatographic columns (a C18 particle-packed column and a silica-based C18 monolithic column) were evaluated [85], observing better performances, in terms of sensitivity, selectivity, and solvent consumption, with the first column. Nevertheless, the direct infusion of protein digest into the MS/MS system could be useful to identify most abundant peptides in a hydrolyzate [75]. This was successfully applied to the analysis of nut traces in commercially available breakfast cereals and biscuits [85]. Since almost all nut allergens belong to the seed storage protein family and have homologue structure, the use of BLAST algorithm has helped to select suitable peptides and transitions for the specific detection of different nuts by SRM and MRM. Indeed, SRM/MRM were applied to the detection of hazelnut allergens [83] and peanut allergens in chocolate [78], cookies [82], rice crispy, and chocolate-based snacks. Alternatively to LC-MS/MS strategy, a Eu-tagged ICP-MS immunoassay was proposed. This methodology is based on a non-competitive sandwich ELISA method with ICP-MS detection of the metal used to tag the antibody. Some advantages of this methodology are the absence of matrix interferences usually producing ion suppression in LC-MS/MS and better LOD [80].

Additionally, marker peptides corresponding to αS1-casein [74] and β-lactoglobulin [75, 76] from milk have been proposed, as shown in Fig. 2 for a chocolate sample. Also Ara h 1, Ara h 2, and Ara h 3/4 from peanut [77-82], and hazelnut allergens [83, 84] have been proposed for the detection of these specific allergens in foodstuffs. Because of the different expression levels of peptides, their simultaneous detection and the selection of different allergenic proteins will increase the confidence in the correct identification of specific allergen traces in analytical samples [79]. Finally, simultaneous analysis of different species has been proposed, as the developed for five nut allergens from cashew-nut, hazelnut, almond, peanut, and walnut in cereals and biscuits [85].

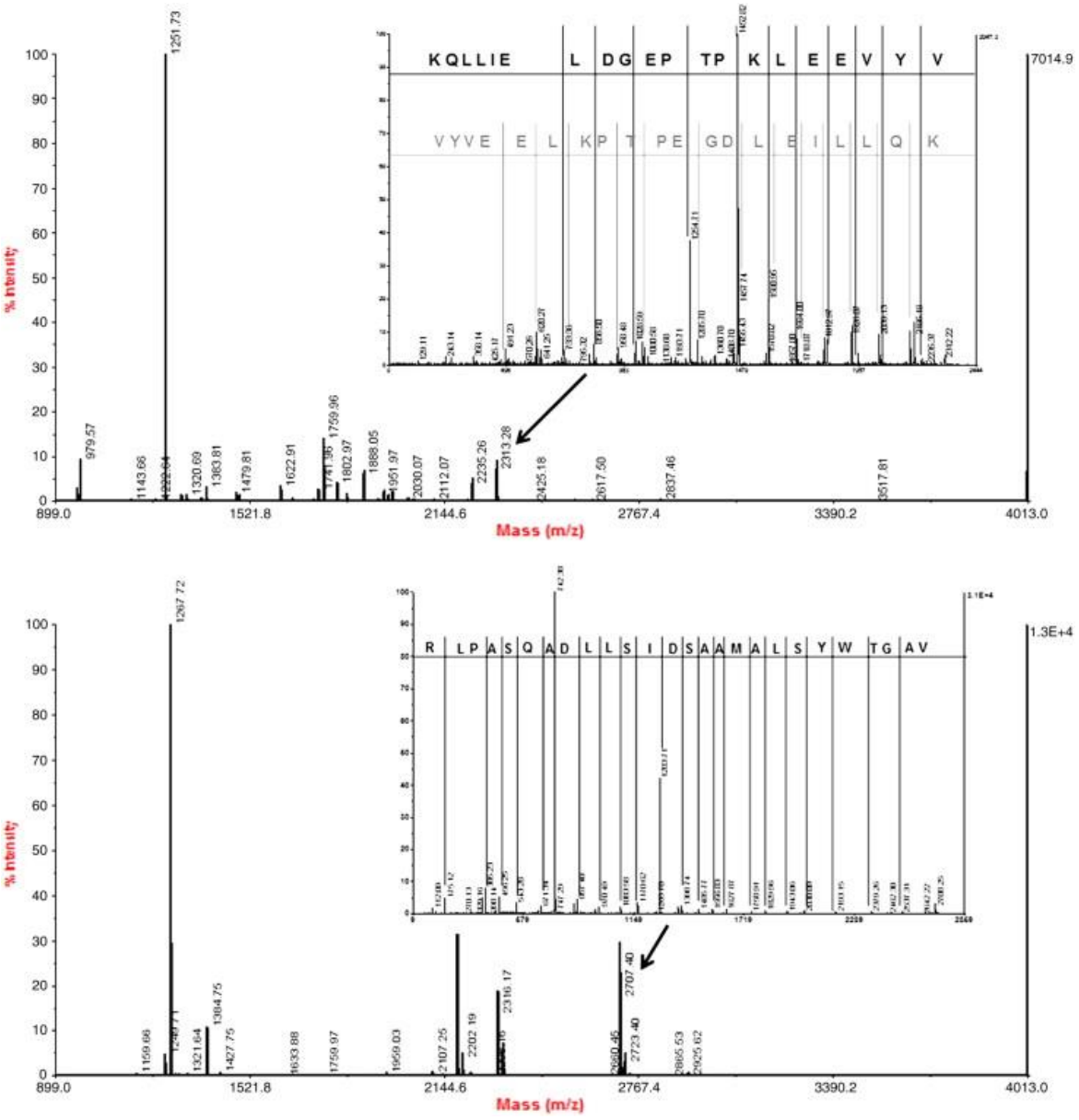


Fig. 2. MALDI-TOF/MS and MS/MS spectra of the chocolate sample. The stable peptides 41Val-Lys60 and 15Val-Arg40 are marked with arrows. Matched y- and b- ions are indicated in black and gray respectively. The amino acid sequence is indicated in the one letter code. Source: [76].

4.2. Examples dealing with bioactive peptides

Epidemiological studies are increasingly linking the prevalence of some diseases to the dietary factor [38]. Humans ingest an average of 50-70 g of food proteins per day. Proteins are hydrolyzed into a high variety of peptides, which can be adsorbed and transported by the blood stream and that can have a significant influence on main human

body systems (cardiovascular, nervous, gastrointestinal or immune) [20]. It is important to highlight that some dietary peptides are extremely potent and even microgram amounts entering to the body circulation can have major pathophysiological effects [135]. The term 'bioactive peptide' refers to peptides of plant or animal origin that brings measurable biological effects at a physiological level, with the caveat that the effect must be beneficial [28]. Around 37 different peptide bioactivities are known, such as antihypertensive, antioxidant, anticancer, opioid, mineral-binding, antimicrobial, immunomodulatory, cytomodulatory, antithrombotic, hypocholesterolemic, *etc.* [136]. Online databases such as BioPep, PepBank, EROP-Moscow, BioPD, PeptideDB, and Peptidome are comprehensive resources of bioactive peptides [21]. Among these peptide bioactivities, the most commonly studied are antihypertensive and antioxidant peptides (see Table 3.). Recently, some excellent reviews devoted to several bioactivities of peptides [24, 25, 48, 137] or, in particular, antihypertensive [28, 38, 138] or antioxidant peptides [139-141] have been published. Studies about bioactive peptides involve their extraction, separation, isolation, identification, and characterization by *in vitro* and *in vivo* studies. In general, animal origin (milk, cheese, pork, egg, fish, ham *etc.*) bioactive peptides are more explored although vegetable origin peptides are recently gaining relevance (see Table 3.). Among vegetable origin peptide sources, soybean is receiving a special attention since in many countries is an important dietary ingredient [20]. As previously explained, bioactive peptides can be present as independent entities or can be part of parental proteins released by their hydrolysis. Since protein hydrolyzates obtained by digestion or fermentation are highly complex matrices, several techniques have been employed to separate and purify bioactive peptides. The use of RP-LC is still the most common although HILIC, AC, and IMAC constitute interesting alternatives. Additionally, appropriate bioactive assay(s) are also needed in order to detect most active fractions. Identification of bioactive peptides is next step. Despite robust Edman degradation peptide sequencer is still in use, high throughput, exact, and quick mass spectrometry techniques recently dominates. Once bioactive peptides are identified, several methods for their characterization are used. General aspects, characteristics, and examples of peptides with selected bioactivities are summarized below.

Table 3. Recent works studying bioactive peptides.

Foodstuffs	Aim of the study	Peptide preparation	Peptide separation	Peptide Detection	Identified peptides	Bioactivity assay	References
Angiotensin I converting enzyme (ACE) inhibitory peptides (antihypertensive peptides)							
Sunflower	Enrichment of antihypertensive peptides by immobilized ACE enzyme.	Digestion *(alcalase / flavourzyme)*	AC RP-LC RP-LC	UV (λ 214 nm)	none	*In vitro* ACE inhibition assay	[142]
Pork meat	Study of the generation of ACE inhibitory peptides after GI, separation, identification, and study of *in vitro* antihypertensive effect.	GI *(pepsin/ pancreatin)*	RP-LC	MALDI-ToF/ToF ESI-MS/MS	12 peptides 10 peptides	*In vitro* ACE inhibition assay	[143]
Pork meat	Investigate the *in vivo* antihypertensive activity of 3 novel peptides.	GI *(pepsin/ pancreatin)*	None	None	none	*In vivo* ACE inhibition assay *(SHR)*	[144]
Milk	Study of different LAB strains. The effect of pH and temperature on antihypertensive activity.	Fermentation *(13 LAB strains)*	RP-LC	UV (λ 210, 280 nm) ESI-MS/MS	27 peptides	*In vitro* ACE inhibition assay	[145]
Milk	Identify peptide sequences released by milk fermentation.	Fermentation *(7 LAB strains)* GI *(pepsin/Corolase PP)*	UF RP-LC	ESI-MS/MS	18 peptides 34 peptides	*In vitro* ACE inhibition assay	[146]
Bovine milk	Effect of temperature on the fermentation, autolysis, and release of antihypertensive peptides in milk.	Fermentation *(4 LAB strains)*	RP-LC	UV (λ 210 nm) ESI-MS/MS	54 peptides	*In vitro* ACE inhibition assay	[147]
Manchego cheese	Identify peptide sequences released during cheese ripening.	Extraction	UF RP-LC	ESI-MS/MS	75 peptides	None	[148]
Wheat bran	Examine and isolate ACE inhibitory peptides produced by autolysis of milled parts of wheat seeds.	Autolysis	RP-LC IEC SEC RP-LC RP-LC	MALDI-ToF-MS Edman degradation peptide sequencer	6 peptides	*In vitro* ACE inhibition assay	[17]

Table 3. Continuation.

Foodstuffs	Aim of the study	Peptide preparation	Peptide separation	Peptide Detection	Identified peptides	Bioactivity assay	References
Antioxidant peptides							
Rice	Study antioxidative properties of rice proteins. Purify and identify antioxidant peptides.	Digestion *(alcalase/chymotrypsin/neutrase/papain/flavorase)*	IEC SEC RP-LC	MALDI-ToF/ToF	2 peptides	DPPH assay Hydroxyl radical scavenging activity Superoxide radical scavenging activity Inhibition of linoleic acid autoxidation Cellular cytotoxicity by MTT assay	[149]
Spanish Chorizo	Isolate and identify peptides and low molecular weight compounds with antioxidant activity.	Extraction	RP-LC RP-LC HILIC	ESI-MS/MS	6 peptides	DPPH assay	[110]
Soybean protein isolate	Release and characterize peptides from soybean protein with antioxidant properties.	Digestion *(pancreatin/trypsin/chymotrypsin)*	UF SEC HILIC	ESI-MS/MS	9 peptides	Trolox assay	[150]
Human milk	Purify and identify novel antioxidant peptides from digested human milk.	GI *(pepsin/ pancreatin)*	UF RP-LC	ESI-MS/MS	20 peptides	ORAC assay Inhibition of linoleic acid autoxidation	[151]
Jinhua Ham	Purify and identify antioxidant peptides from Jinhua ham.	Extraction	SEC RP-LC RP-LC	MALDI-ToF/ToF	1 peptide	Hydroxyl radical scavenging activity DPPH assay Fe^{2+} chelating ability	[152]
Hypocholesterolemic peptides							
Rice	Purify peptides with potent hypocholesterolemic capacity.	Digestion *(alcalase/ neutrase/ papain/ trypsin)*	Separation on resin	UV (λ 220 nm)	none	Micellar cholesterol inhibition assay	[153]
Soybean protein	Purify and identify a hypocholesterolemic peptide.	Digestion *(alcalase)*	Separation on resin SEC RP-LC RP-LC	UV (λ 220 nm) ESI-MS	1 peptide	Micellar cholesterol inhibition assay	[154]

Table 3. Continuation.

Foodstuffs	Aim of the study	Peptide preparation	Peptide separation	Peptide Detection	Identified peptides	Bioactivity assay	References
Bovine casein	Determinate *in vitro* cholesterol lowering reducing effect of different hydrolysates.	Fermentation *(Bb12 strains)* Digestion *(trypsin)*	SEC RP-LC	UV (λ 220 nm) ESI-MS	none	Cholesterol reduction assay	[155]
			Antimicrobial				
Bovine casein	Investigate whatever new antibacterial peptides could be released from bovine casein upon GI.	Digestion *(pepsin/ trypsin/ α/β-chymotrypsin)*	RP-LC RP-LC	ESI-MS ESI-MS/MS Edman degradation peptide sequencer	2 peptides	Antibacterial assay (*Bacillus subtilis /L. innocua/ several gram-positive /negative bacteria)*	[156]
Bovine casein hydrolysate	Isolate antibacterial peptides from commercial casein hydrolysate.	none	IEF SEC RP-LC	UV (λ 230, 214 nm) Edman degradation peptide sequencer	3 peptides	Antibacterial assay (*E. coli/Bacillus subtilis*)	[157]
			Immunomodulating/cytomodulatory				
Mozarella di Bufala Campana Cheese	Identify potential bioactive peptides from whey of Mozarella cheese. Trace the pathway of formation of potential bioactive peptides from extract.	none	UF SPE RP-LC	MALDI-ToF-MS UV (λ 220 nm) ESI-MS ESI-MS/MS	64 peptides	Cell proliferation assay Flow cytometric analysis of DNA content	[158]
			Antithrombotic				
Alaska Pollock frame	Purify and identify immunomodulating peptide from alaska pollock frame hydrolysate.	Digestion *(trypsin)*	IEC SEC Multi RP-LC	ESI-MS/MS	3 peptides	Splenic lymphocytes proliferation assay	[159]
Soybean protein hydrolysate	Isolate peptides from soybean protein hydrolysate inhibiting platelet aggregation.	none	SEC RP-LC IEC RP-LC	ESI-MS Edman degradation peptide sequencer	2 peptides	*In vivo* inhibition of platelet aggregation assay	[160]

Table 3. Continuation.

Foodstuffs	Aim of the study	Peptide preparation	Peptide separation	Peptide Detection	Identified peptides	Bioactivity assay	References
Mutliple bioactivities							
Sunflower	Apply IMAC to purify copper- chelating peptides.	Digestion *(alcalase/ flavourzyme)*	IMAC RP-LC	UV (λ 215 nm)	none	β-carotene oxidation assay	[87]
Sesame	Purify and identify metal chelating peptides from sesame hydrolysate.	Digestion *(papain/ alcalase/ trypsin)*	IMAC RP-LC	ESI-MS/MS	6 peptides	Metal chelating assay Zn and Fe chelating assay ABTS assay	[90]
Parific hake fish	Study antioxidant and ACE inhibitory activities of pacific hake fish hydrolysate. Study the effect of GI on these activities. Investigate intestinal transport of these peptides and their potential bioactivity.	Autolysis	UF RP-LC	UV (λ 214 nm)	none	*In vitro* ACE inhibition assay ABTS assay ORAC assay Caco-2 cell permeability assay Intracellular antioxidative activity by Caco-2 cell Cellular cytotoxicity by MTT assay	[161]
Cocoa	Study antioxidant and ACE inhibitory activities of cocoa autolysate.	Autolysis	None	None	none	*In vitro* ACE inhibition assay FRAP assay β-carotene-linoleate bleaching assay	[162]
Dry-cured ham	Study dry-cured ham as a natural source of antihypertensive and antioxidant peptides.	Extraction	SEC SPE	UV (λ 214 nm)	none	*In vitro* ACE inhibition assay *In vivo* ACE inhibition assay *(SHR)* DPPH assay Superoxide ion extinguishing ability	[163]
Soybean hydrolysate/ natto/tempeh	Purify, characterize, and evaluate biologically active peptides.	Digestion *(pronase/ trypsin/ Glu C protease/ kidney membrane protease)*	Multi RP-LC	ESI-MS/MS	9 peptides	*In vitro* ACE inhibition assay Ferric thiocyanate assay Antithrombotic assay Surface tension assay	[164]

Angiotensin I converting enzyme inhibitory peptides. Antihypertensive peptides are the most extensively studied bioactive peptides. This might be linked to the fact that high blood pressure or hypertension affects about quarter of world's population and it is considered as a significant health problem worldwide. Hypertension is one of the main risk factor in the development of cardiovascular diseases (CVD), a group of disorders of heart and blood vessels [23]. Renin-angiotensin system plays a crucial role in the regulation of blood pressure in human organism. Angiotensin I converting enzyme (ACE) converts angiotensin I into angiotensin II, a potent vasoconstrictor involved in a cascade of mechanisms increasing blood pressure. By the inhibition of ACE, the level of angiotensin II and, as a consequence, blood pressure decreases. Antihypertensive peptides contain few amino acids residues (2-12 amino acids) and commonly hydrophobic amino acids (aromatic or branched side chains) at three C-terminal positions. The presence of W, Y, F, and/or P at the C-terminal was suggested to enhance the effectiveness of peptide inhibition [38]. Despite the inhibition mechanism of ACE inhibitor peptides is still unknown, they represent a healthier and natural alternative to drug counterparts since ACE inhibitor peptides do not present side effects [165]. The affinity of antihypertensive peptides to ACE can also be used for their purification. In fact, ACE enzyme immobilized on a BCL glyoxyl-agarose support was employed for the separation of antihypertensive peptides from the alcalase hydrolysate of sunflower and rapeseed [166] and the alcalase/flavourzyme hydrolysate of sunflower [142]. ACE inhibitor capacity is measured by monitoring the conversion of an appropriate substrate by ACE in the presence or absence of inhibitors. The inhibitory potency is expressed as the IC_{50} value defined as the concentration required for a 50% inhibition of enzyme activity [38]. Among various strategies, a spectrophotometric method measuring the conversion of hippuryl-histidyl-leucine (HHL) to hippuric acid (HA) by ACE is still the most widely employed [137, 167]. On the other hand, an *in vivo* assay based on the measurement of blood pressure in spontaneously hypertensive rats (SHR) after oral administration of a hydrolyzate or peptide is also widely employed. In several occasions, *in vitro* and *in vivo* assays have shown inconsistent results. In fact, the potential of three antihypertensive peptides isolated from gastrointestinal digest of pork meat [168] were *in vivo* assessed with spontaneously SHR. These peptides exerted a makeable *in vivo* antihypertensive effect despite a previous study did not show high ACE inhibitor activity [144]. This could be due to the bioconversion or availability of these peptides in organism, or the influence of these peptides on additional blood pressure regulating system than the major one (renin-angiotensin system).

A variety of food sources of antihypertensive peptides have been investigated (see Table 3.). However, animal origin, including fermented milk and dairy products, are among the most studied. A large set of antihypertensive peptides from milk fermented with a variety of *Lactic acid bacteria* strains (LAB) or enzymes, have been purified and identified by several authors [145-147]. For example, fraction below 3 kDa of fermented milk prior and after the simulated gastrointestinal digestion was separated using RP-LC (see Fig. 3A).

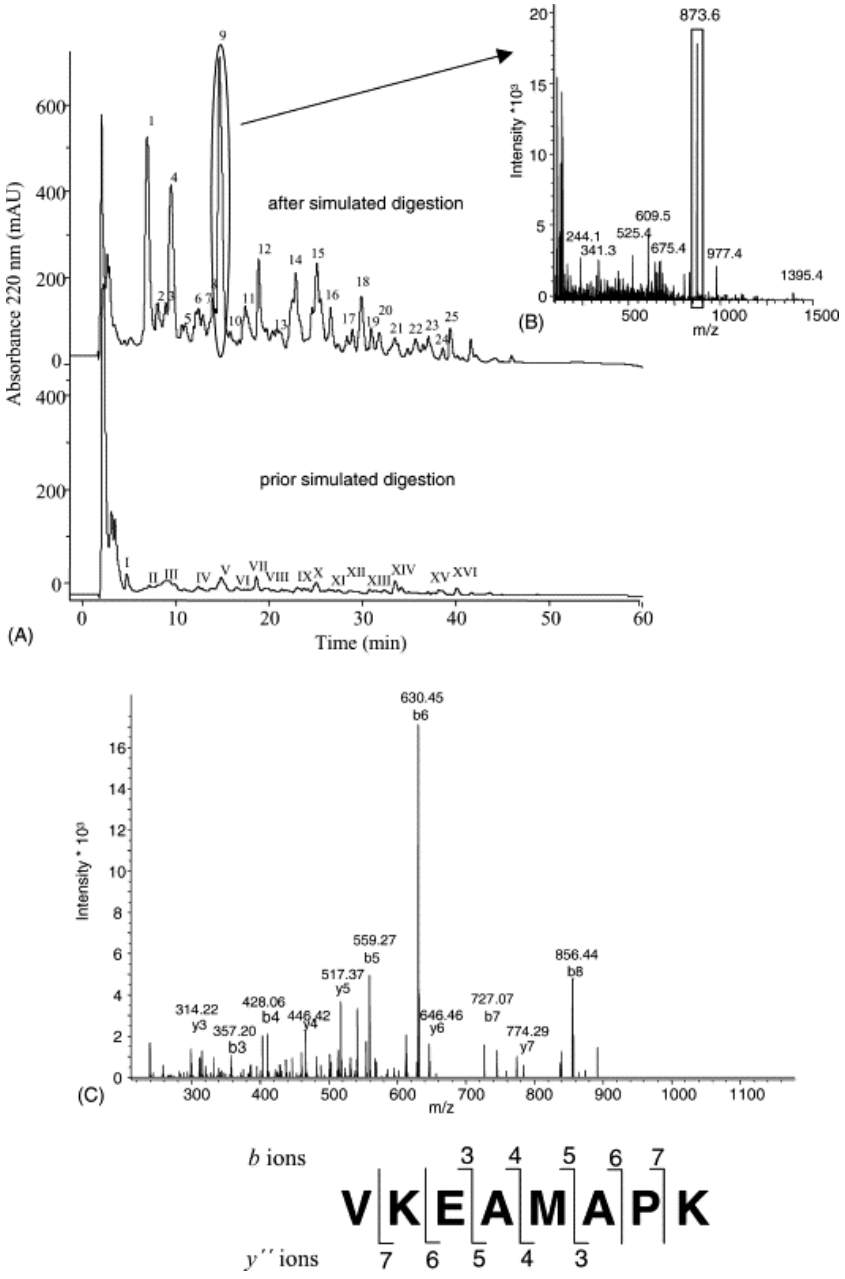


Fig. 3. (A) RP-LC–UV chromatograms of 3 kDa fraction of *fermented* milk (with *L. rhamnosus)* prior and after the simulated gastrointestinal digestion. (B) Mass spectrum of the selected chromatographic peak. (C) MS/MS spectrum of ion *m*/*z* 873.6. The sequence of peptide with the fragment ions observed in the spectrum is presented below. Only the *b* and the *y* fragment ions were labeled. Source: [146].

The MS, MS/MS spectrum obtained for selected peak enabled the identification of peptide β-casein *f*(98-105) (see Fig. 3B, and 3C), and a set of others potential antihypertensive peptides [146]. Antihypertensive peptides formed during Manchego cheese ripening were monitored by HPLC-MS/MS [148]. Epidemiological studies suggested that the consumption of milk and dairy products is inversely related to the risk of hypertension [20]. Indeed, the best characterized ACE inhibitor peptides are casein derived VPP and IPP, also known as lactotripeptides [38]. *In vivo* studies using SHR [20] and around 20 human studies have linked the consumption of products containing lactotripeptides with significant reductions in both systolic and diastolic blood pressure [169]. Consequently, products containing lactopeptides are already commercially available (see Table1.). Nevertheless, despite the great effort invested in the discovery and characterization of antihypertensive peptides, vegetable origin ACE inhibitor peptides are relatively poorly explored. Among them, sunflower and wheat have shown to be good sources of antihypertensive peptides [17, 142].

Antioxidant peptides. Oxidative stress reflexes the increased amount of reactive oxygen species (ROS) with outstripping endogenous antioxidant defense mechanisms [22]. ROS can damage macromolecules such as DNA, proteins, and lipids [141]. Protein oxidation has been linked to the evolution of a variety of diseases (diabetes, atherosclerosis, neurodegenerative disorders, *etc.*) [140]. An antioxidant is a synthetic or natural compound that delays or inhibits oxidation of a substrate when present at low concentration. Strict regulation is needed for synthetic antioxidants since they can possess toxic effects on human's enzyme system [139]. Therefore, safe and widely distributed natural antioxidants are an excellent alternative. The cytotoxicity of natural peptides purified and identified from an alcalase hydrolyzate of rice endosperm was studied. In addition to a higher inhibition of lipid peroxidation than α-tocopherol, no cytotoxic effect on lung fibroblasts or mouse macrophages were observed [149]. The mechanism underlying the antioxidant activity of peptides is still not fully understood. However, it has been shown that antioxidant peptides can be inhibitors of peroxidation, scavengers of free radicals, and chelators of transition metal ions. In order to assess this antioxidant capacity, several methods have been developed. Since none of them can be used as official standardized method, the most usual is the evaluation of antioxidant capacity by various methods in different oxidation conditions [162]. Two groups of methods can be used for the measurement of antioxidant capacity: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) [170]. HAT assays like oxygen absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and β-carotene bleaching

involve a competition reaction between antioxidant and substrate for the generation of peroxyl radicals. ET assays measure the capacity of antioxidant in the reduction of an oxidant used as probe. Trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), and 2,2-diphenyl-1-picrylyhydrazyl radical scavenging capacity (DPPH) assays belong to this group [141]. Evaluation of inhibition of linoleic acid autoxidation, hydroxyl radical or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities are also commonly employed to assess antioxidant capacity of peptides. In these assays, antioxidant peptides activity is related to their composition, structure, and hydrophobicity. Commonly, antioxidant peptides contain aromatic amino acids (F, Y, and W) in addition to other amino acids such as M, K, C, and H [141]. Indeed, a peptide fraction enriched in aromatic amino acids obtained from a soybean protein isolate hydrolyzate showed an antioxidant capacity higher than the observed with other fractions with less content in aromatic amino acids [150]. Moreover, Tsopmo *et al.* [151] suggested that the presence of W residues in the sequence of the most antioxidant peptide in human milk hydrolysate played a significant role. It was demonstrated that it enhanced peroxyl radical scavenging properties and the inhibition of lipid hydroperoxide. Nevertheless, the results obtained by the fractionation of a Spanish chorizo extract using HILIC showed that free amino acids Y, W, M, and F presents as independent entities in some chorizo fractions, had no antioxidant ability. This singular result could be due to a low concentration of these amino acids in the fraction or to the need of being inside a peptide and not as independent entities [110]. Moreover, fractionation of a Jinhua ham extract enabled the purification and identification of antioxidant peptides observing a wider contribution to free radical scavenging activity of more hydrophobic fractions [152].

Hypocholesterolemic peptides. Hypercholesterolemia is another important CVD risk factor [48]. The major preventive strategy for treatment of this disease is to manage blood cholesterol level through diet and drug therapies [155]. Cholesterol, triglycerides, and other lipids are transported to the intestinal epithelium for its absorption by bile acids and phospholipid micelles. Micelles form spontaneously when the concentrations of bile salts and/or phosphatidylcholine are above critical level in bile. Two mechanisms are suggested to cause the removal of cholesterol from micellar aggregates. Since micelles can accommodate a limited level of sterols and lipids, cholesterol can be displaced from the micelle by other sterols like phytosterols. On the other side, cholestyramine, β-sitosterol or hydrophobic peptides can disrupt the micellar structure by binding to bile acids which led to the reduction of micellar-carrying capacity. Unabsorbed cholesterol passes into the colon to be excreted, which result in

lower blood cholesterol level [153]. The suppression of cholesterol micelle solubility in artificially prepared or naturally derived micelles is used for *in vitro* test of hypocholesterolemic efficiency [48]. Hypocholesterolemic peptides have been recognized to contain mostly highly hydrophobic amino acids. Therefore, hydrophobic supports such as DA201-C resin with ethanol elution has been used for the fractionation of soybean and rice bran protein hydrolyzates [153, 154]. In both cases, most hypocholesterolemic peptides appeared in the hydrophobic peptide fraction eluting at 75% (v/v) ethanol. In the case of isolated rice bran proteins, it was possible to observe a positive correlation between low K/R and M/G ratios and hypocholesterolemic capacity. Consequently, some authors suggested that both hydrophobicity and peptide structure could play important roles in the decrease of cholesterol concentration in bile micelles [153][154]. On the other hand, a synergistic effect of hypocholesterolemic peptides was also observed when hypocholesterolemic capacity of a tryptic casein hydrolysate was compared to individual fractions obtained by SEC [155].

Antimicrobial peptides. Antimicrobial peptides inhibit microbe-caused food deterioration and invasion of different pathogens *in vivo* (bacteria, fungi, virus, parasites) [48]. They act either by penetrating and disrupting microbial membrane integrity or by translocating across membranes and acting on internal targets [171]. Antimicrobial peptides are composed of less than 50 amino acids with around 50% hydrophobic amino acids. They are often cationic peptides and have amphipathic 3D structures. Main application areas of antimicrobial peptides are food preservation and therapeutic purpose in health care [48]. Among the broad variety of antimicrobial peptide sources, milk can be credited as a leader. Indeed, two cationic peptides isolated and identified in pepsin hydrolyzate of bovine casein displayed a broad spectrum of activities against several gram-positive (*B. subtilis, L. innocua, L. monocytogenes*) and gram-negative bacteria (*C. freundii, Ent. Aerogenes, E. coli, S. Enteritidis, S. Typhimurium*). Additionally, the high degree of similarity between bovine peptide 99-109 of bovine α_{S1}-casein and the corresponding peptide from sheep, goat, and buffalo α_{S1}-casein indicated probable antimicrobial counterparts in these milk sources. Since identified peptides were obtained by pepsin action, the protection against microbial infection in the gastrointestinal tract was also suggested [156]. On the other hand, peptides generated from α_{S1}-casein, α_{S2}-casein, and κ-casein and possessing high antibacterial activities against *E.coli* and *B.subtilis* were identified in a commercially casein hydrolysate. The amino acid analysis of identified peptides revealed that positive charges of peptides were very important but it did not guarantee antimicrobial activity [157].

Immunomodulatory and cytomodulatory peptides. Immune function plays a significant role in the prevention and control of chronic diseases. The modulation of immune response is difficult and most drugs are not suitable to prevent chronic diseases [159]. Immunomodulatory peptides improve immune cell functions, antibody synthesis, and cytochrome regulation [171]. Cytomodulatory peptides modulate the viability (*e.g.* proliferation) of different cell types and, together with immunomodulatory peptides, might help in the control of tumor development [171]. Potential cytomodulatory peptides have been isolated from Mozzarella di Bufala waste whey [158]. Investigated peptides inhibited significantly cell proliferation, interfered with cell cycle, and exerted a possible pro-apoptotic activity on Caco2 cancer cells. Nevertheless, identification of individual peptides responsible for this cytomodulatory action was impossible due to sample complexity. Instead, the presence of precursors of the widely recognized agonist opioids β-casomorphin 7 and β-casomorphin 5 were evaluated. It was suggested that cell proliferation decreased due to the interaction of identified casomorphin-agonist precursors and specific opioid and somatostain receptors expressed on Caco2 cells [158]. Furthermore, Alaska Pollock frame enhanced splenic lymphocytes proliferation, T lymphocyte proliferation, and macrophage phagocytosis. Purification of Alaska Pollock frame hydrolyzate using IEC, SEC, and multiple RP-LC enabled to purify three bioactive peptides. Identified peptides exhibited high lymphocyte proliferation activities which provided a scientific basis for the preparation of immunomodulating peptides [159].

Antithrombotic peptides. One of CVD complications is related to the tendency to develop thrombosis due to the abnormalities in blood coagulation. Thrombosis is linked to platelet hyperactivity, high levels of hemostatic proteins, defective fibrinolysis, and blood hyperviscosity. Antithrombotic drugs reduce platelet aggregation and enhance fibrinolysis [22]. Due to amino acid sequence similarities between fibrinogen γ-chain from blood and κ- casein from milk and functional similarities between milk and blood coagulation, most investigated antithrombotic peptides are from milk [172]. Recently, two novel peptides from soybean protein hydrolyzate inhibiting rat platelet aggregation have been discovered. Interestingly, during its fractionation by SEC, RP-LC, and IEC it was observed that most fractions yielded some level of antiplatelet effect. Indeed, synthesized counterparts of identified peptides inhibited platelet aggregation in a concentration-dependent manner [160].

Multiple bioactivities peptides. Some peptides can also possess multiple bioactivities. As example, metal-chelating peptides can bind metal elements through certain amino acids (H, M, or C) and, simultaneously, can yield antioxidant activity [48]. Positive correlation between H

content and antioxidant activity of sunflower copper-chelating peptides was reported. Copper, in addition to its role as an essential trace element, can also exhibit pro-oxidative activity. Indeed, copper chelating peptides may be useful in preventing oxidative activity of copper in the digestive tract and oxidative damage of low-density lipoproteins in blood and tissues by copper absorption into the blood stream [87]. Food derived metal-chelating peptides can also enhance *in vivo* absorption of metals and trace elements. A prime example of this kind of metal chelating peptides is caseinophophopeptides (CPPs) [48]. Their calcium absorption ability was already applied in some commercial products (see Table 1.). Recently, tryptic peptides of sesame have also shown to possess metal-chelating activity. Purification of these hydrolyzates enabled to isolate six potent metal chelating peptides. Identified peptides showed high zinc and iron chelating abilities [90]. It is important to point out the effectiveness of IMAC technique in the purification of metal-chelating peptides from food hydrolyzates.

It is also very common for protein hydrolyzates or peptides to possess both antioxidant and antihypertensive activities [161]. It was reported that angiotensin II amplifies oxidative stress and ACE inhibitors intensify antioxidant defense system by inhibition of angiotensin II formation in animals and humans [162]. Peptides obtained from cured ham extract, cocoa, and pacific hake autolyzates showed antihypertensive and antioxidant activities [161-163]. The presence of two bioactivities makes these foods an attractive source of bioactive compounds that can improve cardiovascular health and control related disease [163]. Another attractive source of bioactive peptides turned out to be soybean. Peptides purified and characterized from soybean hydrolyzate, natto, and tempeh have demonstrated a range of biological activities. Antihypertensive, antithrombotic, surface tension, and antioxidant peptides were mainly from glycinin. In this study the use of low specificity proteases produced more bioactive peptides with higher activity than higher specific enzymes [173].

5. Future trends

Isolation of peptides from foods is still a difficult task and a problem to overcome in next years. For that purpose, highly specific, reproducible, and high throughput methods are desirable. Recent popularization of techniques mainly devoted to clinical proteomics like IMAC and AC, have resulted very useful and are gaining acceptance. Although ‘OFFGEL’ isoelectrofocusing has shown to be a complementary tool to obtain peptide fractions, its application in the area of food peptides is still scarce and will spread in the future. Another separation technique that will expand in next years is HILIC. Fractions previously rejected

during RP-LC due to their elution in the void time, can be now easily separated by HILIC. Since HILIC and MS are a perfect marriage, their united expansion in the peptide research field is expected.

The trend in the area of peptide production from foodstuffs is oriented to obtain highly valuable material (bioactive peptides) from cheap sources (use of by-products or wastes), using low cost (autolysis) and/or highly efficient (simulated gastrointestinal digestion) methods. The study of bioactive peptides *in vivo*, together with clinical studies, and further expansion of bioactive peptides in the functional food area are also future trends. Moreover, since most bioactive peptides activities are dose-dependent, a great development of quantification methods is expected in next future which involves overcoming problems related to interferences due to complex food matrices. In the area of biomarker peptides, the lack of proteome databases from different food organisms constitutes a great limitation. This fact, together with huge popularization of MS analysis, will lead to the extension of available food protein database information. In relation to allergens detection using biomarker peptides, the development of methodologies enabling the simultaneous detection of different allergens will increase the confidence in the identification of allergen traces in foodstuffs.

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References for Book chapter part:

[1] N. Sewald, H. Jakubke, Introduction and Background, in: Peptides: Chemistry and Biology, Wiley-VCH Verlag GmbH & Co. KGaA, 2009, 1-4.

[2] K. Suetsuna, Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from Allium sativum L (garlic), J. Nutr. Biochem. 9 (1998) 415-419.

[3] D. H. Lee, J. H. Kim, J. S. Park, Y. J. Choi, J. S. Lee, Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom Tricholoma giganteum, Peptides 25 (2004) 621-627.

[4] K.C. Koo, D.H. Lee, J.H. Kim, H.E. Yu, J.S. Park, J.S. Lee, Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from Pholiota adiposa, J. Microbiol. Biotechn. 16 (2006) 757-763.

[5] H. Korhonen, A. Pihlanto, Bioactive peptides: Production and functionality, Int. Dairy J. 16 (2006) 945-960.

[6] A. Iwaniak, P. Minkiewicz, Biologically active peptides derived from proteins - A review, Pol. J. Food Nutr. 58 (2008) 289-294.

[7] P.B. Kunda, F. Benavente, S. Catalá-Clariana, E. Gímenez, J. Barbosa, V. Sanz-Nebot, Identification of bioactive peptides in a functional yogurt by micro liquid chromatography time-of-flight mass spectrometry assisted by retention time prediction, J. Chromatogr. A 1229 (2012) 121-128.

[8] C. Salakidou, Z. Simov, D. Beshkova, Z. Dimitrov, E. Simova, K. Yoanidou, Biochemical characteristics of yogurt beverage from donkey milk, Bull. Int. Dairy Fed. (2012) 157-160.

[9] H.S. Aloğlu, Z. Oner, Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt, J. Dairy Sci. 94 (2011) 5305-5314.

[10] L. Ong, N.P. Shah, Release and identification of angiotensin-converting enzyme-inhibitory peptides as influenced by ripening temperatures and probiotic adjuncts in Cheddar cheeses, LWT-Food Sci. Technol. 41 (2008) 1555-1566.

[11] Y. Ardo, H. Lilbaek, K.R. Kristiansen, M. Zakora, J. Otte, Identification of large phosphopeptides from Î²-casein that characteristically accumulate during ripening of the semi-hard cheese Herrgard, Int. Dairy J. 17 (2007) 513-524.

[12] U. Butikofer, J. Meyer, R. Sieber, B. Walther, D. Wechsler, Occurrence of the angiotensin-converting enzyme inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties of Swiss origin, J. Dairy Sci. 91 (2008) 29-38.

[13] D. Wang, L. Wang, F. Zhu, J. Zhu, X.D. Chen, L. Zou, M. Saito, L. Li, In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food), Food Chem. 107 (2008) 1421-1428.

[14] B. Gibbs, A. Zougman, R. Masse, C. Mulligan, Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food, Food Res. Int. 37 (2004) 123-131.

[15] C.C. Udenigwe, R.E. Aluko, Food protein-derived bioactive peptides: production, processing, and potential health benefits, J. Food Sci. 77 (2012) R11-R24.

[16] A. Zambrowicz, M. Timmer, A. Polanowski, G. Lubec, T. Trziszka, Manufacturing of peptides exhibiting biological activity, Amino Acids 44 (2013) 315-320.

[17] Y. Nogata, T. Nagamine, M. Yanaka, H. Ohta, angiotensin i converting enzyme inhibitory peptides produced by autolysis reactions from wheat bran, J. Agric. Food Chem. 57 (2009) 6618-6622.

[18] D. Garrett, M. Failla, R. Sarama, Development of an in vitro digestion method to assess carotenoid bioavailability from meals, J. Agric. Food Chem. 47 (1999) 4301-4309.

[19] P. Vlieghe, V. Lisowski, J. Martínez, M. Khrestchatisky, Synthetic therapeutic peptides: science and market, Drug Discov. Today 15 (2010) 40-56.

[20] D. Martínez-Maqueda, B. Miralles, I. Recio, B. Hernández-Ledesma, Antihypertensive peptides from food proteins: a review, Food Funct. 3 (2012) 350-361.

[21] A. Panchaud A, M. Affolter, M. Kussmann, Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects, J. Proteomics 75 (2012) 3546-3559.

[22] K. Erdmann, B.W.Y. Cheung, H. Schroeder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease, J. Nutr. Biochem. 19 (2008) 643-654.

[23] M. Phelan, D. Kerins, The potential role of milk-derived peptides in cardiovascular disease, Food Funct. 2 (2011) 153-167.

[24] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[25] K. Erdmann, B.W. Cheung, H. Schroeder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease, J. Nutr. Biochem. 19 (2008) 643-654.

[26] F. Lalor, P.G. Wall, Health claims regulations Comparison between USA, Japan and European Union, Br. Food J. 113 (2011) 298-313.

[27] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[28] F. De Leo, S. Panarese, R. Gallerani, L.R. Ceci, Angiotensin Converting Enzyme (ACE) inhibitory peptides: production and implementation of functional food, Curr. Pharm. Des. 15 (2009) 3622-3643.

[29] H.J. Issaq, K.C. Chan, J. Blonder, X. Ye, T.D. Veenstra, Separation, detection and quantitation of peptides by liquid chromatography and capillary electrochromatography, J. Chromatogr. A 1216 (2009) 1825-1837.

[30] G. Picariello, G. Mamone, F. Addeo, P. Ferranti, Novel mass spectrometry-based applications of the 'omic' sciences in food technology and biotechnology, Food Technol. Biotechnol. 50 (2012) 286-305.

[31] P. Walstra, Principles of emulsion formation, Chem. Eng. Sci. 48 (1993) 333-349.

[32] C. van der Ven, H. Gruppen, D. de Bont, A. Voragen, Emulsion properties of casein and whey protein hydrolysates and the relation with other hydrolysate characteristics, J. Agric. Food Chem. 49 (2001) 5005-5012.

[33] D. Doucet, S.F. Gauthier, E.A. Foegeding, Rheological characterization of a gel formed during extensive enzymatic hydrolysis, J. Food Sci. 66 (2001) 711-715.

[34] I.M.P.L.V.O. Ferreira, O. Pinho, M.V. Mota, P. Tavares, A. Pereira, M.P. Gonçalves, D. Torres, C. Rocha, J.A. Teixeira, Preparation of ingredients containing an ACE-inhibitory peptide by tryptic hydrolysis of whey protein concentrates, Int. Dairy J. 17 (2007) 481-487.

[35] F. Van Lancker, A. Adams, N. De Kimpe, Chemical modifications of peptides and their impact on food properties, Chem. Rev. 111 (2011) 7876-7903.

[36] R. FitzGerald, G. O'Cuinn, Enzymatic debittering of food protein hydrolysates, Biotechnol. Adv. 24 (2006) 234-237.

[37] B. Saha, K. Hayashi, Debittering of protein hydrolyzates, Biotechnol. Adv. 19 (2001) 355-370.

[38] B. Hernández-Ledesma, M. M. Contreras, I. Recio, Antihypertensive peptides: Production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23-35.

[39] C. Brueckner, D. Imhof, G.K.E. Scriba, Capillary electrophoretic study of the degradation pathways and kinetics of the aspartyl model tetrapeptide Gly-Phe-Asp-GlyOH in alkaline solution, J. Pharm. Biomed. Anal. 76 (2013) 96-103.

[40] O.V. Krokhin, M. Antonovici, W. Ens, J.A. Wilkins, K.G. Standing, Deamidation of -Asn-Gly-sequences during sample preparation for proteomics: Consequences for MALDI and HPLC-MALDI analysis, Anal. Chem. 78 (2006) 6645-6650.

[41] L. Bell, Peptide stability in solids and solutions, Biotechnol. Prog. 13 (1997) 342-346.

[42] P. Butz, A. García, T. Schneider, J. Starke, B. Tauscher, B. Trierweiler, The influence of high pressure on the formation of diketopiperazines and pyroglutamate in peptides and peptide esters, High Pressure Res. 22 (2002) 697-700.

[43] X. Zhang, P. Hojrup, Cyclization of the N-Terminal X-Asn-Gly motif during sample preparation for bottom-up proteomics, Anal. Chem. 82 (2010) 8680-8685.

[44] J. Buhlert, R. Carle, Z. Majer, D. Spitzner, Thermal degradation of peptides and formation of acrylamide, Lett. Org. Chem. 3 (2006) 356-357.

[45] J. Buhlert, R. Carle, Z. Majer, D. Spitzner, Thermal degradation of peptides and formation of acrylamide, Part 2, Lett. Org. Chem. 4 (2007) 329-331.

[46] K.A. Witt, T.J. Gillespie, J.D. Huber, R.D. Egleton, T.P. Davis, Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability, Peptides 22 (2001) 2329-2343.

[47] D. Martínez-Maqueda, B. Hernández-Ledesma, L. Amigo, B. Miralles, J. Gómez-Ruiz, Extraction/fractionation techniques for proteins and peptides and protein digestion, in: proteomics in foods, Springer US, 2013, pp. 21-50.

[48] F. Shahidi, Y. Zhong, Bioactive peptides, J. AOAC Int. 91 (2008) 914-931.

[49] A. Poliwoda, P.P. Wieczorek, Sample pretreatment techniques for oligopeptide analysis from natural sources, Anal. Bioanal. Chem. 393 (2009) 885-897.

[50] B. Cañas, C. Piñeiro, E. Calvo, D. López-Ferrer, J. Manuel Gallardo, Trends in sample preparation for classical and second generation proteomics, J. Chromatogr. A 1153 (2007) 235-258.

[51] J. Rosenfeld, J. Capdevielle, J. Guillemot, P. Ferrara, In-Gel digestion of proteins for internal sequence-analysis after 1-dimensional or 2-dimensional gel-electrophoresis, Anal. Biochem. 203 (1992) 173-179.

[52] A.J. Link, J. Eng, D.M. Schieltz, E. Carmack, G.J. Mize, D.R. Morris, B.M. Garvik, J.R. Yates, Direct analysis of protein complexes using mass spectrometry, Nat. Biotechnol. 17 (1999) 676-682.

[53] M. Nissum, U. Schneider, S. Kuhfuss, C. Obermaier, R. Wildgruber, A. Posch, C. Eckerskorn, In-gel digestion of proteins using a solid-phase extraction microplate, Anal. Chem. 76 (2004) 2040-2045.

[54] J.L. Capelo, R. Carreira, M. Diniz, L. Fernandes, M. Galesio, C. Lodeiro, H.M. Santos, G. Vale, Overview on modern approaches to speed up protein identification workflows relying on enzymatic cleavage and mass spectrometry-based techniques, Anal. Chim. Acta. 650 (2009) 151-159.

[55] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359-U60.

[56] J. Meltretter, A. Schmidt, A. Humeny, C. Becker, M. Pischetsriedert, Analysis of the peptide profile of milk and its changes during thermal treatment and storage, J. Agric. Food Chem. 56 (2008) 2899-2906.

[57] S. Toelstede, A. Dunkel, T. Hofmann, A series of kokumi peptides impart the long-lasting mouthfulness of matured gouda cheese, J. Agric. Food Chem. 57 (2009) 1440-1448.

[58] S. Agboola, S. Chen, J. Zhao, Formation of bitter peptides during ripening of ovine milk cheese made with different coagulants, Lait 84 (2004) 567-578.

[59] M. Kim, Y. Kawamura, C. Lee, Isolation and identification of bitter peptides of tryptic hydrolysate of soybean 11S glycinin by reverse-phase high-performance liquid chromatography, J. Food Sci. 68 (2003) 2416-2422.

[60] M. Zhang, X. Wang, Y. Liu, X. Xu, G. Zhou, Isolation and identification of flavour peptides from Puffer fish (Takifugu obscurus) muscle using an electronic tongue and MALDI-TOF/TOF MS/MS, Food Chem. 135 (2012) 1463-1470.

[61] M.J. Lerma-García, J.M. Herrero-Martínez, G. Ramis-Ramos, C. Mongay-Fernández, E.F. Simo-Alfonso, Prediction of the curing time of Spanish hams using peptide profiles established by capillary zone electrophoresis, Food Chem. 113 (2009) 635-639.

[62] M. Sentandreu, S. Stoeva, M. Aristoy, K. Laib, W. Voelter, F. Toldrá, Identification of small peptides generated in Spanish dry-cured ham, J. Food Sci. 68 (2003) 64-69.

[63] L. Mora, M.A. Sentandreu, K.M. Koistinen, P.D. Fraser, F. Toldrá, P.M. Bramley, Naturally generated small peptides derived from myofibrillar proteins in serrano dry-cured ham, J. Agric. Food Chem. 57 (2009) 3228-3234.

[64] L. Mora, M.L. Valero, M.M. Sánchez del Pino, M.A. Sentandreu, F. Toldrá, Small peptides released from muscle glycolytic enzymes during dry-cured ham processing, J. Proteomics 74 (2011) 442-450.

[65] L. Mora, F. Toldrá, Proteomic identification of small (< 2000 Da) myoglobin peptides generated in dry-cured ham, Food Technol. Biotechnol. 50 (2012) 343-349.

[66] J. López, A. Marina, G. Alvarez, J. Vázquez, Application of proteomics for fast identification of species-specific peptides from marine species, Proteomics 2 (2002) 1658-1665.

[67] M. Carrera, B. Cañas, C. Piñeiro, J. Vázquez, J. M. Gallardo, De novo mass spectrometry sequencing and characterization of species-specific peptides from nucleoside diphosphate kinase B for the classification of commercial fish species belonging to the family Merlucciidae, J. Proteome Res. 6 (2007) 3070-3080.

[68] I. Ortea, B. Cañas, J.M. Gallardo, Mass Spectrometry characterization of species-specific peptides from arginine kinase for the identification of commercially relevant shrimp species, J. Proteome Res. 8 (2009) 5356-5362.

[69] I. Ortea, B. Cañas, J.M. Gallardo, Selected tandem mass spectrometry ion monitoring for the fast identification of seafood species, J. Chromatogr. A 1218 (2011) 4445-4451.

[70] M. Carrera, B. Cañas, D. López-Ferrer, C. Piñeiro, J. Vázquez, J.M. Gallardo, Fast monitoring of species-specific peptide biomarkers using High-Intensity-Focused-Ultrasound-Assisted tryptic digestion and selected MS/MS Ion Monitoring, Anal. Chem. 83 (2011) 5688-5695.

[71] H.H. Grundy, P. Reece, M.D. Sykes, J.A. Clough, N. Audsley, R. Stones, Screening method for the addition of bovine blood-based binding agents to food using liquid chromatography triple quadrupole mass spectrometry, Rapid Commun. Mass Spectrom. 21 (2007) 2919-2925.

[72] M.A. Sentandreu, P.D. Fraser, J. Halket, R. Patel, P.M. Bramley, A proteomic-based approach for detection of chicken in meat mixes, J. Proteome Res. 9 (2010) 3374-3383.

[73] G. Zhang, T. Liu, Q. Wang, L. Chen, J. Lei, J. Luo, G. Ma, Z. Su, Mass spectrometric detection of marker peptides in tryptic digests of gelatin: A new method to differentiate between bovine and porcine gelatin, Food Hydrocoll. 23 (2009) 2001-2007.

[74] D. Weber, P. Raymond, S. Ben-Rejeb, B. Lau, Development of a liquid chromatography-tandem mass spectrometry method using capillary liquid chromatography and nanoelectrospray ionization-quadrupole time-of-flight hybrid mass spectrometer for the detection of milk allergens, J. Agric. Food Chem. 54 (2006) 1604-1610.

[75] P. Ansari, N. Stoppacher, J. Rudolf, R. Schuhmacher, S. Baumgartner, Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 399 (2011) 1105-1115.

[76] T. Cucu, B. De Meulenaer, B. Kerkaert, I. Vandenberghe, B. Devreese, MALDI based identification of whey protein derived tryptic marker peptides that resist protein glycation, Food Res. Int. 47 (2012) 23-30.

[77] H. Chassaigne, J.V. Norgaard, A.J. van Hengel, Proteomics-based approach to detect and identify major allergens in processed peanuts by capillary LC-Q-TOF (MS/MS), J. Agric. Food Chem. 55 (2007) 4461-4473.

[78] K.J. Shefcheck, J.H. Callahan, S.M. Musser, Confirmation of peanut protein using peptide markers in dark chocolate using liquid chromatography-tandem mass spectrometry (LC-MS/MS), J. Agric. Food Chem. 54 (2006) 7953-7959.

[79] M. Careri, A. Costa, L. Elviri, J. Lagos, A. Mangia, M. Terenghi, A. Cereti, L.P. Garoffo, Use of specific peptide biomarkers for quantitative confirmation of hidden allergenic peanut proteins Ara h 2 and Ara Ill 3/4 for food control by liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 389 (2007) 1901-1907.

[80] M. Careri, L. Elviri, M. Maffini, A. Mangia, C. Mucchino, M. Terenghi, Determination of peanut allergens in cereal-chocolate-based snacks: metal-tag inductively coupled plasma mass spectrometry immunoassay versus liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 807-811.

[81] M. Careri, L. Elviri, J.B. Lagos, A. Mangia, F. Speroni, M. Terenghi, Selective and rapid immunomagnetic bead-based sample treatment for the liquid chromatography-electrospray ion-trap mass spectrometry detection of Ara h3/4 peanut protein in foods, J. Chromatogr. A 1206 (2008) 89-94.

[82] R. Pedreschi, J. Norgaard, A. Maquet, Current challenges in detecting food allergens by shotgun and targeted proteomic approaches: a case study on traces of peanut allergens in baked cookies, Nutrients 4 (2012) 132-150.

[83] P. Ansari, N. Stoppacher, S. Baumgartner, Marker peptide selection for the determination of hazelnut by LC-MS/MS and occurrence in other nuts, Anal. Bioanal. Chem. 402 (2012) 2607-2615.

[84] T. Cucu, B. De Meulenaer, B. Devreese, MALDI-based identification of stable hazelnut protein derived tryptic marker peptides, Food Addit. Contam. Part A-Chem. 29 (2012) 1821-1831.

[85] C. Bignardi, L. Elviri, A. Penna, M. Careri, A. Mangia, Particle-packed column versus silica-based monolithic column for liquid chromatography-electrospray-linear ion trap-tandem mass spectrometry multiallergen trace analysis in foods, J. Chromatogr. A 1217 (2010) 7579-7585.

[86] M. Carrera, B. Cañas, J.M. Gallardo, Rapid direct detection of the major fish allergen, parvalbumin, by selected MS/MS ion monitoring mass spectrometry, J. Proteomics 75 (2012) 3211-3220.

[87] C. Megias, J. Pedroche, M.M. Yust, J. Girón-Calle, M. Alaiz, F. Millan, J. Vioque, Affinity purification of copper-chelating peptides from sunflower protein hydrolysates, J. Agric. Food Chem. 55 (2007) 6509-6514.

[88] M. Lund, Y. Ardo, Purification and identification of water-soluble phosphopeptides from cheese using Fe(III) affinity chromatography and mass spectrometry, J. Agric. Food Chem. 52 (2004) 6616-6622.

[89] Y. Lv, Q. Liu, X. Bao, W. Tang, B. Yang, S. Guo, Identification and characteristics of iron-chelating peptides from soybean protein hydrolysates using IMAC-Fe3+, J. Agric. Food Chem. 57 (2009) 4593-4597.

[90] C. Wang, B. Li, J. Ao, Separation and identification of zinc-chelating peptides from sesame protein hydrolysate using IMAC-Zn2+ and LC-MS/MS, Food Chem. 134 (2012) 1231-1238.

[91] F. Shen, Y. Hu, P. Guan, X. Ren, Ti4+-phosphate functionalized cellulose for phosphopeptides enrichment and its application in rice phosphoproteome analysis, J. Chromatogr. B 902 (2012) 108-115.

[92] S. Gedela, N.R. Medicherla, Chromatographic techniques for the separation of peptides: Application to proteomics, Chromatographia 65 (2007) 511-518.

[93] M. Lund, Y. Ardo, Purification and identification of water-soluble phosphopeptides from cheese using Fe(III) affinity chromatography and mass spectrometry, J. Agric. Food Chem. 52 (2004) 6616-6622.

[94] H. Liu, X.L. Bao, Y. Lv, J.T. Xu, S.T. Guo, Separation and evaluation of soybean protein hydrolysates prepared by immobilized metal ion affinity chromatography with different metal ions, J. Chromatogr. Sci. 50 (2012) 714-720.

[95] I. Neverova, J. Van Eyk, Role of chromatographic techniques in proteomic analysis, J. Chromatogr. B 815 (2005) 51-63.

[96] P.J. Boersema, N. Divecha, A.J.R. Heck, S. Mohammed, Evaluation and optimization of ZIC-HILIC-RP as an alternative MudPIT strategy, J. Proteome Res. 6 (2007) 937-946.

[97] W. Jian, R.W. Edom, Y. Xu, N. Weng, Recent advances in application of hydrophilic interaction chromatography for quantitative bioanalysis, J. Sep. Sci. 33 (2010) 681-697.

[98] J. Bernal, A.M. Ares, J. Pol, S.K. Wiedmer, Hydrophilic interaction liquid chromatography in food analysis, J. Chromatogr. A 1218 (2011) 7438-7452.

[99] T. Sonntag, C. Kunert, A. Dunkel, T. Hofmann, Sensory-guided identification of n-(1-methyl-4-oxoimidazolidin-2-ylidene)-alpha-amino acids as contributors to the thick-sour and mouth-drying orosensation of stewed beef juice, J. Agric. Food Chem. 58 (2010) 6341-6350.

[100] S. Toelstede, T. Hofmann, Sensomics mapping and identification of the key bitter metabolites in Gouda cheese, J. Agric. Food Chem. 56 (2008) 2795-2804.

[101] L. Mora, M.A. Sentandreu, F. Toldrá, Contents of creatine, creatinine and carnosine in porcine muscles of different metabolic types, Meat Sci. 79 (2008) 709-715.

[102] C.J. van Platerink, H.M. Janssen, J. Haverkamp, Application of at-line two-dimensional liquid chromatography-mass spectrometry for identification of small hydrophilic angiotensin I-inhibiting peptides in milk hydrolysates, Anal. Bioanal. Chem. 391 (2008) 299-307.

[103] G. Picariello, P. Ferranti, G. Mamone, P. Roepstorff, F. Addeo, Identification of N-linked glycoproteins in human milk by hydrophilic interaction liquid chromatography and mass spectrometry, Proteomics 8 (2008) 3833-3847.

[104] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231-247.

[105] J. Chenau, S. Michelland, J. Sidibe, M. Seve, Peptides OFFGEL electrophoresis: a suitable pre-analytical step for complex eukaryotic samples fractionation compatible with quantitative iTRAQ labeling, Proteome Sci. 6 (2008) 9.

[106] J.M. Cunliffe, T.D. Maloney, Fused-core particle technology as an alternative to sub-2-alpha m particles to achieve high separation efficiency with low backpressure, J. Sep. Sci. 30 (2007) 3104-3109.

[107] Y. Saito, K. Jinno, T. Greibrokk, Capillary-columns in liquid chromatography: between conventional columns and microchips, J. Sep. Sci. 27 (2004) 1379-1390.

[108] P. Hemstrom, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 1784-1821.

[109] J. Tang, M. Gao, C. Deng, X. Zhang, Recent development of multi-dimensional chromatography strategies in proteome research, J. Chromatogr. B 866 (2008) 123-132.

[110] J.M. Broncano, J. Otte, M.J. Petron, V. Parra, M.L. Timon, Isolation and identification of low molecular weight antioxidant compounds from fermented "chorizo" sausages, Meat Sci. 90 (2012) 494-501.

[111] H. Schlichtherle-Cerny, M. Affolter, C. Cerny, Hydrophilic interaction liquid chromatography coupled to electrospray mass spectrometry of small polar compounds in food analysis, Anal. Chem. 75 (2003) 2349-2354.

[112] C. Simó, C. Barbas, A. Cifuentes, Capillary electrophoresis-mass spectrometry in food analysis, Electrophoresis 26 (2005) 1306-1318.

[113] V. Kasicka, Recent developments in CE and CEC of peptides (2009-2011), Electrophoresis 33 (2012) 48-73.

[114] C. Hughes, B. Ma, G.A. Lajoie, De Novo sequencing methods in proteomics, Methods Mol. Biol. 604 (2010) 105-121.

[115] G. Mamone, G. Picariello, S. Caira, F. Addeo, P. Ferranti, Analysis of food proteins and peptides by mass spectrometry-based techniques, J. Chromatogr. A 1216 (2009) 7130-7142.

[116] M. Holcapek, R. Jirasko, M. Lisa, Recent developments in liquid chromatography-mass spectrometry and related techniques, J. Chromatogr. A 1259 (2012) 3-15.

[117] M.C. García, A.C. Hogenboom, H. Zappey, H. Irth, Effect of the mobile phase composition on the separation and detection of intact proteins by reversed-phase liquid chromatography - electrospray mass spectrometry, J. Chromatogr. A 957 (2002) 187-199.

[118] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature 422 (2003) 198-207.

[119] P. Edman, Method for determination of the amino acid sequence in peptides, Acta Chem. Scand. 4 (1950) 283-293.

[120] H. Steen, M. Mann, The ABC's (and XYZ's) of peptide sequencing, Nat. Rev. Mol. Cell Biol. 5 (2004) 699-711.

[121] V. Wysocki, K. Resing, Q. Zhang, G. Cheng, Mass spectrometry of peptides and proteins, Methods 35 (2005) 211-222.

[122] E.L. de Graaf, A.F.M. Altelaar, B. van Breukelen, S. Mohammed, A.J.R. Heck, Improving SRM assay development: a global comparison between triple quadrupole, ion trap, and higher energy CID peptide fragmentation spectra, J. Proteome Res. 10 (2011) 4334-4341.

[123] M.M. Savitski, F. Kjeldsen, M.L. Nielsen, R.A. Zubarev, Relative specificities of water and ammonia losses from backbone fragments in collision-activated dissociation, J. Proteome Res. 6 (2007) 2669-2673.

[124] B. Ma, K.Z. Zhang, C. Hendrie, C.Z. Liang, M. Li, A. Doherty-Kirby, G. Lajoie, PEAKS: powerful software for peptide de novo sequencing by tandem mass spectrometry, Rapid Comm. Mass Spectrom. 17 (2003) 2337-2342.

[125] J.K. Eng, A.L. Mccormack, J.R. Yates, An approach to correlate tandem mass-spectral data of peptides with amino-acid-sequences in a protein database, J. Am. Soc. Mass Spectrom. 5 (1994) 976-989.

[126] A.I. Nesvizhskii, O. Vitek, R. Aebersold, Analysis and validation of proteomic data generated by tandem mass spectrometry, Nat. Methods 4 (2007) 787-797.

[127] A. D'Alessandro, L. Zolla, We are what we eat: food safety and proteomics, J. Proteome Res. 11 (2012) 26-36.

[128] M. Gobbetti, L. Stepaniak, M. De Angelis, A. Corsetti, R. Di Cagno, Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy processing, Crit. Rev. Food Sci. Nutr. 42 (2002) 223-239.

[129] M. Kim, Y. Kawamura, C. Lee, Isolation and identification of bitter peptides of tryptic hydrolysate of soybean 11S glycinin by reverse-phase high-performance liquid chromatography, J. Food Sci. 68 (2003) 2416-2422.

[130] M.A. Sentandreu, S. Stoeva, M.C. Aristoy, K. Laib, W. Voelter, F. Toldrá, Identification of small peptides generated in Spanish dry-cured ham, J. Food Sci. 68 (2003) 64-69.

[131] A.I. Sancho, E.N.C. Mills, Proteomic approaches for qualitative and quantitative characterisation of food allergens, Reg. Toxicol. Pharmacol. 58 (2010) S42-S46.

[132] Food and Drug Administration, Food allergen labeling and consumer protection act of 2004, Public Law, USA (2004) 108-282.

[133] P.G. Righetti, E. Boschetti, L. Lomas, A. Citterio, Protein Equalizer (TM) technology: The quest for a "democratic proteome", Proteomics 6 (2006) 3980-3992.

[134] P.G. Righetti, E. Boschetti, A. Zanella, E. Fasoli, A. Citterio, Plucking, pillaging and plundering proteomes with combinatorial peptide ligand libraries, J. Chromatogr. A 1217 (2010) 893-900.

[135] G. Załoga, R. Siddiqui, Biologically active dietary peptides, Mini-Rev. Med. Chem. 4 (2004) 815-821.

[136] P. Minkiewicz, J. Dziuba, A. Iwaniak, M. Dziuba, M. Darewicz, BIOPEP database and other programs for processing bioactive peptide sequences, J. AOAC Int. 91 (2008) 965-980.

[137] M.C. García, P. Puchalska, C. Esteve, M.L. Marina, Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities, Talanta 106 (2013) 328-349.

[138] P. Puchalska, M. Marina, M. García, Isolation and characterization of peptides with antihypertensive activitiy in foodstuffs, Crit. Rev. Food Sci. Nutr. (DOI 10.1080/10408398.2012.664829).

[139] A. Pihlanto, Antioxidative peptides derived from milk proteins, Int. Dairy J. 16 (2006) 1306-1314.

[140] R.J. Elias, S.S. Kellerby, E.A. Decker, Antioxidant activity of proteins and peptides, Crit. Rev. Food Sci. Nutr. 48 (2008) 430-441.

[141] B.H. Sarmadi, A. Ismail, Antioxidative peptides from food proteins: A review, Peptides 31 (2010) 1949-1956.

[142] C. Megias, J. Pedroche, M. M. Yust, M. Alaiz, J. Girón-Calle, F. Millan, J. Vioque, Purification of angiotensin converting enzyme inhibitory peptides from sunflower protein hydrolysates by reverse-phase chromatography following affinity purification, LWT-Food Sci. Technol. 42 (2009) 228-232.

[143] E. Escudero, M. A. Sentandreu, K. Arihara, F. Toldrá, Angiotensin I-converting enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat, J. Agric. Food Chem. 58 (2010) 2895-2901.

[144] E. Escudero, F. Toldrá, M. A. Sentandreu, H. Nishimura, K. Arihara, Antihypertensive activity of peptides identified in the in vitro gastrointestinal digest of pork meat, Meat Sci. 91 (2012) 382-384.

[145] M.S. Nielsen, T. Martinussen, B. Flambard, K.I. Sorensen, J. Otte, Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time, Int. Dairy J. 19 (2009) 155-165.

[146] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion, J. Chromatogr. A 1049 (2004) 107-114.

[147] J. Otte, T. Lenhard, B. Flambard, K.I. Sorensen, Influence of fermentation temperature and autolysis on ACE-inhibitory activity and peptide profiles of milk fermented by selected strains of Lactobacillus helveticus and Lactococcus lactis, Int. Dairy J. 21 (2011) 229-238.

[148] J. Gómez-Ruiz, M. Ramos, I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1054 (2004) 269-277.

[149] J. Zhang, H. Zhang, L. Wang, X. Guo, X. Wang, H. Yao, Isolation and identification of antioxidative peptides from rice endosperm protein enzymatic hydrolysate by consecutive chromatography and MALDI-TOF/TOF MS/MS, Food Chem. 119 (2010) 226-234.

[150] C. Beermann, M. Euler, J. Herzberg, B. Stahl, Anti-oxidative capacity of enzymatically released peptides from soybean protein isolate, Eur. Food Res. Technol. 229 (2009) 637-644.

[151] A. Tsopmo, A. Romanowski, L. Banda, J.C. Lavoie, H. Jenssen, J.K. Friel, Novel anti-oxidative peptides from enzymatic digestion of human milk, Food Chem. 126 (2011) 1138-1143.

[152] C. Zhu, W. Zhang, G. Zhou, X. Xu, Z. Kang, Y. Yin, Isolation and identification of antioxidant peptides from Jinhua Ham, J. Agric. Food Chem. 61 (2013) 1265-1271.

[153] H. Zhang, W.H. Yokoyama, H. Zhang, Concentration-dependent displacement of cholesterol in micelles by hydrophobic rice bran protein hydrolysates, J. Sci. Food Agric. 92 (2012) 1395-1401.

[154] F. Zhong, X. Zhang, J. Ma, C.F. Shoemaker, Fractionation and identification of a novel hypocholesterolemic peptide derived from soy protein Alcalase hydrolysates, Food Res. Int. 40 (2007) 756-762.

[155] O.A. Alhaj, A.D. Kanekanian, A.C. Peters, A.S. Tatham, Hypocholesterolaemic effect of Bifidobacterium animalis subsp lactis (Bb12) and trypsin casein hydrolysate, Food Chem. 123 (2010) 430-435.

[156] K. McCann, B. Shiell, W. Michalski, A. Lee, J. Wan, H. Roginski, M. Coventry, Isolation and characterisation of a novel antibacterial peptide from bovine alpha(s1)-casein, Int. Dairy J. 16 (2006) 316-323.

[157] H.A. Elbarbary, A.M. Abdou, Y. Nakamura, E.Y. Park, H.A. Mohamed, K. Sato, Identification of novel antibacterial peptides isolated from a commercially available casein hydrolysate by autofocusing technique, Biofactors 38 (2012) 309-315.

[158] C. De Simone, G. Picariello, G. Mamone, P. Stiuso, A. Dicitore, D. Vanacore, L. Chianese, F. Addeo, P. Ferranti, Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey, J. Pept. Sci. 15 (2009) 251-258.

[159] H. Hou, Y. Fan, B. Li, C. Xue, G. Yu, Z. Zhang, X. Zhao, Purification and identification of immunomodulating peptides from enzymatic hydrolysates of Alaska pollock frame, Food Chem. 134 (2012) 821-828.

[160] K. Lee, S. Kim, SSGE and DEE, new peptides isolated from a soy protein hydrolysate that inhibit platelet aggregation, Food Chem. 90 (2005) 389-393.

[161] A.G.P. Samaranayaka, D.D. Kitts, E.C.Y. Li-Chan, Antioxidative and angiotensin-1-converting enzyme inhibitory potential of a pacific hake (merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 Cell permeation, J. Agric. Food Chem. 58 (2010) 1535-1542.

[162] B. Sarmadi, A. Ismail, M. Hamid, Antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of cocoa (Theobroma cacao L.) autolysates, Food Res. Int. 44 (2011) 290-296.

[163] E. Escudero, M. Aristoy, H. Nishimura, K. Arihara, F. Toldrá, Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham, Meat Sci. 91 (2012) 306-311.

[164] B.F. Gibbs, A. Zougman, R. Masse, C. Mulligan, Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food, Food Res. Int. 37 (2004) 123-131.

[165] G. Li, G. Le, Y. Shi, S. Shrestha, Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, Nutr. Res. 24 (2004) 469-486.

[166] C. Megias, J. Pedroche, M.D.M. Yust, M. Alaiz, J. Girón-Calle, F. Millan, J. Vioque, Affinity purification of angiotensin converting enzyme inhibitory peptides using immobilized ACE, J. Agric. Food Chem. 54 (2006) 7120-7124.

[167] D. Cushman, H. Cheung, Spectrophotometric assay and properties of angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637.

[168] E. Escudero, M. A. Sentandreu, K. Arihara, F. Toldrá, Angiotensin i-converting enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat, J. Agric. Food Chem. 58 (2010) 2895-2901.

[169] E. Boelsma, J. Kloek, Lactotripeptides and antihypertensive effects: a critical review, Br. J. Nutr. 101 (2009) 776-786.

[170] D. Huang, B. Ou, R. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (2005) 1841-1856.

[171] M. Danquah, D. Agyei, Pharmaceutical applications of bioactive peptides, OA Biotechnology. 29 (2012) 1-7.

[172] K. Rutherfurd, H. Gill, Peptides affecting coagulation, Br. J. Nutr. 84 (2000) S99-S102.

[173] B. Gibbs, A. Zougman, R. Masse, C. Mulligan, Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food, Food Res. Int. 37 (2004) 123-131.

Article 1

Isolation and characterization of peptides with antihypertensive activity in foodstuffs

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Abstract

Hypertension is one of the main causes of cardiovascular diseases. Synthetic drugs inhibiting ACE activity present high effectiveness in the treatment of hypertension but cause undesirable side effects. Unlike these synthetic drugs, antihypertensive peptides do not show any adverse effect. These peptides are naturally present in some foods and since hypertension is closely related to modern diet habits, the interest for this kind of foods is increasing. Different methods for the purification, isolation, and characterization of antihypertensive peptides in foods have been developed. Nevertheless, there is no revision work summarizing and comparing these strategies. In this review, *in vivo* and *in vitro* pathways to obtain antihypertensive peptides have been summarized. The ACE mechanism and the methodologies developed to assay the ACE inhibitory activity have also been described. Moreover, a comprehensive overview on the isolation, purification, and identification techniques focusing on the discovery of new antihypertensive peptides with high activity has been included. Finally, it is worthy to highlight that the quantitation of antihypertensive peptides in foods is a new trend since genotype and processing conditions could affect their presence. Analytical methodologies using mass spectrometry constitute an interesting option for this purpose.

Keywords:

ACE; Chromatography; Bioactivity; Peptide; Protein; Enzyme

1. Introduction

Traditionally, the dietetic value of a protein was evaluated from its nutritional quality, mainly by the presence of antinutrients and availability of essential amino acids. A new aspect to take into account is the possibility of generating bioactive peptides. Bioactive dietary components are defined as `*food components that can affect biological processes or substrates and hence have an impact on body function or condition and ultimately health*' [1]. Since any consumed dietary component in enough quantity could be described by this definition, two caveats should be added: the component should impart a measurable effect at a realistic physiological level and the measured 'bioactivity' has to show a potential beneficial health effect [1, 2]. Bioactive peptides can be naturally presented in foods but the most usual scenario is that they are encrypted in parent proteins [2-5].

Several bioactive peptides from different origins such as milk [6-8] and soybean [5] have been released, isolated, characterized, and briefly reviewed. Moreover, peptides showing numerous bioactivities such as antihypertensive, antilipemic, anticarcinogenic, antioxidative, antimicrobial, antiamnestic, opiate, antithrombotic, osteoprotective, vasodilatative, immunomodulating were thoroughly described [3-5, 9]. According to the BIOPEP database, 37 different types of bioactivities have been gathered for more than 1950 peptides [10]. Among them, peptides with antihypertensive activity are the most prevalent.

Hypertension or high blood pressure is attributed by World Health Organization (WHO) as the fundamental source of cardiovascular mortality. Worldwide high blood pressure was estimated to be the cause of 7.6 million premature deaths (13.5% of the total premature deaths) [11]. Additionally, hypertension along with other cardiovascular risk factors (high cholesterol, high Body Mass Index, low fruit and vegetable intake, smoking, and alcohol intake) were established to be the cause of about 83-89% ischaemic heart disease cases and 70-76% of strokes in the world [12]. Furthermore, hypertension can lead to cardiac arrhythmia, coronary heart disease, heart and renal failure, disability and death [13] and, in accordance to the World Hypertension League (WHL), over 50% of the hypertension population is unaware of their condition [14]. Hypertension can be treated with distinct medications such as nitrates, diuretics, β-blockers, α-adrenergic antagonist, vasodilators, dopamine agonists, calcium channel blockers (CCBs), and angiotensin converting enzyme (ACE) inhibitors [15]. Among them, ACE inhibitors are mostly employed for showing greater effectiveness and lower side effects [16].

First ACE inhibitor was described by Ferreira *et al.* (1970). It was a bradykinin potentiator and was isolated from snake (*Bothrops jararaca*) venom [17]. The first synthetic ACE inhibitor adopted for hypertension therapy was [2S]-1-[3-mercapto-2-methylpropionyl]-L-proline (captopril) [18]. Afterwards, several other synthetic ACE inhibitors were employed for treatment of hypertension (enalapril, lisinopril, alecepril or fosinopril) although they provoke adverse effects such as skin rashes, cough, angioedema, taste disturbances, hypotension, reduced renal function, increased potassium levels, and fetal abnormalities [19, 20]. Unlike these drugs, antihypertensive peptides do not yield any adverse effect but are usually less potent in comparison to synthetic substances [21]. Indeed, seven dipeptides isolated from garlic showed decreasing systolic blood pressure (SBP) after oral administration of 200 mg/kg in spontaneously hypertensive rats (SHRs). However, none of these peptides lowered SBP as much as captopril which was used as a positive control test [22]. There are some exceptions to this fact such as a peptide isolated from tuna frame protein peptic hydrolysate and the milk peptides VPP and IPP that exert antihypertensive effects comparable with captopril [21, 23].

Since hypertension is closely related to modern diet habits, interest in functional foods with antihypertensive activity is worth great consideration. Therefore, the aim of this work has been to review the methodologies used to isolate, purify, identify, and characterize food peptides with antihypertensive activity.

2. ACE and blood pressure (BP)

Several interacting biochemical pathways are associated with the control of blood pressure (BP) in living organisms, the *renin-angiotensin* system being the most important. Additionally, kinin-nitric oxide system, endothelin converting enzyme system, and neutral endopeptidase system are also recognized to have influence on BP.

Renin-angiotensin system is shown in Fig. 1. Angiotensinogen is the first link of the reaction chain in the renin-angiotensin system. It is the precursor of Angiotensin I (Ang I- DRVYIHPFHL). In fact, it converts to Ang I in the presence of renin (E.C. 3.4.23.15) in the bloodstream. On the other hand, Ang I hydrolyzes by the removal of the C-terminal dipeptide HL to Angiotensin II (Ang II- DRVYIHPF) through the action of angiotensin I converting enzyme (ACE; kinases II peptidyldipeptide hydrolase). Afterwards, Ang II is distributed in the blood until it is inactivated by aminopeptidase A (E.C. 3.4.11.7) or N (E.C. 3.4.11.2) enzymes and it's converted to Angiotensin III and IV (RVYIHPF and VYIHPF, respectively). Ang II peptide causes vasoconstriction by the activation of the AT1 receptor (AT1R) which leads to

increase in the BP. Furthermore, Ang II negatively affects kidney retaining salts and water, causing an increase in extracellular fluid volume and, in a consequence, an increase in the BP [20, 24, 25].

Moreover, ACE also removes a dipeptide from C-terminus of bradykinin (RPPGFSPFR) resulting in the inactivation of this vasodilator. Therefore, ACE inhibitors decrease the BP not only by lowering the level of Ang II but also by increasing the level of bradykinin. Since the inhibition of ACE causes an effective decrease in BP, most antihypertensive drugs employ this mechanism for the treatment of hypertension.

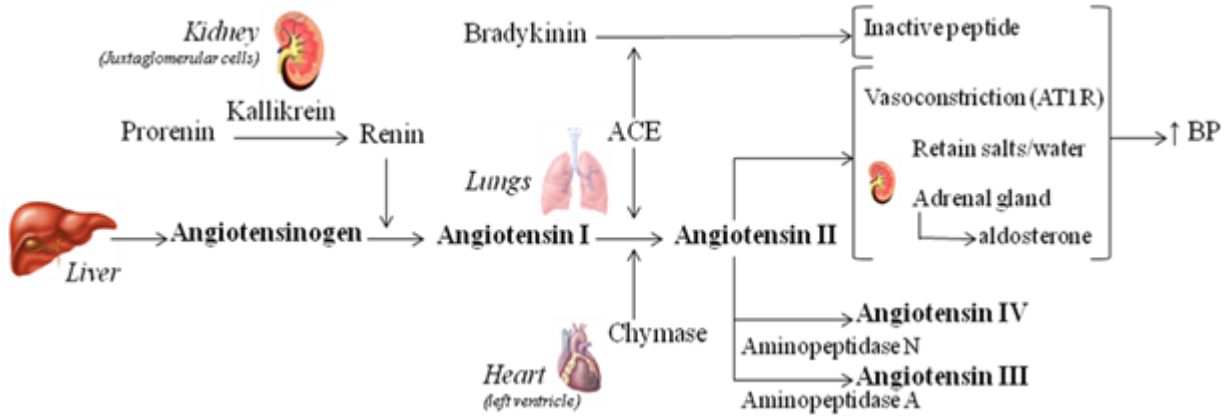

Fig. 1. Scheme of Renin- Angiotensin System.

3. Release of antihypertensive peptides from foodstuffs

Antihypertensive peptides used to be encrypted in a parent protein from which they need to be released in order to exert its ability to inhibit ACE. Two main proteolytic pathways can be distinguished, *in vivo* and *in vitro*. The first one involves the *in vivo* digestion of the parent protein by the action of gastrointestinal enzymes while the second one involves food or protein processing before its ingestion. Moreover, some bioactive peptides cannot be liberated by gastrointestinal enzymes and so have to be synthetically produced, added to foods, and supplied as functional foods [26].

The first antihypertensive peptide isolated and identified from food was described in 1982 by Maruyama and Suzuki [27]. Casein from bovine milk was subjected to hydrolysis by trypsin and purified by several chromatographic steps. A peptide with 12 amino acids and sequence

FFVAPFPEVFGK was identified. The knowledge about preparation, purification, and identification of antihypertensive peptides from food steadily increased since this first discovery, especially in the case of milk derived peptides [28-31]. In this case, advances have even enabled the development of commercial milk products enriched with antihypertensive peptides (Table 1) [29, 32].

In addition to milk and dairy products, several other foodstuffs have been examined as potential sources of peptides with ACE inhibition activity. Marine foods (shrimps, sea cucumber, blue mussel), fishes (alaska pollock, bonito, salmon, pacific hake), meat (pork, bullfrog, chicken), vegetable foods (soybean, wheat products, rice, garlic, aramanth grain), mushrooms or processed products (miso paste, douche, wakame, royal jelly, soybean sauce or paste) are some examples. The most common way to induce the *in vitro* release of antihypertensive peptides is enzymatic digestion (hydrolysis) and fermentation with bacterial organisms.

Table 1. Commercially available milk products enriched with antihypertensive peptides. Adapted from: [29, 32].

Brand name	Company, country	Bioactive peptide
Ameal S ®	Calpis Co., Japan	VPP, IPP
BioZate ®	Davisco, USA	----
Calpis ®	Calpis Co., Japan	VPP, IPP
Casein DP ®	Kanebo Ltd., Japan	FFVAPFEVFGK
C12 peptide ®	DMV International, Holland	FFVAPFEVFGK
Danten ®	Danone, France	----
Evolus ®	Valio, Finland	VPP, IPP

With regards to enzymatic digestion, the composition of hydrolysate depends on several parameters such as the enzyme to substrate ratio, hydrolysis time, pH, temperature of hydrolysis, *etc.* but it mostly depends on the type of proteolytic enzyme. Most commonly used enzymes are pepsin [21, 33], thermolysin [34, 35], and alcalase [36-38]. They cleave peptide bonds near to hydrophobic amino acid residues, resulting in peptides with the most favorable amino acid residues for antihypertensive activity at the C-terminal position [37, 39].

Different strategies have been followed to increase antihypertensive activity. Pepsin treatment followed by digestion with pancreatin [40-42], corolase PP [43] or trypsin (E.C. 3.4.21.4) with chymotrypsin (E.C. 3.4.21.1) [43-45] has usually been employed to obtain smaller peptides with greater antihypertensive effects. Moreover, since these enzymes are present during gastrointestinal digestion, it is possible to assess whether these peptides will be inactivated during this process or not. Quiros *et al.* [46] attempted to promote the release of bioactive peptides from ovalbumin with chymotrypsin, trypsin, and pepsin using high hydrostatic pressures observing that antihypertensive effect of certain peptides improved when pressures of 200-400 MPa were employed. Another strategy for increasing antihypertensive activity was explored by Jia *et al.* [47]. They evaluated the effect of ultrasonic irradiation on the hydrolysis and the ACE inhibitory activity of defatted wheat germ protein (DWGP). Results suggested that this approach improved enzymatic hydrolysis by promoting the release of peptides. Moreover, some authors have demonstrated an increase in the antihypertensive activity of foodstuffs by the combination of bacterial fermentation and enzymatic digestion. Tonouchi *et al.* [48] observed these results when using different enzymes to digest a Danish skim milk-cheese previously fermented with *Lactococcus*. Similarly, Hernández-Ledesma *et al.* [49] found a higher number of antihypertensive peptides when a milk sample fermented with *Lactobacillus rhamnosus* was subjected to simulated gastrointestinal digestion. Chobert *et al.* [50] compared the antihypertensive activity of peptides obtained from ovine milk by tryptic digestion and fermentation with different bacterial strains. Fermentation yielded higher ACE inhibitory activity than digestion, probably because fermentation yielded peptides with lower molecular masses [50].

On some occasions, the foodstuffs contain antihypertensive peptides that are not encrypted in any protein and fermentation or digestion is not necessary. For example, few peptides which exerted antihypertensive activity were detected in garlic (*allium sativum* L) [22], in various kinds of mushrooms (*Pholiota adiposa, Tricholoma giganteum*) [51, 52], in soypaste [53], and in different kind of cheeses (gouda, manchego, and varieties of Spanish and Swiss cheeses) [54-57]. These peptides can simply be extracted with water or alcohols like ethanol or methanol. With this in mind, it is possible to differentiate between processed and unprocessed products. Unprocessed products are garlic or mushrooms while processed products comprised soypaste and cheese. The manufacture of these products involves the use of enzymes or bacterial organisms but, in no case, they are used to release antihypertensive peptides.

The other way to release peptides without the addition of bacterial organisms or enzymes is autolyzation. Autolyzation involves the employment of proteolytic enzymes which are already ingredients of foodstuffs. This approach was followed for the preparation of hydrolysates of bonito bowels [58, 59], pacific hake fish [60] or wheat bran [61]. A similar approach was also used when proteins of oyster and blue mussel were fermented without any addition of bacterial organism for 6 months at 20°C in salty conditions. In both cases, antihypertensive peptides were obtained after long-term fermentations [62, 63].

4. Determination of ACE inhibitory activity of food peptides

The general framework of the experimental investigation for the production, purification, and identification of antihypertensive peptides is presented in Fig. 2. Work strategies commonly consist of the release of peptides, and the isolation, purification, identification, and determination of the amino acid sequence. After each step, the screening of ACE inhibitory activity is crucial to select the experimental conditions or fractions with the most potential antihypertensive abilities.

ACE inhibition activity is expressed using an IC_{50} index which represents the required concentration of a particular substance in order to inhibit 50% of the ACE activity. Different assays have been developed to determine the ACE inhibition value *in vitro*. Initially, the assays were based on the employment of Ang I or bradykinin as an ACE substrate and measurement of the generated product was done by radiochromatography, colorimetry or radioimmunoassays in the presence or absence of the inhibitor. Nevertheless, the problem related to the interferences produced by other peptidases that were degradating substrates or products of ACE, led to erroneous results. Then, assays using artificial substrate started to play a considerable role since they were inexpensive, easy to obtain, not liable to be hydrolyzed by peptidases and presented a higher dissociation constant for ACE [64]. Released compounds by the action of ACE could be quantified -through a spectrophotometric [65], fluorometric [66], HPLC [67], CE [68] or by a radiometric method [69].

Nowadays, the most broadly spread method for the determination of ACE inhibition activity is that developed by Cushman and Cheung (1971) [70]. It is based on the reaction between hippuryl-L-histydyl-L-leucine (HHL) used as substrate and ACE and shows a subsequent formation of hippuric acid (HA). The ACE activity is directly related to the extent of HA liberated from HHL.

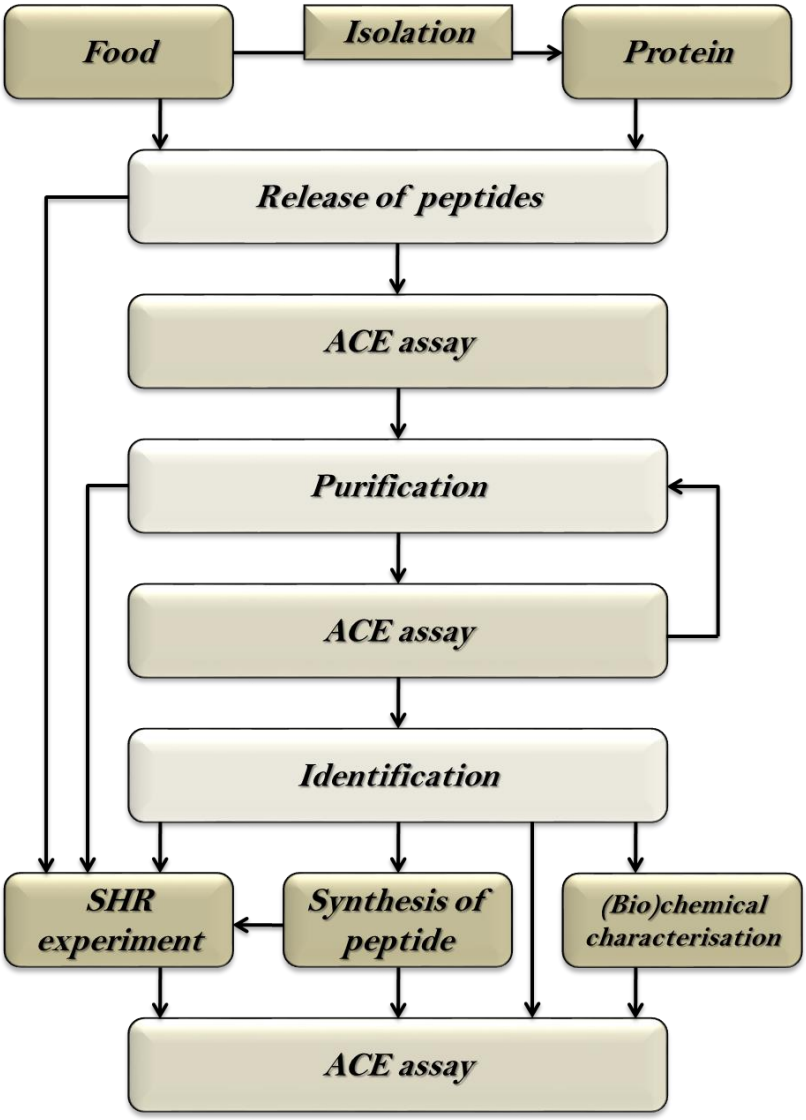

Fig. 2. Framework of production, purification, and identification of bioactive peptides with antihypertensive activity.

The extent of this reaction in the presence or absence of inhibitory peptides is evaluated by measuring the amount of formed HA from its absorbance at 228 nm. In this first approach ACE was acetone extracted from rabbit lung. Further modification of this procedure employed pure ACE from a rabbit's lung in place of their acetone extract [71, 72]. Despite the high selectivity of this reaction, the assay had low sensitivity since unhydrolyzed HHL was co-extracted with HA [28, 64]. Furthermore, long incubation time (around 30 min) was required to obtain enough product amounts to be quantified. Therefore, numerous modifications appeared in the literature, and, as a consequence, the obtained IC_{50} values differed significantly due to comparison amongst themselves not being possible [73]. In fact, the IC_{50} of hydrolysates obtained by digestion of an insect protein with four different enzymes was determined by applying two

different ACE assays. One method was based on the spectrophotometric measurement of FAPGG [2-furanacryloyl-phenylalanyl-glycyl-glycine], used as a substrate, whilst an HPLC method (which adopted DTG [dansyltriglycine] as a substrate) was employed in the second approach. When using the FAPGG method, IC_{50} values were 3.935 ± 0.014 and 0.214 ± 0.179 mg/mL for the nonhydrolyzed and hydrolyzed extract, respectively. The HPLC method yielded IC_{50} values of 22.465 ± 0.615 and 4.969 ± 0.622 mg/mL (with 50 μL of ACE extract) and 43.220 ± 12.66 and 1.253 ± 0.120 mg/mL (with 25 μL of ACE extract), respectively, for the nonhydrolyzed and hydrolyzed extract [74].

As well as to the *in vitro* determination of IC_{50} values, additional experiments are very frequently included to confirm ACE inhibitory activity. Indeed, experiments using spontaneously hypertensive rats (SHR) have also been used for assaying ACE inhibition in living organisms. This type of experiment is usually focused on short and/or long-term administration studies. Nevertheless, results obtained by *in vivo* studies sometimes significantly differ from the results observed by *in vitro* assays. These differences could be justified by the bioavailability of peptides [28, 29]. As an example, a peptide (β- lactosin B, ALPM) derived from a commercial whey product that presented weak ACE inhibitory activity (IC_{50}=928 μM), surprisingly showed a noticeable decrease of SBP after 8 h oral administration (2 mg/mL) to SHRs [75]. In other occasion, Fujita and Yoshikawa [76] compared the ACE inhibitory activity of LKPNM and LKP peptides (obtained by digestion from dried bonito with thermolysin) with captopril using an *in vitro* and an *in vivo* study. The *in vitro* study yielded much lower ACE inhibitory activity for peptides while the *in vivo* study demonstrated that peptides were more effective for reducing BP than captopril [76]. The differences between the two approaches to assess ACE inhibitory activity also appeared when Yamamoto *et al.* [30] purified and characterized a dipeptide from a yoghurt-like product. The IC_{50} value of this dipeptide was estimated as 720 μM which would classify it as a peptide with moderate antihypertensive activity. However, the same peptide provoked similar *in vivo* antihypertensive activity to IPP and VPP, which are generally categorized as peptides with very high activity (IC_{50} values, 5.15 and 9.13 μM, respectively) [30].

In addition to the estimation of the IC_{50} value, some authors also pay attention to additional measurements such as simulated gastrointestinal digestion or Caco2 cell monolayer transport. These experiments can yield information on the bioavailability of target compounds and will be discussed later. Additionally, the activity of peptides may be affected by factors such as the

amino acid composition, hydrophobicity, size, stability, processing or the mechanism of action [77]. In fact, ACE inhibition activity is significantly influenced by the position of proline in the amino acid sequence, by protein hydrophobicity, and by the size of released peptides.

The following examples demonstrate this point. Different protein sources were hydrolyzed in the same conditions: soybean protein, wheat gluten, caseinate, and whey proteins. The IC_{50} for these hydrolysates was 180, 340, 100, and 200 μg/mL, respectively. High antihypertensive activity of casein (100 μg/mL) could be attributed as much for their high hydrophobicity as for the high amount of encrypted prolines in its primary structure. Despite wheat gluten, also containing high amounts of proline residues but with lower hydrophobicity, yielded a significantly high IC_{50} value (340 μg/mL) [73]. In addition to the IC_{50}, another important parameter to take into account is the degree of hydrolysis (DH). This parameter is commonly calculated by the o-phtaldialdehyde (OPA) method [38, 78]. Nevertheless, other methods and techniques have also been employed for this purpose, such as SDS-PAGE [79], calculation of α-amino nitrogen and total nitrogen, [80] or the calculation of the relative peak area in regards to whole protein [50]. Yak milk casein was hydrolyzed by alcalase at pH 8 and 55°C in times ranging from 0 to 340 min. It was observed that at 240 min of hydrolyzing, the ACE inhibitory activity reached the maximum level and DH was correlated with it. After this time, DH was too high and inhibition activity decreased due to the hydrolysis of small peptides with antihypertensive activity [80]. However, when the same yak milk casein was hydrolyzed with various enzymes (trypsin, pepsin, alcalase, flavourzyme, papain, and neutrase) at their optimal pH and temperature and at different times till 12 h, the DH was not correlated with the ACE inhibition activity. ACE inhibition activity was the poorest when using flavourzyme despite its high DH. Inversely, the most promising antihypertensive activities were obtained with papain and neutrase which showed low DH [78]. Similar results were also observed when milk was fermented by 13 different strains of lactic acid bacteria [81] and when a soybean protein isolate was hydrolyzed by different enzymes (alcalase, flavourzyme, trypsin, chymotrypsin, and pepsin) [38].

5. Isolation and purification of bioactive peptides from foodstuffs

The purification of a hydrolysate showing antihypertensive activity is one of the most important steps in the framework presented in Fig. 2. The purification pathway could significantly influence the number of identified peptides, their activity and characteristics (*e.g.* size of the peptide and their composition), and their properties. Generally, Liquid

Chromatography (LC) is the most often employed technique. Different chromatographic modes can be selected from the base of the properties of ACE inhibitory peptides. After each chromatographic step, fractions with the highest *in vitro* ACE inhibitory activity are lyophilized and subjected to the next chromatographic step till pure peptide/s is/are obtained.

Table 2 summarizes the methods that have been employed for the release and purification of peptides with antihypertensive properties. Despite there being some general approaches that are more or less common in all procedures, the number of purification steps in each case depends on the complexity of the sample and the dynamic range and abundance of peptides [90, 135].

Generally, the first step in the purification process is separation based on the size of the peptide using either ultrafiltration (UF) or size-exclusion chromatography (SEC). UF is a low-pressure technique where the solution is processed through a semi permeable membrane and molecules are isolated by molecule size. Moreover, UF also enables the concentration and enrichment of fractions by the removal of the solvent. What is more, it is quite easy to use, it does not require special equipment, and can be used at cold room temperature [136]. UF enables the separation of small antihypertensive peptides from bigger molecules, such as unproteolyzed proteins and other interferences, thus in many cases it is the first step towards purification. Despite the fact that membranes with Mw in the range of 1-30 kDa have been tried, smaller cut-off membranes are preferred. For example, a hydrolysate of sea cucumber gelatin was subjected to UF using membranes with cut-offs of 10, 5, and 1 kDa observing IC_{50} values of 0.72, 0.47, and 0.35 mg/mL, respectively. Based on the ACE inhibition activity, the fraction containing molecules smaller than 1 kDa were purified [122]. However, very low Mw cut-off membranes can sometimes result in a loss of activity [60, 96, 137, 138].

Table 2. Purification of peptides with antihypertensive activity (*- indicates articles in which not all data were shown)

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
MILK AND DAIRY PRODUCTS				
Milk	Fermentation/ digestion	Several bacterial strains or *Lactbacillus rhamnosus* and pepsin, corolase PP	UF: M_{WCO} 3 kDa RP-LC: Widepore C18, 250 x 4.6 mm; 0-45% B in 60 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; UV detection (220 nm)	[49]
Milk	Fermentation	Strains *Enterococcus faecalis*	Centrifugation (20,000g, 10 min, 10°C) / filtration (Whatman no. 40) RP-LC: Widepore C18, 250 x 4.6 mm; 0-45% B in 60 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; MS detection	[82]
Milk	Fermentation	Strains *Enterococcus faecalis*	Extraction followed by centrifugation (20000g, 10 min, 10˚C) and filtration (Whatman no. 40) UF: M_{WCO} 3 kDa RP-LC: Prep Nova Pak® HR C18, 300 x 7.8 mm; 0-35% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm) RP-LC: Prep Nova Pak® HR C18, 300 x 7.8 mm; 20-35% B in 40 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm)	[83]
Goat milk*	Digestion	Alcalase	RP-LC: Resource RP; linear gradient of 0.05% TFA and 84% ACN+0.05% TFA, 40 min; 1 mL/min; UV detection (220 nm) Caco2 cell RP-LC: Zorbax 5 C18, 2.1 x 250 mm; 0-30% in 60 min; 30-80% in 10 min; A: 0.05% TFA; B: ACN + 0.04% TFA; 350 µL/min; UV detection (220 nm)	[84]
Ovine milk	Simulated gastrointestinal digestion	Pepsin, trypsin with chymotrypsin or pepsin and corolase PP	UF: Centrifugation/ filtration (Whatman no. 40); M_{WCO} 3 kDa RP-LC: Prep Nova Pak® HR C18, 300 x 7.8 mm; 0-40% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm) CE: bare fused silica capillary, 76 cm x 50 µm; 0.9 M HFo, pH=2.0; 18 kV; MS detection	[43]
Ovine β-lacto-globulin from skimmed milk	Digestion	Trypsin	RP-LC: Nucleosil C18, 250 x 4 mm; 10-100% B in 23 min; A: 0.11% TFA; B: 80% ACN + 0.09% TFA; 0.6 mL/min	[50]
Yak milk casein	Digestion	Trypsin, pepsin, alcalase, flavourzyme, papain or neutrase	UF: M_{WCO} 10 kDa and 6 kDa SEC: Sephadex G- 25, 26 x 800 mm; elution with water; 0.6 mL/min; UV detection (215 nm); RP-LC: Shim-pack PREP-ODS C18, 20 x 250 mm; 10-60% B in 35 min; A: 0.05% TFA; B: ACN + 0.05% TFA; 6 mL/min; UV detection (215 nm) RP-LC: C18, 4.6 x 250 mm; 20-50% B in 20 min; A: 0.05% TFA; B: ACN + 0.05% TFA; 1 mL/min; UV detection (215 nm)	[78]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Yak milk casein	Digestion	Alcalase	UF: M_{WCO} 10 kDa and 6 kDa IEC: DE-52, 1.6 x 30 cm; 0-0.4 mM NaCl in 5 mM PBS, pH 8.0; 24 mL/h; UV detection (220 nm) SEC: Sephadex G-25, 2.5 x 100 cm; elution with water; 16 mL/h; UV detection (220 nm) RP-LC: Zorbax Eclipse XDB-C18, 10 x 400 mm; 0–60% B (B: ACN + 0.1% TFA) in 45 min; 1 mL/min; UV detection (214 nm) RP-LC: Zorbax Eclipse XDB-C18, 2.1 x 150 mm; A: 0.1% TFA; B: ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm)	[80]
Casein	Fermentation	*Lactobacillus helveticus CP790*	RP-LC: µ-Bondashere C18, 3.9 x 150 mm; 0-40% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RP-LC: µ-Bondashere C18, 3.9 x 150 mm; 90-30% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)	[85]
Skimmed milk	Fermentation	Strains *Lactobacillus helveticus JCM1004*	RP-LC: YMC-Pack ODS-AP-303, 4.6 x 250 mm; 0-100% B; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm) RP-LC: ODS-100S, 3.9 x 150 mm; 0-40% B in 40 min, 40-70% B in 28 min, 70-100% B in 22 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm) RP-LC: µBondasphere C18, 3.9 x 150 mm; 0-30% B in 45 min, 30-65% B in 25 min, 65-100% B in 20 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.8% TFA; 1 mL/min; UV detection (215 nm)	[86]
Sheep milk yoghurt	Fermentation	Different bacterial cultures	RP-LC: Nucleosil C18, 250 x 4 mm; 0% B in 10 min; 0-80% B in 80 min; 100% B in 10 min; A: 0.1% TFA; B: 60% ACN + 0.09% TFA; 0.8 mL/min; UV detection (214 nm) RP-LC: Nucleosil C18, 250 x 4 mm; 0% B in 10 min; 0-80% B in 80 min; 100% B in 10 min; A: 0.1% TFA; B: 60% ACN + 0.09% TFA; 0.2 mL/min; UV detection (278 nm)	[87]
Yoghurt	Fermentation	Different bacterial cultures	RP-LC: C-18 monomeric, 250 x 4.6 mm; 0-100% B in 90 min; A: 0.1% TFA; B: 90% ACN + 0.1% TFA; 0.75 mL/min; UV detection (214 nm) CE: Coated capillary, 50 cm x 50 µm; 30 mM Na_3BO_3 and 17 mM PBS, pH=8.2; 15 kV, 30 min; 20˚C; UV detection (214 nm)	[88]
Yoghurt-like product	Fermentation	*Lactobacillus helveticus CPN4*	Extraction in Sep-pak C-18 cartridges; elution with different ACN ratios 10-50% B (ACN + 0.1% TFA) RP-LC: µ-Bondasphere C18, 3.9 x 150 mm; 100-60% in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RP-LC: µ-Bondasphere C18, 3.9 x 150 mm; 5-20% B in 60 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)	[30]
Caprine Kefir*	Fermentation	Different bacteria strains	Centrifugation/ filtration/ UF 12,000 x g, 10 min, 5°C/ filter Whatman no. 40/ M_{WCO} 3 kDa RP-LC: 0-30% B in 70 min; A: 0.1% TFA; B: ACN + 0.08% TFA; UV detection (214 nm) RP-LC: 8-20% B in 45 min; A: 0.1% TFA; B: ACN + 0.08% TFA; UV detection (214 nm)	[89]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Cheese	Fermentation/ digestion	*Lactococcus starter culture* (MM100)/ protease N amino, Umamizyme and Flavourzyme	Dialysis: Molecular porous membrane tubing (Spectra/Por 3; M_{WCO} 3.5) against water; 48 h; 4˚C RP-LC: YMC-Pack R&D ODS, 20 x 250 mm; 0-70% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm) RP-LC: TSK-gel ODS 80Ts, 20 x 250 mm; 0-30% B (B: ACN + 0.1% TFA) in 50 min; 7.5 mL/min; UV detection (214 nm) SEC: Superdex Peptide HR 10/30, 10 x 300 mm; elution with water + 0.1% TFA; 0.5 mL/min; UV detection (214 nm) RP-LC: CAPCELL PAK C18 MG, 4.6 x 250 mm; elution with 12% ACN + 0.1% TFA; 0.4 mL/min; UV detection (214 nm)	[48]
Gouda cheese	Extraction	----------	Hydrophobic chromatography in Wakogel LP-40c18 resin; 15-90% ACN RP-LC: Superiorex ODS, 4.6 x 150 mm; 0-100% B in 30 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm) SEC: Superdex Peptide HR 10/30, 1 x 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm) CE: Coated capillary, 24 cm x 25 μm; BGE: 0.1 M PBS, pH=2.5; 10 kV, 15 min; UV detection (200 nm)	[57]
Several Spanish cheeses	Extraction	----------	UF: M_{WCO} 1 kDa RP-LC: Hi-Pore C18, 250 x 4.6 mm; 0-40% B in 60 min; 40-70% B in 5 min; A: 0.037% TFA; B: ACN + 0.027% TFA; 0.8 mL/min; UV detection (214 nm)	[55]
Manchego cheese	Fermentation /ripening	Different bacterial strains	Extraction/ centrifugation/ filtration/ centrifugation/ filtration UF: M_{WCO} 3 kDa cIEC: HiLoadt 26/10 SP Sepharose Fast Flow; 0% B in 20 min; 0- 30% B in 40 min; 30-100% B in 10 min; 100% B in 10 min; A: 10mM HFo; B: 5M NH_4OH; 5 mL/min; UV detection (280 nm) RP-LC:C18 Prep-Nova Pak HR, 300 x 7.8 mm; 5- 50% B in 60 min; 60- 100% B in 10 min; 100% B in 10 min; A: 0.1% TFA; B: 80% ACN + 0.08% TFA; 6 mL/min; UV detection (214, 280 nm) RP-LC: Hi-Pore C18, 250 x 4.6 mm; 10- 25% B in 30 min or 18-23% B in 25 min or 20-24% B in 25 min; A: 0.1% TFA; B: 80% ACN + 0.08% TFA; 0.8 mL/min; UV detection (214, 280 nm)	[54]
Manchego cheese	Fermentation	Several bacterial strains	UF: M_{WCO} 3 kDa RP-LC: Widepore C18, 250 x 4.6 mm; 0-40% A in 60 min; A: 0.037% TFA; B: 80% ACN + 0.027% TFA; 0.8 mL/min; UV detection (220 nm)	[90]
Enzyme – modified cheese	Digestion	Neutrase, Lactobacillus casei enzyme or Debitrase® DBP20	RP-LC: Delta Pak C18, 30 x 150 mm; 20-40% B in 15 min; 40-60% B in 15 min; 60-100% B in 5 min; 100-20% B in 35 min; A: 0.1% TFA; B: 40% ACN + 0.08% TFA; 0.5 mL/min; UV detection (214 nm)	[91]
Cheese whey protein	Digestion	Trypsin, proteinase K, actinase E, thermolysin, papain, chymotrypsin or pepsin	Hydrophobic chromatography in LiChropep RP-18 resin (25-40 μm); 0-90% MetOH Hydrophobic chromatography in LiChropep RP-18 resin (25-40 μm); 30-42% MetOH RP-LC: Superiorex ODS, 4.6 x 150 mm; 0-100% B in 30 min; 100% B in 10 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm) SEC: Superdex peptide HR 10/30, 1 x 30 cm; elution with 0.1% TFA; 0.5 mL/min; UV detection (214 nm) CE: Coated capillary, 24 cm x 25 μm; 0.1 M PBS, pH=2.5; 10 kV; UV detection (200 nm)	[92]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Commercial whey product*	Not shown	Not shown	Extraction with Wakogel LP-40C18 resin (20-40 µm); elution with 90% EtOH SEC: Sephadex G-15, 2.6 x 90 cm; 0.05% TFA; 0.5 mL/min; UV detection (214 nm) RP-LC: Wakosil-II 5C18, 4.6 x 150 mm; 0- 80% B in 15 min; A: 10% ACN + 0.05% TFA; B: 60% ACN + 0.05% TFA; 0.5 mL/min; UV detection (214 nm) CE: Coated capillary, 17 cm x 25 µm; 0.1 M PBS, pH=2.5; 10 kV, 15 min; 20˚C; UV detection (200 nm)	[75]
Sodium caseinate	Fermentation	*Lactobacillus (Lb.) helveticus NCC 2765*	SEC: Superdex 75 HR 10/30; 50 mM NH_4Ac (pH 7) for 65 min; 0.5 mL/min; UV detection (215, 280 nm) RP-LC: C8 208TP54; 0% B in 5 min, 0-50% B in 60 min, 50-100% B in 1 min, 100% B in 4 min; A: 0.05% TFA; B: 80% ACN + 0.045% TFA; 0.8 mL/min; UV detection (215 nm)	[93]
α-lactalbumin[1] and β-casein[2]	Digestion	Thermolysin	SEC: Superdex™ 30 prep grade, 2.6 x 61 cm; 0.1M NH_4HCO_3, pH 8.0; 2.5 mL/min; UV detection (280 nm) RP-LC: Nucleosil 300-S C18, 4.6 x 250 mm; [1]0-80% B (10-90 min); [2]30-55% B (90 min) or [2]2-40% B (80 min); A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (210 and 280 nm) RP-LC: Nucleosil 300-S C18, 4.6 x 250 mm; [1] 20-45% B (80 min) or [1]20-50% B (80 min); A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (210 and 280 nm)	[94]
			PLANT ORIGIN PRODUCTS	
Soybean	Digestion	Pepsin	IEC: Dowex 50 W, 45 x 200 mm; elution with 5 % NH_4OH SEC: Sephadex G-25, 26 x 1400 mm; water; 30 mL/min IEC: Sephadex C-25, 20 x 500 mm; 0- 3% NaCl; 30 mL/min RP-LC: Develosil ODS-5, 4.6 x 250 mm; 0- 16% B (B: ACN + 0.05% TFA) in 60 min; 1 mL/min; UV detection (220 nm)	[95]
Soybean	Fermentation	*Aspergillus oryzae*	UF: M_{WCO} 3, 10, 30 kDa cIEC: HiPreP 16/10 SP FF, 16 x 100 mm; 0-100% B in 40 min: A: 10 mM NaAc, pH 4.0; B: 20 mM NaAc (pH 4.0) in 1 M NaCl; 5 mL/min; UV detection (214 nm) Desalination: Cellulose dialysis membrane, M_{WCO} 100 SEC: Superdex Peptide 10/300 GL, 10 x 300 mm; elution with 30% ACN in 40 min; 0.36 mL/min; UV detection (214 nm) RP-LC: µBondapak™ C18, 4.6 x 300 mm; 0-100% B in 40 min; A: 0.1% TFA; B: 40% ACN + 0.1% TFA; 1 mL/min; UV detection (214 nm) RP-LC: µBondapak™ C18, 4.6 x 300 mm; 0-100% B in 40 min; A: 25 % ACN + 0.1% TFA; B: 35% ACN + 0.1% TFA; 1 mL/min; UV detection (214 nm)	[96]
Soybean protein	Digestion	Mature D3 protease	Desalination by electrodialyzation SEC: Superdex Peptide HR 10/300; elution with 0.05 % TFA; 0.5 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5C18 AR 4.6/250; 0-50% B in 50 min; A: 0.05% TFA; B: ACN + 0.065% TFA; 0.75 mL/min; UV detection (215 nm)	[73]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Glycinin from soybean*	Digestion	Protease P, trypsin, chymotrypsin or ginger protease	RP-LC: C-18 Shimpak, 250 x 4.6 mm; 0-35% B; A: 0.1% TFA; B: 70% ACN + 0.05% TFA; 0.7 mL/min; UV detection (230 nm) RP-LC: C-18 Shimpak, 250 x 4.6 mm; 0-100% B; A: 50 mM NH_4Ac; B: 50 mM NH_4Ac/ ACN (50:50); UV detection (230 nm) RP-LC: C-18 Shimpak, 250 x 4.6 mm; 0-35% B; A: 0.1% TFA; B: 70% ACN + 0.05% TFA; 0.7 mL/min; UV detection (230 nm)	[97]
Steamed soybean mixed with roasted wheat	Fermentation	*Tane koji* rich in *A. sojae*	Desalination by electrodialyzing RP-LC: SP-120-40/60-ODS-B, 150 x 1000 mm; 0-100% B in 25 h; water- 0.1% TFA-ACN; 45 mL/min; UV detection (220 nm) RP-LC: Cosmosil-5C18-ARII, 20 x 250 mm; 0-100% B in 90 min; water- 0.1% TFA-ACN; 5 mL/min; UV detection (220 nm) RP-LC: C30 Develosil RPAQUEOUS-AR, 20 x 250 mm; 0-100% B in 90 min; water-ACN; 5 mL/min; UV detection (220 nm)	[98]
Wheat bran*	Autolysis	Endogenous proteases	RP-LC: LiChroprep RP-18, 2.5 x 25 cm; 10-95% EtOH IEC: AG MP-1 resin, 3 x 20 cm SEC: Superdex 75HR, 10 x 30 cm; elution with 30% ACN + 0.1% TFA; 0.5 mL/min; UV detection (220 nm) RP-LC: Jupiter C4, 10 x 250 mm; 0-35% ACN + 0.1% TFA in 30 min; 4 mL/min RP-LC: Jupiter C18, 10 x 250 mm; 0-35% ACN + 0.1% TFA in 30 min; 4 mL/min	[61]
Wheat gliandin	Digestion	Pepsin and protease M	IEC: SP-Toyopearl 550C, 2.6 x 40 cm; 0-0.5M NaCl in 5 mM NaAc, pH 3.5; 1 mL/min; UV detection (220 nm) SEC: Bio-gel P-2, 1.6 x 100 cm; elution with water; 0.33 mL/min; UV detection (220 nm) RP-LC: TSK-GEL ODS 120T, 4.6 x 250 mm; 0-30% ACN + 0.01% TFA; 1 mL/min; UV detection (220 nm)	[99]
White wheat, wholemeal wheat, rye flours*	Fermentation	Different sourdoughs	Extraction with 30 mL 50 mM Tris-HCl, pH 8.8 at 4 °C and centrifugation at 20,000*g*, 20 min RP-FPLC: 5% B 16 min; 5-46% B 46 min; A: 0.05% TFA; B: ACN+0.05%; 1 mL/min; UV detection (214 nm)	[100]
Rice*	Digestion	Alcalase	Desalination with an ion exchange resin SEC: Sephadex G-15, 1.8 x 60 cm; 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm) RP-LC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 250 mm; 0- 60% B (B: ACN + 0.1% TFA) in 60 min; 1 mL/min; UV detection (220 nm) RP-LC: Sephasil Peptide C2/C18 ST 4.6/250, 4.6 x 250 mm; 10-30% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min; UV detection (220 nm)	[101]
Rice	Fermentation	*Monascus* strains	Separation in SEPABEADS SP825 and elution with different EtOH percentages: 10-70% SEC: Sephadex G-25, 1.2 x 142.5 cm; elution with water; UV detection (220 nm) RP-LC: Cosmosil 5 C18-AR-300; 0–50% B (B: ACN + 0.05% TFA) in 50 min; 0.5 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5 C18-AR-300 or Cosmosil 5Ph-AR-300; ACN + 0.05% TFA; 0.25 mL/min; UV detection (220 nm)	[102]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Mush-room *Tricho-loma agiganteum*	Extraction	----------	UF: M_{WCO} 5 kDa SEC: Sephadex G-25, 3.0 x 35 cm; elution with water; 12 mL/min RP-LC: µBondapack C18; 0-100 % B; A: 0.1% TFA; B: ACN RP-LC: µBondapack C18; 0- 100% B; A: 0.1% TFA; B: ACN RP-LC: Nova-pak C18; 0- 100% B; A: 0.1% TFA; B: ACN	[51]
Mushroom *Pholiota adiposa**	Extraction	----------	UF: M_{WCO} 5 kDa SEC: Sephadex G-25, 3.0 x 80 cm; elution with water; 24 mL/min RP-LC: µBondapack C18; 0-100% B; A: 0.1% TFA; B: ACN RP-LC: µVydac protein/peptide 218Tp; 0-100% B; A: 0.1% TFA; B: ACN	[52]
α-zein*	Digestion	Thermolysin	DEAE-Tyopearl 650 M, 2.6 x 100 cm; 0-0.3 M NaCl in 5mM Tris-HCl (pH 8); 3 mL/min; UV detection (254 nm) Desalination with Sephadex LH-20, 1.6 x 100 cm RP-LC: C-18 Capcellpak, 1.5 x 25 cm; 10-60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm) RP-LC: C-18 Capcellpak, 1.5 x 25 cm; ACN + 0.1% TFA; 8 mL/min; UV detection (210 nm) cIEC: Senshupak SCN-1251, 0.46 x 25 cm; 20 mM/pH 4.0- 50 mM/pH 6.3 NH_4Ac SP-Toyopearl 650 M, 2.6 x 100 cm; 20 mM/pH 4.0- 50 mM/pH 6.3 NH_4Ac; 3 mL/min; UV detection (254 nm) RP-LC: C-18 Capcellpak, 1.5 x 25 cm; 10-60% B (B: ACN + 0.1% TFA); 8 mL/min; UV detection (210 nm) RP-LC: C-18 Capcellpak, 1.5 x 25 cm; 5-30% B (ACN + 0.1% TFA); UV detection (210 nm)	[103]
Urea denaturated Z19 α-zein	Digestion	Thermolysin	RP-LC: YMC-GEL C4, 4.6 x 110 mm; 0 -30% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm) RP-LC: YMC-GEL C18, 4.6 x 250 mm; 0-15% or 0-25% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm)	[104]
Urea denaturated total α-zein			RP-LC: YMC-GEL C18, 4.6 x 250 mm; 0-30% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm)	
Corn gluten meal*	Digestion	Protamex, neutrase, alcalase or trypsin	UF: M_{WCO} 5 kDa SEC: Bio-Rad P-2, 700 x 15 mm; 2 mM PBS, pH 8.0; 0.25 mL/min; UV detection (220 nm) RP-LC: µ-Bondapak C 18, 300 x 7.8 mm; 0–40% B (B: ACN + 0.1% TFA); 3 mL/min; UV detection (220 nm)	[36]
Spinach Rubisco	Simulated gastrointestinal digestion	Pepsin and pancreatin	RP-LC: Cosmosil 5C18-AR-II, 20 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 10 mL/min; UV detection (230 nm) RP-LC: 5PE-MS, 4.6 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm) RP-LC: Cosmosil 5 CN-R, 4.6 x 250 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm) RL-LC: 5NPE, 4.6 x 150 mm; 1%/min B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (230 nm)	[42]
Amaranth (*Amaranthu shypocho-ndriacus*) grain	Digestion	Alcalase	SEC: Sephadex G-200, 1.4 x 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (280 nm) SEC: Sephadex G-15, 1.4 x 29 cm; 0.4 M NaCl + 20 mM 2-MER; 0.4 mL/min; UV detection (214 nm) RP-LC: Nucleosil 100 C18 RP, 4.6 x 250 mm; 0-30% B (B: ACN + 0.1% TFA) in 60 min; 2 mL/min; UV detection (214 nm)	[105]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Buckwheat	Digestion	Pepsin, chymotrypsin, trypsin	SEC: Superdex Peptide HR 10/30, 1 x 30 cm; elution with 30% ACN + 0.1% TFA; 0.3 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5Ph, 4.6 x 250 mm; 5-35% B (B: ACN + 0.1% TFA) in 30 min; 0.3 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5C18-ARII, 4.6 x 250 mm; 5-35% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm)	[45]
Garlic (*Allium sativum L)*	Extraction	----------	cIEC: Dowex 50WX4, 2.5 x 30 cm; elution with 2 N NH_4OH SEC: Sephadex G-25, 2.5 x 150 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min RP-LC: Develosil ODS-5, 4.6 x 150 cm; 0-8% B (B: ACN + 0.05% TFA) in 1 h; 1 mL/min; UV detection (220 nm)	[22]
Mung bean protein	Digestion	Alcalase	UF: M_{WCO} 6 kDa SEC: Sephadex G-15, 1.8 x 60 cm; elution with 20 mM NaAc, pH 4.0; 0.4 mL/min; UV detection (220 nm) RP-LC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 250 mm; 0-100% B in 60 min; A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm) RP-LC: Sephasil Peptide C18 ST 4.6/250, 4.6 x 250 mm; 10-80% B in 40 min; A: 0.1% TFA; B: 60% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm)	[106]
Sesame protein hydrolysate	Hydrolysis	Thermolysin	SEC: Bio-Gel P-2, 15 x 820 mm; elution with 10% EtOH; 0.18 mL/min; UV detection (210 nm) RP-LC.: Develosil ODS-10, 20 x 250 mm; 5% B in 20 min; 5-40% B in 60 min; B, ACN + 1% TFA; 10 mL/min; UV detection (210 nm) RP-LC: Develosil C-30-UG-5, 10 x 250 mm; ACN + 1% TFA; 4 mL/min; UV detection (210 nm) RP-LC: Develosil Ph-UG-5, 10 x 250 mm; 6% ACN + 1% TFA; 4 mL/min; UV detection (210 nm)	[107]
Alfalfa white protein*	Hydrolysation at pilot plant scale by Delvolase® in enzymatic membrane reactor		SEC: Superdex Peptide HR 10/300, 10 x 300 mm; elution with 30% ACN + 0.1% TFA; 0.2 mL/min; UV detection (226 nm) RP-LC: C18, 4.6 x 250 mm; 0-28% B in 50 min, 28-47% B in 20 min; B, ACN + 0.1% TFA; UV detection (226 nm)	[108]
MEAT AND CHICKEN				
Chicken bone	Digestion	Pepsin	SEC: TSK gel G2000SWXL, 7.8 x 300 mm; elution with 0.2 M PBS, pH 7.0; 1 mL/min; UV detection (225 nm) RP-LC: Inertsil ODS-2;0–35% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (225 nm) RP-LC: Inertsil ODS-2; 8-14% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm) RP-LC: Cosmosil 5PE-MSl; elution with 10% ACN and 5% ACN; 0.5 mL/min; UV detection (215 nm)	[109]
Chicken leg	Digestion	*Aspergillus oryzae* protease, protease FP/ protease A amano G/ protease N, pepsin and trypsin/ chymotrypsin	UF: M_{WCO} 3 kDa RP-LC: C18 ODS, 22 x 250 mm and 4.6 x 250 mm; 8-40% B in 40 min or 8-40 % B in 64 min (B: ACN + 0.1% TFA); 1 mL/min; UV detection (220 nm)	[110]
Chicken leg bone	Digestion	Alcalase	UF: M_{WCO} 5 kDa SEC: Superdex™ Peptide HR 10/30, 10 x 300 mm; elution with water; 0.5 mL/min; UV detection (220 nm)	[111]
Chicken muscle[1] and ovalbumin[2]	Digestion	Thermolysin[1] and pepsin, trypsin, chymotrypsin or thermolysin[2]	RP-LC: Cosmosil 5C18-AR, 20 x 250 mm; 0-50% B (B: ACN + 0.1% TFA) in 50 min; 10 mL/min; UV detection (215[1] or 230[2] nm) RP-LC: Cosmosil 5 Ph, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min RP-LC: Cosmosil 5CN-R, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1% TFA) in 40 min; 1 mL/min RP-LC: Cosmosil 5C18-AR, 20 x 250 mm; 0-40% B (B: ACN + 10 mM PBS, pH 7.0) in 40 min; 1 mL/min	[112]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Porcine skeletal muscle	Digestion	Trypsin, α-chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain or pepsin	RP-LC: CAPCELL PAK C18 UG120, 4.6 x 150 mm; 0-100% B; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RP-LC: CAPCELL PAK C18 UG120, 4.6 x 150 mm; 0-100% B; A: 0.015% NH_4OH; B: ACN + 0.015% NH_4OH; 1 mL/min; UV detection (215 nm)	[34]
Porcine skeletal muscle troponin	Digestion	Pepsin	AEC: DE53, 16 x 150 mm; 0–300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm) Desalination: Sep-Pak Plus C18; elution with 50% ACN RP-LC: Cosmosil 5C18 ARII, 4.5 x 150 mm; 1–80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5C18 ARII, 4.5 x 150 mm; elution with 12 or 16% ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) SEC: TSK-gel G2000SWXL, 7.8 x 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5PEMS, 4.6 x 250 mm; elution with 12 or 15% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm);	[113]
Porcine hemoglobin	Digestion	Pepsin, trypsin or papain	SEC: Sephadex LH-20, 2.6 x 90 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm) RP-LC: Hypersil BDS C18, 4.6 x 250 mm; 0% B in 5 min; 0-50% B in 40 min; 100% B in 10 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm) RP-LC: Hypersil BDS C18, 4.6 x 250 mm; 0% B in 3 min; 0-40% B in 15 min; 100% B in 10 min; A: 10% ACN + 0.1% TFA; B: 90% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)	[114]
Porcine myosin B	Digestion	Pepsin	SEC: Superdex^{TM} 30, 1.6 x 90 cm; elution with 20 mM NaAc (pH 7.0) + 150 mM NaCl; 0.45 mL/min Desalination: SEP-PAK Plus C18; elution with 50% ACN RP-LC: Inertsil ODS-2, 4.6 x 250 mm; 1–80% B and:1-50% B (ACN + 0.1% TFA); 0.5 mL/min; UV detection (225 nm) RP-LC: Comosil 5PE-MS, 4.6 x 250 mm; elution with ACN at different proportions and flow-rates (0.1-0.5 mL/min); UV detection (225 nm)	[115]
Pork loin	Digestion	Pepsin	AEC: DE53, 16 x 150 mm; 0- 300 mM NaCl in 20 mM Tris-acetate, pH 7.5; 1.13 mL/min; UV detection (215 nm) Desalination with Sep-Pak Plus C18; elution with 50% ACN RP-LC: Cosmosil 5C18 AR-II, 4.5 x 150 mm; 1-80% B (B: ACN + 0.1% TFA); 0.5 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5C18 AR-II , 4.5 x 150 mm; elution with 12% ACN + 0.1% TFA; 0.5 mL/min; UV detection (215 nm) SEC: TSK-gel G2000 SWXL, 7.8 x 300 mm; elution with 20 mM PBS, pH 7.0; 0.5 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5PE-MS, 4.6 x 250 mm; elution with 12% ACN + 0.1 % TFA; 1 mL/min; UV detection (215 nm)	[116]
Pork meat	Simulated gastrointestinal digestion	Pepsin and pancreatin	RP-LC: Symmetry C18, 4.6 x 250 mm; 1% B in 5 min and 1-100% B in 80 min; water-0.1-0.085% TFA-ACN; 0.8 mL/min;	[40]
Bullfrog (*Rana catesbeiana Shaw*) muscle	Digestion	Alcalase, α-chymotrypsin, neutrase, papain, pepsin or trypsin	IEC: HiPrep 16/10 CM FF; 0–2 M NaCl in 20 mM PBS, pH 4.0; 62 mL/ h; UV detection (215 nm) RP-LC: Primesphere 10 C18, 10 x 250 mm; 0–35% B (B: ACN + 0.1% TFA) in 35 min; 1.2 mL/ min; UV detection (215 nm) RP-LC: SynChropak RP-P-100, 4.6 x 250 mm; elution with 15% ACN + 0.1% TFA in 20 min; 1.2 mL/min; UV detection (215 nm)	[37]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Beef rump	Digestion	Thermolysin, proteinase A or protease type XIII and their combination	UF: M_{WCO} 10 kDa SEC: Sephadex G-25, 2.6 cm x 1 m; elution eith 20 mM PBS, pH 7.4; 1.6 mL/min RP-LC: C18, 25 x 0.46 cm; 0% B in 10 min, 0-65% B in 20 min, 100% B in 10 min; A: 0.1% TFA; B: ACN + 0.07% TFA; 0.8 mL/min; UV detection (214 nm)	[79]
Bovine α_{S2}-casein	Digestion	Trypsin	RP-LC: XTerra C18, 4.6 x 250 mm; 1.6% B in 3 min; 1.6-40% B in 87 min (B:ACN + 0.1% TFA); 1 mL/min; UV detection (210-300 nm)	[117]
Bovine lactoferrin	Digestion	Pepsin or trypsin and chymotrypsin	RP-LC: Capcell PAK C18, 4.6 x 150 mm; 0-45% B in 25 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (230 nm)	[118]
Hen ovotrans-fferin			RP-LC: TSK gel ODS 80-Ts, 4.6 x 150 mm; 0-45% B in 25 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 1 mL/min; UV detection (230 nm)	[119]
			SEAFOOD	
Antarctic krill (*Euphausia superba*) tail meat	Digestion	Thermoase PC10F	cIEC: HiPrep 16/10 SP XL, 16 x 100 mm; 0-1 M NaCl + 26.5 mM HFo; 2 mL/min; UV detection (214 nm) SEC: Superdex Peptide 10/300 GL, 10 x 300 mm; 0.9 mL/min; UV detection (214 nm); RP-LC: ODS-80TM, 4.6 x 75 mm; 0- 40% ACN + 0.1% TFA; 0.5 mL/min; UV detection (214 nm) RP-LC: μRP-nLC C2/C18 SC 2.1/10, 2.1 x 100 mm; 7-13% ACN in 35 min; UV detection (214 nm)	[120]
Blue mussel *(Mytilus edulis)*	Fermentation	Salty conditions for 6 months	SEC: Sephadex G-75, 2.5 x 90 cm; elution with 50 mM PBS, pH 7.0; 60 mL/h; UV detection (280 nm) IEC: SP-Sephadex C-25, 2.5 x 45 cm; 0-1 M NaCl in 20 mM NaAc, pH 4.0; UV detection (280 nm) RP-LC: Nucleosil 100-7 ODS C18, 10 x 250 mm; 0-40% B (B: ACN + 0.1% TFA); 2 mL/min; UV detection (215 nm) RP-LC: Nucleosil 100-7 ODS C18, 10 x 250 mm; 0-25% B (B: ACN + 0.1% TFA); 2 ml/min; UV detection (215 nm)	[63]
Marine shrimp *(Acetes chinensis)*	Fermentation	*Labtobacillus fermentum SM 605*	UF: M_{WCO} 3 kDa SEC: Sephadex G-15, 1.6 x 80 cm; elution with water; 25 mL/min; UV detection (220 nm) RP-LC: HIQ sil C18-10, 4.6 x 250 mm; 0-50% B (B: MetOH + 0.1% TFA) in 50 min; 0.8 mL/min; UV detection (214 nm)	[121]
Gelatin of sea cucumber	Digestion	Bromelain and alcalase	UF: M_{WCO} 1, 5, 10 kDa IEC: SP Sephadex C-25, 16 x 300 mm; 0-0.15 M NaCl in 20 mM NaAc (pH 4); 0.4 mL/min; UV detection (220 nm) SEC: Sephadex G-15, 16 x 300 mm; elution with water; 0.3 mL/min; UV detection (220 nm) RP-LC.: Zorbax C18, 1 x 250 mm; 0-10% ACN in 15 min; 0.8 mL/min; UV detection (220 nm) RP-LC: Zorbax SB C18, 4.6 x 250 mm; 0-10% ACN in 10 min; 0.8 mL/min; UV detection (220 nm)	[122]
Sea cucumber	Digestion	Bromelain and alcalase	UF: Mwco 2 kDa SEC: Sephadex G-25, 1.6 x 30 cm; elution with water; 0.6 mL/min; UV detection (220 nm) IEC: SP Sephadex C-25, 2.6 x 30 cm; 0–1 M NaCl in 20 mM NaAc, pH 4.0; 0.6 mL/min; UV detection (220 nm) SEC: Sephadex G-25, 1.6 x 100 cm RP-LC: Zorbax C18, 9.4 x 250 mm; 5–40% B (B: ACN + 0.1% TFA) in 40 min; 0.8 mL/min; UV detection (220 nm)	[123]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Oyster*	Fermentation	Salty conditions for 6 months	IEC: SP-Sephadex C-25, 4.0 x 40 cm; 0-2 M NaCl in 20 mM NaAc, pH 4.0; 60 mL/h; UV detection (215 nm) Desalination by electrodialyzation SEC: Sephadex G-50, 2.5 x 98 cm; elution with water; 60 mL/h; UV detection (215 nm) SEC: elution with water; 60 mL/min; UV detection (215 nm) RP-LC: Nucleosil 100-3 ODS C18; 0-11% B (B: ACN + 0.1% TFA); 1 mL/min; UV detection (215 nm)	[62]
Oyster	Digestion	Pepsin	Filtration: M_{wco} 10 kDa SEC: Sephadex LH-20, 2.7 x 80 cm; elution with 30% MetOH; 0.5 mL/min; UV detection (280 nm) RP-LC: Hypersil BDS C18, 4.6 x 210 mm; 0–100% B in 40 min; 100% B in 10 min; A: 0.1% TFA; B: ACN; 1 mL/min; UV detection (215 nm)	[124]
Wakame (*Undaria pinnatifida*)	Digestion	Pepsin	Dialysis against water in cellulose tubing for 2 days IEC: Dowex 50W, 2.6 x 20 cm; elution with 3.7% NH_4OH SEC: Sephadex C-25, 2 x 50 cm; elution with 1.5% NaCl; 70 mL/h RP-LC: Develosil C18 ODS-5, 4.6 x 250 cm; 0-25% B (B: ACN + 0.05% TFA) in 2 h; 1 mL/min; UV detection (220 nm)	[125]
Wakame (*Undaria pinnatitida)*	Digestion	Protease S "amano"	Extraction with 1-butanol RP-LC: µBondasphere C18, 300 x 30 mm; 0-35% B in 140 min; A: 0.1% TFA; B: ACN + 0.07% TFA; 30 mL/min; UV detection (220 nm) RP-LC: XTerraRP18, 150 x 4.6 mm; 0% B in 10 min, 0-20% B in 40 min; A: 50 mM NH_4Ac (pH 10) + 1% ACN; B: 50 mM NH_4Ac (pH 10) + 95% ACN; 1 mL/min; UV detection (220 nm) RP-LC: ^{1}C.: ODP50-4D, 150 x 4.6 mm; 0-20% B in 30 min; A: 50 mM NH_4OH (pH 10) + 1% ACN; B: 50 mM , NH_4OH NH_4OH (pH 10) + 95% ACN; 0.5 mL/min; UV detection (220 nm); ^{2}C.: XTerra RP18, 150 x 4.6 mm; 0-30% B in 40 min; A: 0.1% TFA/0.07% TFA in ACN (99/1); B: 0.1% TFA/0.07% TFA in ACN (5/95); 1 mL/min; UV detection (220 nm)	[126]
Wakame (*Undaria pinnatifida*)	Extraction	----------	Dialysis against water (10 L) in cellulose tubular membrane (90 cm) IEC: Dowex 50W, 45 x 450 mm; elution with NH_4OH SEC: Sephadex G-25, 2.6 x 140 cm; elution with 0.1 M PBS, pH 7.0; 30 mL/min RP-LC: Develosil ODS-5, 4.6 x 250 mm; 0-25% B (B: ACN + 0.05% TFA) in 180 min; 1 mL/min; UV detection (220 nm) RP-LC: Asahipack CG-320HQ, 7.6 x 300 mm; elution with 25 % ACN in 50 mM NH_4Ac, pH 6.8; 0.5 mL/min	[127]
			FISH	
Tuna dark muscle	Digestion	Alcalase, neutrase, pepsin, papain, α-chymotrypsin or trypsin	UF: M_{WCO} 3 kDa IEC: HiPrep 16/10 DEAE FF; 0–2 M NaCl in NaAc (pH 4); 2.0 mL/min; UV detection (280 nm) RP-LC: ODS C18 Primesphere 10, 20 x 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 55 min; 2.0 mL/min; UV detection (215 nm) RP-LC: Synchropak RPP-100, 4.6 x 250 mm; elution with 20% ACN + 0.1% TFA; 1 mL/min; UV detection (215 nm)	[33]
Tuna	Digestion	Alcalase, α-chymotrypsin, papain pepsin, neutrase or trypsin	UF: M_{WCO} 1, 5, 10 kDa IEC: Hiprep 16/10 DEAE FF; 0–2 M NaCl in 20 mM NaAc, pH 4.0; 62 mL/h; UV detection (280 nm) RP-LC: Primesphere 10 C18, 20 x 250 mm; 0–50% B (B: ACN + 0.1% TFA) in 20 min; 2 mL/min; UV detection (215 nm) RP-LC: Synchropak RPP-100, 4.6 x 250 mm; elution with 15% ACN + 0.1% TFA; 1.2 mL/min; UV detection (215 nm)	[21]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Upstream chum salmon muscle	Digestion	Thermolysin	RP-LC: ODS, Comsosil 140,C18- OPN, 44 x 370; elution with 10, 25, 50, 99.5% EtOH SEC: Sephadex G-25, 16 x 650 mm; elution with water RP-LC: Mightysil RP-18, 4.6 x 250 mm; elution with 10% ACN + 0.1% TFA; 1 mL/min; UV detection (220 nm)	[128]
Salmon muscle	Digestion	Papain	Extraction with 1-butanol Separation in silica gel; PSQ 100B, 1380 x 100 mm; elution with $CHCl_3$: MetOH: water: CH_3COOH (65:25:4:1, 31 L) Separation in silica gel, 400 x 80 mm; elution with $CHCl_3$:2-propanol:water: CH_3COOH IEC: Amberlite CG50-type, 400 x 80 mm; elution with water; water:MetOH; MetOH; MetOH:2M HCl RP-LC: XTerra MS C18, 100 x 4.6 mm; 5–30% B (B: ACN + 0.1% HFo) in 30 min; 0.2 mL/min Methylation: 10% sodium methoxide in MetOH (50 mL); 16 h, RT; refluxing (4.5 h) Separation in Silica gel 60, 600 x 20 mm; elution with $CHCl_3$:2-propanol at different ratios	[129]
Dried bonito	Digestion	Pepsin, chymotrypsin, trypsin, thermolysin	RP-LC: YMC-Pack ODS-AQ, SH-343-5, 20 x 250 mm; 1-41% B (B: ACN + 0.1% TFA) in 40 min; 10 mL/min; UV detection (230 nm) RP-LC: Cosmosil 5Ph, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5CN-R, 4.6 x 250 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm) RP-LC: Cosmosil 5C18, 4.6 x 150 mm; 0-40% B (B: ACN + 0.1 % TFA) in 40 min; 1 mL/min; UV detection (215 nm)	[35]
Bonito bowels	Autolysis	Endogenous proteases	UF: M_{wco} 6 kDa Purification with Sep-Pak Plus C18; elution with 15% ACN Purification with Toyopak IC-SP M; elution with 10 mM PBS, pH 9.0 RP-LC: RP-18(e), 100 mm x 250 mm; 0-30% B (B: ACN + 0.05% TFA); 4 mL/min; UV detection (210 nm) RP-LC: RP-18(e), 4 mm x 250 mm; 0-30% B (B: ACN + 0.05% TFA); 1 mL/min; UV detection (210 nm) SEC: Asahipak GS-220 and GS-320, 7.6 mm x 500 mm; 50 mM NH_4Ac; 1 mL/min; UV detection (210 nm) IEC: SP-2SW, 4.6 mm x 250 mm; 0-0.5 M NaCl in 20 mM PBS, pH 6.0; UV detection (210 nm) RP-LC: RP-18(e), 4 mm x 250 mm; elution with 7% ACN + 0.05% TFA; UV detection (210 nm)	[59]
Alaska pollack (*Theragra-chalcogra-mma*) skin	Digestion	Alcalase, pronase E and collagenase	SEC: Sephadex G-25, 2.5 x 90 cm; elution with water; 0.5 mL/min; UV detection (220, 280 nm) cIEC: SP-Sephadex C-25, 2.5 x 45 cm; 0-1 M NaCl in 20 mM NaAc, pH 4.0; 2 mL/min; UV detection (220, 280 nm) SEC: Sephadex G-15; elution with water; 0.5 mL/min; UV detection (220, 280 nm) RP-LC: ODS C18; 10-50% B in 40 min; A: 0.1% TFA; B: ACN + 0.1% TFA; 2 mL/min; UV detection (215 nm) CE: Coated capillary, 24 cm x 25 μm; 0.1 M PBS, pH=2.5; 10 kV; UV detection (200 nm)	[130]
Yellowfin sole frame	Digestion	α-chymotrypsin	UF: M_{WCO} 5, 10, 30 kDa IEC: SP-Sephadex C-25, 35 x 350 mm; 0-2 M NaCl in 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 and 280 nm) SEC: OHpak SB-803 HQ, 8.0 x 300 mm; 20 mM NaAc, pH 4.0; 1 mL/min; UV detection (215 nm) RP-LC: SP Nucleosil 100- 7 C18, 1 x 250 mm; 0-19% B (B: ACN + 0.1% TFA) in 40 min; 2.0 mL/min; UV detection (215 nm) RP-LC: Zorbax SB C18, 4.6 x 250 mm; 0-19% B (B: ACN + 0.1% TFA) in 30 min; 0.5 mL/min; UV detection (215 nm)	[131]

Table 2. Continuation

Source of peptide	Release of peptides	Employed enzyme(s) or bacterial strains	Purification steps	References
Pacific hake fish	Autolysis	Endogenous proteases	UF: M_{WCO} 1, 3, 10 kDa RP-LC: Jupiter C12 Proteo 90 Å, 250 x 4.6 mm; 0-25% B in 25 min and 25- 80% B in 5 min; water- 0.05% TFA-ACN; 1 mL/min; UV detection (214 nm)	[60]
			PROCESSED PRODUCTS AND OTHERS	
Fermented soybean paste	Extraction	----------	RP-LC: JAIGEL-A-343-10, 250 x 20 mm; 98% B in 5 min; 96% B in 20 min; 65% B in 30 min; 5 mL/min; B, ACN; UV detection (214 nm) IEC: JAIGEL-ES-502CP, 20 x 100 mm; elution with 0.01M sodium succinate buffer (pH 4.3) in 20% ACN; 4 mL/min; UV detection (214 nm) RP-LC: JAIGELODS-A-343-10, 250 x 20 mm; elution with 0.05% TFA/ACN (95:5, v/v); 5 mL/min; UV detection (214 nm) IEC: Shodex Asahipak ES-2502N-7C, 100 x 7.6 mm; elution with 20 mM Tris-HCl (pH 7.5) + 125 mM NaCl; 1 mL/min; UV detection (214 nm)	[53]
Salt-free soy sauce	Fermentation	*Aspergillus oryzae*	Extraction: Sep-Pak Plus C18; elution with 35% ACN + 0.1% TFA RP-LC: Cosmosil 5C18-ARII, 4.6 x 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 65 min; 0.4 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5C18-AR300, 4.6 x 250 mm; 5–35% B (B: ACN + 0.1% TFA) in 65 min; 0.3 mL/min; UV detection (220 nm)	[132]
Douchi	Fermentation and ripening	*Aspergillus egypticus* culture	Extraction/centrifugation (3,000g, 10 min)/filtration SEC: Sephadex- G25, 10 x 750 mm; elution with PBS; 0.2 mL/min; UV detection (220 and 280 nm) RP-LC: Vydac 218TP54; 0-60% B (B: ACN + 0.1 % TFA) in 60 min; 1 mL/min; UV detection (220 nm)	[133]
Miso paste with addition of casein	Digestion	Porcine pepsin A	UF: M_{WCO} 3 kDa RP-LC: Prep Nova Paks HR C18, 300 x 7.8 mm; 0- 21% B in 30 min, 21- 35% B in 40 min, 35-70% B in 5 min; A: 0.1% TFA; B: ACN + 0.08% TFA; 4 mL/min; UV detection (214 nm)	[134]
Royal jelly[b]	Digestion	Pepsin, chymotrypsin, trypsin	SEC: Superdex Peptide HR 10/30, 1 x 30 cm; elution with 30% ACN + 0.1% TFA; 0.3 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5Ph, 4.6 x 250 mm; [a]5-35% B (B: ACN + 0.1% TFA) in 30 min and [b]1-40% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm) RP-LC: Cosmosil 5C18-ARII, 4.6 x 250 mm; 5-35% B (B: ACN + 0.1% TFA) in 60 min; 0.3 mL/min; UV detection (220 nm)	[45] [44]
Egg	Simulated gastrointestinal digestion	Pepsin and pancreatin	UF: M_{WCO} 3 kDa cIEC: HiPreP 16/10 SP FF, 16 x 100 mm; Eq.: 10 mM NH_4Ac pH 4; Elution: 0.5 M NH_4HCO_3; 5 mL/min SEC: Superdex peptide 10/300GL, 10 x 300-310 mm	[41]

In fact, results obtained when a pacific hake protein hydrolysate was ultrafiltrated through membranes cut-off at 10, 3, and 1 Mw indicated that the fraction with the highest ACE inhibition activity was that obtained when the hydrolysate passed through the 3 kDa cut-off membrane [60].

SEC (also known as Gel-Filtration Chromatography (GFC) when an aqueous solution system is used and Gel-Permeation Chromatography (GPC) with a non-aqueous solution system) is also frequently used for the purification of peptides. SEC tends to be used at the beginning of the purification path, as is UF, as well as in the middle of protocols for removing interferences. SEC is quick, easy to use, universal, and compatible with physiological conditions. SEC is also useful for estimating the Mw range or for desalting. Nevertheless, the separation of a target peptide from a closely related peptide mixture is practically impossible and additional SEC separations using stationary phases with different pore diameters are needed [139]. Among SEC columns, porous silica base TSK-gel SW [109, 113, 116] and polyhydroxymethacrylate base OHpak [131] are preferred. For low and medium pressure SEC, dextran base Sephadex or agarose/dextran base Superdex columns are mainly employed. Among Superdex columns, those with an Mw ranging from 100 to 7000 like the Peptide 10/300 GL column [41, 96, 120] and the Peptide HR 10/30 column [44, 45, 48, 57, 92, 111] were mostly chosen. Regarding Sephadex columns, the ones most commonly used were Sephadex G-25 (Mw range, 1000-5000) and Sephadex G-15 (Mw ≤ 1500). Other less used Sephadex columns are G-50 (Mw range, 1500–30000) [62], G-75 (Mw range, 30000–80000) [63], and G-200 (Mw range, 5000–250000) [105]. Tovar-Pérez *et al.* [105] purified alcalase aramanth albumin and globulin protein hydrolysates using sequentially Sephadex G-200 and G-15 columns. Albumin hydrolysate eluted in 18 h in one broad peak (M_r<1.35 kDa) using the G-200 column while globulin hydrolysate eluted in 5 h in two separated fractions. Afterwards, fractions were individually separated in a G-15 column. The peaks corresponding to the albumin hydrolysate were observed at Mw of 4.70 and 0.55 kDa and peaks corresponding to the globulin hydrolysate were observed at Mw of 7.50, 4.70, 0.55, and 0.40 kDa [105].

An alternative and complementary chromatographic mode for the purification of ACE inhibitory peptides is Ion Exchange Chromatography (IEC). IEC is mainly employed as a further purification step after or between UF or SEC purification. Cation exchange resins with negatively charged groups like sulfopropyl (SP), methyl sulfonate (S), and carboxymethyl (CM) and anion exchange resins positively charged with quaternary ammonium (Qa),

quaternary aminoethyl (QAE) or DEAE (diethylaminoethyl) are mostly employed [140]. Since antihypertensive peptides contain mainly hydrophobic amino acids, whose pI's are between 5-7, both cation exchange (cIEC) and anion exchange (AEC) can be employed. When AEC is used, the pH tends to be around 7.5 and binding peptides are negatively charged, while in cIEC the pH is maintained at acidic level (4.0) to retain positively charged peptides. AEC purification methods focus more on columns with weak ion-exchange ligands, such as DEAE [21, 33] or DE [80, 113, 116], while cIEC methods mainly prefer strong ion-exchange ligands as SP. In both cases, peptides are eluted by increasing the eluent ion strength using NaCl gradients at a constant pH [141]. cIEC with isocratic elution has also been possible through the use of a sodium succinate buffer in 20% ACN [53], an ammonia solution [22, 95, 125, 127] or an ammonium carbonate buffer [41].

Reserved-Phase Chromatography (RP-LC) is the dominate technique in the purification of peptides with antihypertensive activity [141]. Generally, RP-LC is employed at the end of the purification protocol after UF, SEC or IEC separations. However, there are also examples in which this has been the only technique employed in the purification [35, 50, 85-87, 97, 104, 112, 118, 119].

There is a large number of RP-LC columns that can be used in the separation and purification of peptides where column support, bonded phase, pore size, particle size, and column dimension should be taken into consideration. Porous silica-based supports are the first choice since they offer good mechanical stability and a wide range of selectivity through the bonding of different phases. C_4-C_{12} phases are typically used with high hydrophobic samples like large peptides and small hydrophilic proteins, while C_{18} phases prefer slightly more hydrophilic analytes and are the perfect choice for small peptides. Moreover, phases such as cyano, hexyl, phenyl, hexyl/phenyl, perfluorinated are also available [142]. Alternatively, polymeric reserved phases such as polystyrene divinylbenzene, withstand a wide range of pHs and have also been employed.

Mobile phases consist of mixtures of water with an organic modifier being acetonitrile and alcohols such as methanol, ethanol or isopropanol [142] the most popular options. Gradient elution by the increased concentration of the organic modifier is the option most commonly used. Mobile phases are usually prepared at acidic pH. Moreover, the addition of ion-pairing agents is also very useful to increase hydrophobicity of peptides, as it creates a pair with positively charged peptides. Trifluoroacetic acid (TFA) is usually the first choice because it is

transparent to UV light, does not block amino groups (therefore, derivatization of peptides is possible), is highly volatile (therefore, it is easy to remove by lyophylization), and easily miscible with most organic mobile phases. Other ion-pairing agents like acetic acid, formic acid, phosphoric acid, heptafluorobutyric acid (HFBA) or quaternary ammonium salts can be alternatives to TFA.

Online detection during purification was mainly performed using UV absorption at 210-220 nm (absorbance wavelength of peptide bonds). Moreover, in some cases wavelengths of 254 nm, where phenylalanine residues absorbs, and 275-280 nm, where aromatic residues (tyrosine, tryptophan) absorb, are also employed [141].

Moreover, additional steps involving liquid-liquid extraction, desalination or dialysis are also employed for the purification of antihypertensive peptides. Desalination of samples is usually conducted by electrodialysis [62, 63, 73, 98] or by solid-phase extraction [113, 115, 116].

6. Identification and characterization of bioactive peptides from foodstuffs

Isolated and purified peptides possessing the most potential antihypertensive activity at the end of the framework (see Fig. 2) are next identified and characterized. Characterization mostly involves the determination of the amino acid sequence and the IC_{50} value. Moreover, in some cases, additional information like the amino acid composition, molecular weight, molecular weight distribution, peptide content, molecular structure, and purity are also determined. Table 3 summarizes the peptides that have been identified from foodstuffs and the kind of characterization that has been performed.

Amino acid sequence determination can be carried out by mass spectrometry (MS) or by Edman degradation sequencing. Edman degradation is based on the sequential elimination of N-terminal amino acids by chemical procedures. However, this method is time consuming and requires highly purified samples (free of salts, detergents, and nonvolatile additives such as urea). Edman degradation can be performed manually or it can be fully automated using special automated protein/peptide sequencers [87, 96, 97, 102, 118, 119].

Table 3. Characterization of purified peptides with antihypertensive activity

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	References
Goat milk	TGPIPN, SLPQ, SQPK	316, 330, 354 μM	Edman degradation sequencing	[84]
Bonito bowels	YRPY, GHF, VRP, IKP, LRP, IRP	320, 1100, 2.2, 2.5, 1.0, 1.8 μM	Automated protein sequencing by Edman degradation	[59]
Yoghurt	8 peptides	1.56- 12.41 μg/mL	Automated protein/peptide sequencing by Edman degradation	[88]
Beef rump	VLAQYK	23.2 μg/mL	Peptide sequencing by Edman degradation	[79]
Spinach Rubisco	MRWRD, MRW, LRIPVA, IAYKPAG	2.1, 0.6, 0.38, 4.2 μM	Automated protein sequencing by Edman degradation	[42]
Cheese whey protein	VYPFPG, GKP, IPA, FP, VYP, TPVVVPPFLQP	221, 352, 141, 315, 288, 749 μM	Automated protein sequencing by Edman degradation	[92]
Rice	IY, VVY, VF, VW	4.0, 22.0, 49.7, 3.1 μM	Gas/liquid phase protein sequencing by Edman degradation	[102]
Porcine myosin B	KRVIQY, VKAGF	6.1, 20.3 μM or 4.9, 10.6 μg/mL	Protein sequencing	[115]
Porcine skeletal muscle troponin	EKERERQ, KRQKYDI	552.5, 26.2 μM	Protein sequencing	[113]
Salt-free soy sauce	AF, FI, IF	165, NI, 65.8 μmol/L	Protein sequencing	[132]
Chicken bone	YYRA	33.9 μg/mL	Protein sequencing	[109]
Pork loin	VKKVLGNP	28.5 μM	Protein sequencing	[116]
Casein	10 peptides	22-> 1000 μM	Protein sequencing	[85]
Dried bonito	8 peptides	3.7- 62 μM	Protein sequencing	[35]
Chicken muscle	LKA, LKP, LAP, IKW, FQKPKR, FKGRYYP, IVGRPRHQG	0.32- 14 μM	Protein sequencing	[112]
Ovalbumin	FGRCVSP, ERKIKVYL, FFGRCVSP, LW, FCF, NIFYCP	0.4- 15 μM		
Bovine lactoferrin	LRPVAA	4.14 μM	Gas-phase sequencing	[118]
Hen ovotransferrin	KVREGTTY	102.8 μM	Gas-phase sequencing	[119]
Sheep milk yoghurt	12 peptides	----------	Liquid-phase protein/peptide sequencing	[87]
Miso paste with casein	RYLGY; AYFYPEL; YQKFPQY	0.71, 6.58, 20.08 μM	Amino acid sequencing by RP-LC-MS/MS	[134]
Caprine Kefir	PYVRYL, LVYPFTGPIPN	2.4, 27.9 μM	Amino acid sequencing by RP-LC-MS/MS	[89]
Milk	LHLPLP	----------	Amino acid sequencing by HPLC-MS/MS	[82]
Manchego cheese	75 peptides	13.4- > 1000 μM	Amino acid sequencing by HPLC-MS/MS	[90]
Milk	40 peptides *	----------	Amino acid sequencing by HPLC-MS/MS	[49]
White wheat, wholemeal wheat, rye flours	14 peptides	0.19 - 0.45 mg/mL	Amino acid sequencing by nLC-ESI-MS/MS	[100]
Ovine β-lactoglobulin from skimmed milk	21 peptides**	30-71.2 %	Amino acid sequencing by LC-MS/MS	[50]
Fermented milk	27 peptides	----------	Amino acid sequencing by LC-MS/MS	[81]
Soybean protein	8 peptides	21- > 10000 μM	Amino acid sequencing by ESI-MS/MS	[73]

Table 3. Continuation

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	References
Sodium caseinate	21 peptides	39- > 1000 μM and 15-650 μM	Amino acid sequencing by ESI- MS/MS	[93]
Marine shrimp *(acetes chinensis)*	DP, GTG, ST	2.15, 5.54, 4.03 μM	Mw determination and amino acid sequencing by ESI-MS/MS	[121]
Yak milk casein	YQKFPQY, LPQNIPPL, SKVLPVPQK, LPYPYY, FLPYPYY	----------	Mw determination and amino acid sequencing by ESI-MS/MS	[78]
Egg	VDF, LPF, MPF, YTAGV, ERYPI, IPF, TTI	6.59-27.38 μM	Amino acid sequencing by LC-ESI- MS/MS	[41]
Several Spanish cheeses	41 major peptides**	113.1- 2419.4 μM	Amino acid sequencing by RP-LC and off-line MS/MS	[55]
Ovine milk	IAK,VR, EKDERF, KDERF, YIPIQY, LPYPY	10.0- 848.0 μM	Amino acid sequencing by CE-ESI-IT-MS	[43]
Milk	8 peptides	5.2- >1500 μM	Amino acid sequencing by ESI-Q-IT-MS	[46]
Porcine hemoglobin	LGFPTTKTYFPHF, VVYPWT	4.92, 6.02 μM	Amino acid sequencing by MALDI-ToF/MS and ESI-MS/MS	[114]
Enzyme –modified cheese	13 peptides	----------	Mw and amino acid sequencing by API-MS	[91]
Salmon muscle	20 di- and tri-peptides	Dipeptides: 1.2- 86 % Tripeptides: 7.5- 59%	Amino acid sequencing by LC-ESI-MS and ^{1}H NMR	[129]
Chicken leg bone	GAVGPSG, AVKQPAVVYP, AATENM, DMSVF, EGGPKP, ANSSIL, AITAKL, IGNTLI, NLAPFL, EIAKLM	----------	Amino acid sequencing by LC/MS/MS	[111]
Gelatin of sea cucumber	----------	0.0142 mg/mL	Hydrolysis/derivatization/Automatic amino acid analysis, Mw distribution by ESI-IT-MS	[122]
Corn gluten meal	AY	14.2 μM	Amino acid sequencing by HPLC-ESI-MS and determination of amino acid composition by hydrolysis/OPA derivatization/fluorescence detection	[36]
Rice	TQVY	18.2 μM	Determination of amino acid composition by hydrolysis/OPA derivatization/automatic amino acid analysis and amino acid sequencing by MALDI-ToF-MS/MS	[101]
Garlic (*Allium sativum L)*	SY, GY, FY, NY, SF, GF, NF	66.3, 72.1, 3.74, 32.6, 130.2, 277.9, 46.3 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automatic protein sequencing by Edman degradation and FAB-MS	[22]
Wakame (*Undaria pinnatifida*)	AIYK, YKYY, KFYG, YNKL	213, 64.2, 90.5, 21 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation and FAB-MS	[125]
α-zein	Among 3 with high activity: LRP, LSP, LQP	0.29, 1.7, 2.0 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation and FAB-MS	[103]

Table 3. Continuation

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	References
Soybean	IA , YLAGNQ, FFL, IYLL, VMDKPQG	153, 14, 37, 42, 39 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	[95]
Wheat gliadin	IAP	2.7 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	[99]
Royal jelly	FY, KF, IF, IVY, IMY, DGL, TKY, LTF, FNF, AVL, GLY	1.67-930 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automatic protein sequencing by Edman degradation	[44]
Yoghurt- like product	YP	720 μM	Determination of amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	[30]
Buckwheat	VK, FY, AY, LF, YV, YQ, YQY, PSY, LGI, ITF, INSQ	4-628 μM	Determination of amino acid composition by HPLC and automated protein sequencing by Edman degradation	[45]
Urea denaturated Z19 α-zein	17 peptides**	1.9- 57 μM	Determination of amino acid composition and automated protein sequencing by Edman degradation	[104]
Urea denaturated total α-zein	27 peptides**	3.9- 100 μM		
Mung bean protein	KDYRL, VTPALR, KLPAGTLF	26.5, 82.4, 13.4 μM	Determination of amino acid composition by hydolysis/derivatization/automatic amino acid analysis, determination of Mw and amino acid sequencing by MALDI-ToF MS	[106]
Oyster	----------	0.0874 mg/mL	Mw determination by SEC	[62]
Yak milk casein	PPEIN, PLPLL	0.29, 0.25 mg/mL	Mw determination by LC-MS and amino acid sequencing by LC-ESI-MS/MS	[80]
Soybean	LVQGS	22 μg/mL (43.7 μM)	Mw determination by MALDI-ToF-MS and liquid-phase peptide sequencing by Edman degradation	[96]
Porcine skeletal muscle	MNPPK, ITTNP	945.5, 549 μM	Mw determination by FAB-MS and automated protein sequencing by Edman degradation	[34]
Manchego cheese	22 peptides	23.7- 100 %	Mw determination and amino acid sequencing by ESI-MS/MS	[54]
Alfalfa white protein	VW	1.1 μM	Mw determination and amino acid sequencing by ESI-MS	[108]
Tuna dark muscle	WPEAAELMMEVDP	21.6 μM	Mw determination and amino acid sequencing by ESI-MS	[33]
Bullfrog (*Rana catesbeiana Shaw*) muscle	GAAELPCSADWW	0.95 μM	Mw determination and amino acid sequencing by ESI-MS	[37]
Sea cucumber (*Acaudina molpadioidea*)	MEGAQEAQGD	15.9 μM	Mw determination and amino acid sequencing by nESI-MS/MS	[122]

Table 3. Continuation

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	References
Tuna	GDLGKTTTVSNWSPPKYKDTP	11.28 μM	Mw determination and amino acid sequencing by ESI-Q-ToF-MS	[21]
Pork meat	ER, EPR, PER, KLP, AGLP, GPR, NVR, PGR, VGPR, RPR, PAGPR, PAGPVG	382- >1000 μM	Amino acid identification by nLC-ESI-MS/MS	[40]
	MMVPI, IGGSI, KAPVA, PTPVP, YPGIA, NIIPA, MYPGIA, VIPEL, INDPF, VLPEI	46.56- >1000 μM	Amino acid sequencing and Mw determination by MALDI-ToF/ToF	
Wheat bran	LQP, IQP, LRP, VY, IY, TF	2.2, 3.8, 0.21, 21, 3.4, 18 μM	Mw determination by MALDI-ToF-MS and automatic protein sequencing-HPLC	[61]
Pholiota adiposa	GEGGP	254 μM	Mw determination by MALDI-MS and automated protein sequencing by Edman degradation	[52]
Yellowfin sole frame	MIFPGAGGPEL	28.7 μg/mL	Mw determination by SEC and automated protein sequencing by Edman degradation	[131]
Blue mussel *(Mytilus edulis)*	EVMAGNLYPG	19.34 μg/mL	Mw determination by SEC and automated protein sequencing by Edman degradation	[63]
Oyster (*Crassostrea talienwhanensis Crosse*)	VVYPWTQRF	66 μmol/L	Mw determination by LC-MS (LC-APCI-QqQ-MS) and automated protein sequencing by Edman degradation	[124]
Chicken leg	GA(Hyp)GLHypGP, GA(Hyp)GPAGPGGI(Hyp)GERG, GL(Hyp)GSRGE RGL(Hyp)G, GI(Hyp) GERGPVGPSG	29.4, 45.6, 60.8, 43.4 μM	Mw determination by LC-ESI-Q-MS and protein sequencing	[110]
Skimmed milk	VPP, IPP	9.13, 5.15 μM	Determination of peptide content, amino acid content by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	[86]
Steamed soybean mixed with roasted wheat	AW, GW, AY, SY, GY, AF, VP, AI, VG	10- 1100 μg/mL	Amino acid analysis and determination of molecular structure by ^{1}H NMR; ^{13}C NMR; LC-MS	[98]
Antarctic krill (*Euphausia superba*) tail meat	VW, LKY, ITRY, VFER	12.9, 10.1, 236.9, 152.8 μM or 2.75, 4.26, 130.7, 84 μg/mL	Mw determination by SEC, quantitation of peptides by UPLC-ESI-MS, and protein sequencing	[120]
α-lactalbumin and β-casein	9 peptides	1->76 μM	Amino acid sequencing by LC-MS/MS and automatic Edman degradation	[94]
Glycinin from soybean	VLIVP	1.69 μM	Determination of amino acid composition by hydrolysis/derivatization/HPLC, Mw by MALDI-ToF, and gas- phase protein sequencing by Edman degradation	[97]

Table 3. Continuation

Source of peptide(s)	Identified peptide(s)	IC_{50} [units]	Characterization	References
Alaska pollack (*Theragra chalcogramma*) skin	GPM, GPL	17.13, 2.65 μM	Determination of Mw distribution by SEC, amino acid composition by hydrolysis/amino acid analysis, and automated protein sequencing by Edman degradation	[130]
Cheese	LQP, MAP	3.4, 0.8 μM	Determination of Mw by LC/MSD, amino acid composition by hydrolysation/inspissation/amino acid analysis, and automated protein sequencing by Edman degradation	[48]
Gouda cheese	RPKHPIKHQ, RPKHPIKHQGLPQ, YPFPGPIPN, MPFPKYPVQPF	13.4, -- , 14.8,- - μM	Determination of Mw by FAB-MS, amino acid composition by hydrolysis/HPLC and protein sequencing	[57]
Sesame protein hydrolysate	LVY, LSA, LQP, LKY, IVY, VIY, MLPAY	0.33-5.80 μg/ mL	Determination of amino acid composition by hydrolysis/amino acid analysis, peptide content by LC/MS/MS and protein sequencing by ToF-MS/MS	[107]
Wakame (*Undaria pinnatifida*)	10 dipeptides YH, KW, KY, KF, FY, VW, VF, IY, IW, VY	2.7- 43.7 μmol/L	Determination of Mw by FAB-MS, amino acid composition by hydrolysis/amino acid analysis, and automated protein sequencing by Edman degradation	[127]
Commercial whey product	ALPM	928 μM	Determination of Mw by FAB-MS, amino acid composition by hydrolysis/HPLC, and protein sequencing by automatic Edman degradation	[75]
Mushroom *Tricholoma giganteum*	GEP	0.04 mg	Determination of Mw by LC-MS, amino acid composition by hydrolysis/fluorometric analysis, and automated protein sequencing by Edman degradation	[51]
Fermented soybean paste	HHL	2.2 μg/mL	Determination of Mw by SEC, amino acid composition by HPLC, protein sequencing	[53]
Upstream chum salmon muscle	WA, VW, WM, MW, IW, LW	2.5- 277.3 μM	Determination of Mw by ESI-MS, amino acid composition by hydrolysis/amino acid analysis and automated protein sequencing by Edman degradation	[128]

Table 3: NI- no inhibition; (*)- in the article authors did not show which peptides are antihypertensive although optimization of the fermentation procedure in order to obtain them was based on ACE inhibitory activity; (**)- among identified peptides only for selected the ACE inhibitory activity were measured; (***)- in review one letter abbreviations for amino acids were adopted, however due to the lack of abbreviation for a non-protein amino acid in this system, three letter abbreviation was used: hydroxyproline (Hyp).

MS is a powerful technique widely employed for the characterization of bioactive peptides. In addition to the amino acid sequence, MS can also yield accurate information on molecular masses, peptide purity or post-translational modifications, *etc.* [141]. MALDI (matrix assisted laser desorption and ionization), ESI (electrospray ionization) and, less frequently, FAB (Fast Atom Bombardment) have been the ionization sources employed. LC and, less frequently, capillary electrophoresis (CE) are sometimes needed previously to the MS analysis. An alternative technique to determine molecular structure is NMR. NMR has been used for tripeptides of salmon muscle hydrolysate (^{1}H NMR) [129] and dipeptides of steamed soybean mixed with roasted wheat hydrolysate (^{1}H NMR and ^{13}C NMR) [98].

Another strategy for peptide characterization is to determine its amino acid composition. This is determined by the chemical hydrolysis of peptides and the amino acid analysis using an automatic analyzer. Other options for the amino acid analysis have been peptide hydrolysis with HCl and phenol, followed by RP-LC separation and UV detection [57, 75].

Although a full relationship between structure and the ACE inhibitory properties of antihypertensive peptides has still not been established, it is important to highlight some common features for antihypertensive peptides. In addition to low molecular weight and short sequences (2-12 amino acid residues [13], antihypertensive peptides contain a significant amount of hydrophobic amino acids especially at C-terminal position [26]. The presence at C-terminal position of proline (P) or a positive charge of lysine (K) (ε- amino group) or arginine (R) (guanidine group) enhances the potency of antihypertensive peptides [6, 9, 13, 26]. This fact could be related to the bioavailability of antihypertensive peptides since it has been demonstrated that peptides including proline at the end of the sequence are particularly resistant to *in vivo* proteolysis [143].

Nevertheless, the presence of *in vitro* antihypertensive activity of isolated peptides does not involve the activity *in vivo*. In fact, orally administered peptides need to reach the target cardiovascular system in an active form. Before reaching the cardiovascular system however, orally delivered peptides have to resist the gastrointestinal tract digestion and be transported in bioactive form [144]. Primary digestion of peptides starts in the stomach with the action of pepsin in acidic conditions. Following that, peptides are digested in the luminal phase of the small intestine at an alkaline pH by the action of pancreatic proteases like trypsin, α-chymotrypsin, elastase, and carboxypeptidase A and B [144]. Next, peptides resisting gastrointestinal digestion are subjected to the intestinal brush border membrane where a variety

of peptidases can further hydrolyze the ACE inhibitory peptide. Generally, peptides resisting this step can be transported to the blood circulation [145].

In order to demonstrate peptide bioavailability, additional (bio)chemical characterization is needed. Several measurements of the stability of the purified antihypertensive peptides against gastrointestinal enzymatic digestion can be carried out. The pure peptide can be submitted to a simulated gastrointestinal digestion using different enzyme systems. A combination of trypsin-chymotrypsin [100], pepsin-pancreatin [93], pepsin-corolase PP [89], pepsin-trypsin [52] or pepsin-trypsin-protease N [51] enzymes have been employed for this purpose. This procedure has been assayed with peptides isolated from sea cucumber [123], rice [102], oyster [124], porcine hemoglobin [114] and wakame [126] hydrolysates. Resistance to intestinal digestion can also be demonstrated by the use of a model system such as Caco2 cells. Caco2 cells in a monolayers format display a variety of intestinal enzymes and transporters and have been employed as a model of intestine epithelium [28]. Geerlings *et al.* [84] purified three peptides (TGPIPN, SLPQ, and SQPK) from goat milk hydrolysate, which all had similar IC_{50} values. All peptides were subjected to the Caco2 monolayer experiment but only TGPIPN was found to pass through the Caco2 monolayer intact (albeit in a small quantity). Therefore, the intake of goat milk hydrolysate by SHR over 12 weeks had resulted in a decrease of SBP [84]. Nevertheless, it is important to highlight that this model could yield erroneous conclusions since the Caco2 model is tighter than intestinal mammalian tissue. Therefore, some molecules exerting *in vivo* activity could not show sufficient absorption in this model [146].

Furthermore, since ACE cleaves the C-terminal of oligopeptides with wide specificity, antihypertensive peptides reaching the cardiovascular system also need to resist ACE action. In relation to this fact, peptides can be divided into three groups: inhibitor type, substrate type, and pro-drug type [112]. *'Inhibitor type'* peptides are not affected when they are preincubated with ACE. *'Substrate type'* peptides show a decrease in activity when they are exposed to ACE whereas *'Pro-drug type'* peptides are transformed to a true inhibitor by ACE or gastrointestinal proteases [144, 147]. True inhibitor type [36, 48, 123], substrate type [113, 116], and pro-drug inhibitor [148] peptides have been found in different hydrolysates.

One of the attempts to understand the inhibition site and to explore the inhibition mechanism of antihypertensive peptides is the measurement of the inhibition mode of peptides. The overall pattern for ACE inhibition was investigated by the incubation of inhibitory peptides with different concentrations of HHL and by measuring of the ACE inhibitory activity. The majority

of antihypertensive peptides inhibit ACE following a competitive mode although noncompetitive inhibition has also been found [147]. Structure- activity correlation is influenced by the three C-terminal residues of the antihypertensive peptide where the substrate or competitive inhibitors, which constantly contain hydrophobic (aromatic or branched-side chains) residues, are preferred. However, the most favorable are aromatic amino acid residues and proline [106]. Competitive ACE inhibitor peptides have been found inter alia in porcine skeletal muscle troponin [113], soybean glycinin [97], *Pholiota adiposa* [52], oyster [62], mushroom *tricholoma giganteum* [51], and porcine hemoglobin [114] hydrolysates. Noncompetitive ACE inhibitor peptides have been found in oyster [124], tuna dark muscle [33], bullfrog muscle [37], pork loin [116], bovine lactoferrin [118], and hen ovotransferrin hydrolysates [119].

7. Quantification of peptides with antihypertensive activity

Since the first discovery of antihypertensive peptides from foodstuffs, studies in the area of ACE inhibitory peptides were mainly focused on isolation, purification, identification, and characterization of these peptides. In the last years, a new trend has been the quantitative analysis of particular peptides with high IC_{50} values. In fact, the recent rapid development of functional foods which contain antihypertensive peptides requires established standardized methodologies for the quantification of peptides including stability studies in complex biological matrices. In this cases, quantitative determination of antihypertensive peptides is essential to assess safety, product activity, and healthy claims [149, 150]. Furthermore, the amount of encrypted peptides could varied within crop varieties and the amount of released peptides could depends on hydrolysis and storage conditions [46, 87]. Moreover, considering that functional foods could become widespread, quantitative information will be essential to establish regulations controlling the addition of antihypertensive peptides to commercial foodstuffs [149, 150].

Generally, the quantification of selected antihypertensive peptides is carried out by MS with previous HPLC separation, however some other attempts can also be found in literature. The quantification of particular peptides which possess antihypertensive activity was made on the standard calibration curve of corresponding synthetic peptides injected into the LC-MS system. By this methodology, the concentration of seven dipeptides in wakame [126] and eight dipeptides in salmon muscle [129] hydrolysates were estimated. Fig. 3 shows the chromatograms and the mass spectra corresponding to the antihypertensive peptide FY in a

synthetic standard and in the hydrolysate of wakame. Since the mass spectrum obtained with the synthetic peptide was identical to that observed in the hydrolysate, this was used for the quantitation of the peptide in wakame by LC-MS (Sato *et al.*, 2002). A similar approach has also been used for the determination of three peptides in goat milk hydrolysate [84].

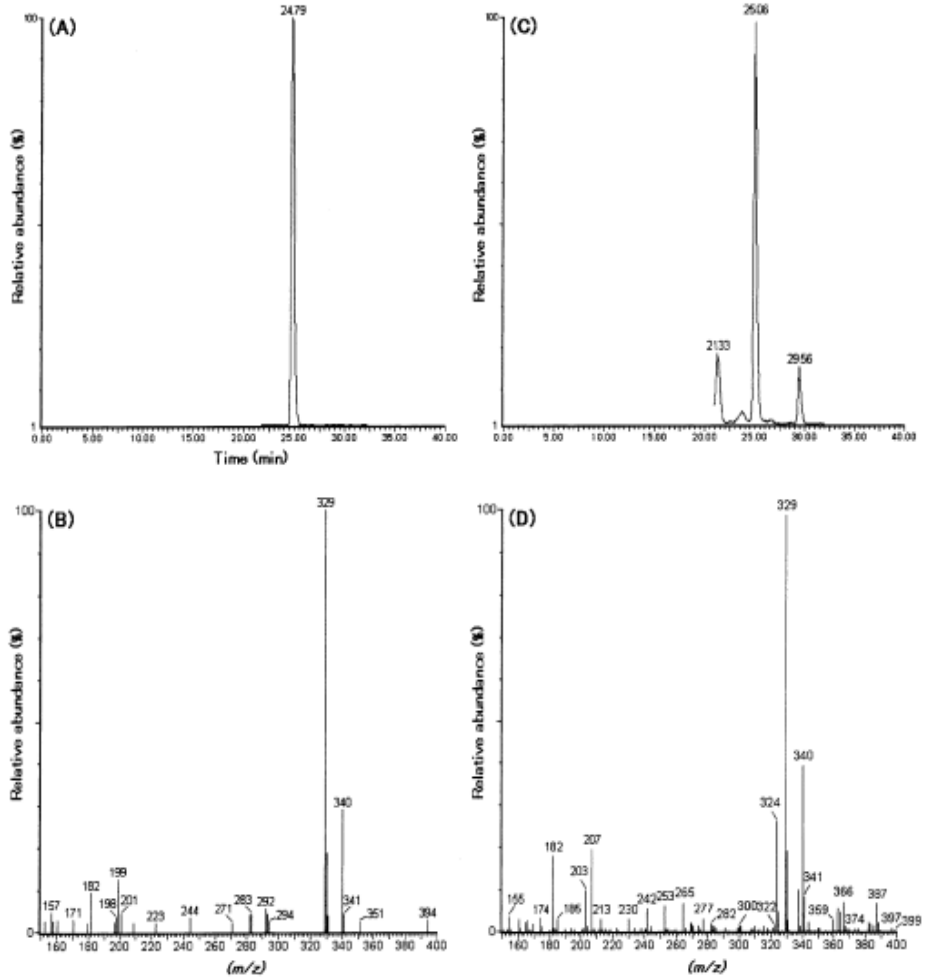

Fig. 3. Chromatograms (A, C) and mass spectra (B, D) obtained by LC-MS and corresponding to FY: (A, B) synthetic FY; (C, D) FY in the hydrolysate of wakame. LC conditions: Column, Xterra MS C18, 150 x 2.1 mm; gradient, 3-20% in 40 min; mobile phases, A: water + 0.05% TFA; B: ACN+0.05% TFA; flow-rate, 0.2 mL/min; MS conditions: cone voltage: +30V; capillary voltage: 3 kV; desolvation temp.: 300 °C; source block temp.: 100°C; desolvation gas flow: 350 L/min; cone gas flow: 50 L/min. Source: [126].

Quantification of LHLPLP peptide in fermented milk has been performed by HPLC-MS and HPLC-MS/MS. The developed method was validated by the determination of repeatability, reproducibility, linearity, and recovery. Calibration was performed based on the peak areas of the precursor and its adducts in the MS experiments and on the 0peak area of the most abundant product ions after precursor fragmentation by MS/MS analysis. The limits of detection and quantification determined by MS/MS were 7 µg/mL and 25 µg/mL, respectively [82]. Similarly,

the LKPNM antihypertensive peptide was determined in bonito muscle hydrolysates by HPLC-MS and HPLC-MS/MS. Validation of the method by measuring specificity, linearity, accuracy, precision, and reproducibility was also presented [151]. Next, the quantification of nine antihypertensive dipeptides in fermented soybean seasonings and soybean sauces was performed by LC-MS/MS [98]. A comparative study of the concentration of IPP and VPP in Swiss cheeses and non-Swiss cheeses [152] and in cheeses with different ripening degrees [56] using HPLC-MS^3 and PPPP as an internal standard revealed that there were large variations among individual loaves from various producers. Moreover, high concentrations of both peptides often occurred in these cheeses produced from raw milk which was matured over a long period of time. The same peptides were also quantified in miso paste by LC-MS using internal standard methodology. In this case isotopes ($^{13}C_5$)Val($^{13}C_5$)Pro-Pro and Ile-($^{13}C_5$)Pro-Pro were involved [153]. HPLC with UV detection has also been employed for the quantitation of antihypertensive peptides in foodstuffs. Yamamoto *et al.* [30] used it for the quantitation of YP in yoghurt like products using a synthetic peptide for the calibration [30]. The same methodology was employed in the quantification of IY, VY and IVY in Protease N treated Royal Jelly [154].

Moreover, targeted peptides are usually presented in highly complex matrices and at low concentration. These complex matrices could compromise the determination of these minor components. MRM (multiple reaction monitoring) assays could be the technique of choice in these cases. Nevertheless, only one work in which antihypertensive peptides were quantified in rat plasma after their administration was found [155]. To our knowledge, there is no work that had used this methodology to quantify antihypertensive peptides in foodstuffs. Since the MRM assay offers reliable quantification for low abundance analytes in complex matrices, it can be a potential tool for overcoming these future challenges.

8. Conclusions

The role of antihypertensive peptides derived from foodstuffs becomes increasingly appreciated since hypertension is a serious problem, especially in highly-developed countries. The knowledge about ACE inhibitory peptides in the last years improved at the same rate as the specifications of their biochemistry, bioavailability, properties, and mechanisms of inhibition. In addition to this, the number of identified peptides with certain ACE inhibition activity from various sources increased considerably. Antihypertensive peptides usually contain 2-12 amino acids and significant amounts of hydrophobic residues. Secondly, the selection of

an appropriate source of protein with a suitable releasing technique is crucial in the production of antihypertensive peptides. The most frequently involved technique is enzymatic digestion where the use of enzymes with low specificity is essential. Techniques such as fermentation, autolysis or simple extraction in the case of naturally presented antihypertensive peptides were also found in literature to be an alternative. Different ACE assays have been employed to evaluate IC_{50} values. Nevertheless, the significant differences among obtained results demand a standardized method for measuring antihypertensive activity. Purification paths generally depend on the complexity of the hydrolysate although some common features can be extracted. UF or SEC are commonly used as a first stage purification. IEC, as much cIEC as AEC, is also frequently used in the purification path. RP-LC can be used both at the end of the purification stage or as well as the only technique used in the purification of peptides. Some others methods, such as desalination, liquid-liquid extraction, solid phase extraction or capillary electrophoresis also randomly appears in the purification paths of antihypertensive peptides. Peptide identification has been perfomed by Edman degradation despite MS is now the preferred technique. Antihypertensive peptide characterization also includes (bio)chemical tests to assess bioactivity. Namely, gastrointestinal digestion, Caco2 monolayer, preincubation with ACE or inhibition mode are employed to check inter alia the bioavailability of peptides. Quantitative analysis of some targeted peptides is becoming more usual being HPLC-MS the preferred technique for this purpose.

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References for Article 1:

[1] J. Schrezenmeir, H. Korhonen, C. Williams, H. Gill, N. Shah, Beneficial natural bioactive substances in milk and colostrum - Occurrence, biochemical and technological characteristics of bioactive substances - Physiological effects and potential health benefits - Foreword, Br. J. Nutr. 84 (2000) S1-S1.

[2] N.P. Moller, K.E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, Bioactive peptides and proteins from foods: indication for health effects, Eur. J. Nutr. 47 (2008) 171-182.

[3] A. Iwaniak, P. Minkiewicz, Biologically active peptides derived from proteins - A review, Pol. J. Food Nutr. 58 (2008) 289-294.

[4] H. Korhonen, A. Pihlanto, Food-derived bioactive peptides - Opportunities for designing future foods, Curr. Pharm. Des. 9 (2003) 1297-1308.

[5] W. Wang, E. De Mejia, A new frontier in soy bioactive peptides that may prevent age-related chronic diseases, Compr. Rev. Food Sci. F. 4 (2005) 63-78.

[6] B. Hernández-Ledesma, I. Recio, L. Amigo, Beta-Lactoglobulin as source of bioactive peptides, Amino Acids 35 (2008) 257-265.

[7] A.R. Madureira, T. Tavares, A.M.P. Gomes, M.E. Pintado, F.X. Malcata, Invited review: Physiological properties of bioactive peptides obtained from whey proteins, J. Dairy Sci. 93 (2010) 437-455.

[8] M. Zimecki, M.L. Kruzel, Milk-derived proteins and peptides of potential therapeutic and nutritive value, J. Exp. Ther. Oncol. 6 (2007) 89-106.

[9] D.D. Kitts, K. Weiler, Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery, Curr. Pharm. Des. 9 (2003) 1309-1323.

[10] P. Minkiewicz, J. Dziuba, A. Iwaniak, M. Dziuba, M. Darewicz, BIOPEP database and other programs for processing bioactive peptide sequences, J. AOAC Int. 91 (2008) 965-980.

[11] C.M.M. Lawes, S. Vander Hoorn, A. Rodgers, Int Soc Hypertens, Global burden of blood-pressure-related disease, 2001, Lancet 371 (2008) 1513-1518.

[12] M. Ezzati, S. Vander Hoorn, A. Rodgers, A. López, C. Mathers, C. Murray, Comparative Risk Assessment Collab, Estimates of global and regional potential health gains from reducing multiple major risk factors, Lancet 362 (2003) 271-280.

[13] B.A. Murray, R.J. FitzGerald, Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production, Curr. Pharm. Des. 13 (2007) 773-791.

[14] A. Chockalingam, World hypertension day and global awareness, Can. J. Cardiol. 24 (2008) 441-444.

[15] M.I. Pérez, V.M. Musini, Pharmacological interventions for hypertensive emergencies: a Cochrane systematic review, J. Hum. Hypertens. 22 (2008) 596-607.

[16] L.M. Souza, R. Riera, H. Saconato, A. Demathe, A.N. Atallah, Oral drugs for hypertensive urgencies: systematic review and meta-analysis, Sao Paulo Med. J. 127 (2009) 366-372.

[17] S. Ferreira, D. Bartelt, L. Greene, Isolation of bradykinin-potentiating peptides from bothrops-jararaca venom, Biochemistry (N. Y.). 9 (1970) 2583-&.

[18] D. Cushman, M. Ondetti, History of the design of captopril and related inhibitors of Angiotensin Converting Enzyme, Hypertension 17 (1991) 589-592.

[19] A.B. Atkinson, J.I.S. Robertson, Captopril in the treatment of clinical hypertension and cardiac-failure, Lancet 2 (1979) 836-839.

[20] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[21] S. Lee, Z. Qian, S. Kim, A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Food Chem. 118 (2010) 96-102.

[22] K. Suetsuna, Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from Allium sativum L (garlic), J. Nutr. Biochem. 9 (1998) 415-419.

[23] A.S. Pina, A.C.A. Roque, Studies on the molecular recognition between bioactive peptides and angiotensin-converting enzyme, J. Mol. Recognit. 22 (2009) 162-168.

[24] Z. Chen, C. Peng, R. Jiao, Y.M. Wong, N. Yang, Y. Huang, Anti-hypertensive nutraceuticals and functional foods, J. Agric. Food Chem. 57 (2009) 4485-4499.

[25] R.E. Schmieder, K.F. Hilgers, M.P. Schlaich, B.M.W. Schmidt, Renin-angiotensin system and cardiovascular risk, Lancet 369 (2007) 1208-1219.

[26] H. Meisel, Biochemical properties of regulatory peptides derived from milk proteins, Biopolymers 43 (1997) 119-128.

[27] S. Maruyama, H. Suzuki, A Peptide Inhibitor of angiotensin-I converting enzyme in the tryptic hydrolysate of casein, Agric. Biol. Chem. 46 (1982) 1393-1394.

[28] R. López-Fandiño, J. Otte, J. van Camp, Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity, Int. Dairy J. 16 (2006) 1277-1293.

[29] I. Ricci, R. Artacho, M. Olalla, Milk protein peptides with angiotensin I-converting enzyme inhibitory (ACE I) activity, Crit. Rev. Food Sci. Nutr. 50 (2010) 390-402.

[30] N. Yamamoto, M. Maeno, T. Takano, Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4, J. Dairy Sci. 82 (1999) 1388-1393.

[31] N. Yamamoto, T. Takano, Antihypertensive peptides derived from milk proteins, Nahrung-Food 43 (1999) 159-164.

[32] C.R. Sirtori, C. Galli, J.W. Anderson, A. Arnoldi, Nutritional and nutraceutical approaches to dyslipidemia and atherosclerosis prevention: Focus on dietary proteins, Atherosclerosis 203 (2009) 8-17.

[33] Z.J. Qian, J.Y. Je, S.K. Kim, Antihypertensive effect of angiotensin i converting enzyme-inhibitory peptide from hydrolysates of Bigeye tuna dark muscle, Thunnus obesus, J. Agric. Food Chem. 55 (2007) 8398-8403.

[34] K. Arihara, Y. Nakashima, T. Mukai, S. Ishikawa, M. Itoh, Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins, Meat Sci. 57 (2001) 319-324.

[35] K. Yokoyama, H. Chiba, M. Yoshikawa, Peptide inhibitors for angiotensin-i-converting enzyme from thermolysin digest of dried bonito, Biosci. Biotech. Bioch. 56 (1992) 1541-1545.

[36] Y. Yang Y, G. Tao , P. Liu, J. Liu, Peptide with angiotensin I-converting enzyme inhibitory activity from hydrolyzed corn gluten meal, J. Agric. Food Chem. 55 (2007) 7891-7895.

[37] Z. Qian, W. Jung, S. Lee, H. Byun, S. Kim, Antihypertensive effect of an angiotensin I-converting enzyme inhibitory peptide from bullfrog (Rana catesbeiana Shaw) muscle protein in spontaneously hypertensive rats, Process Biochem. 42 (2007) 1443-1448.

[38] W. Chiang, M. Tsou, Z. Tsai, T. Tsai, Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor, Food Chem. 98 (2006) 725-732.

[39] J. Otte, S.M. Shalaby, M. Zakora, A.H. Pripp, S.A. EI-Shabrawy, Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme, and time of hydrolysis, Int. Dairy J. 17 (2007) 488-503.

[40] E. Escudero, M. A. Sentandreu, K. Arihara, F. Toldrá, Angiotensin I-Converting Enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat, J. Agric. Food Chem. 58 (2010) 2895-2901.

[41] K. Majumder, J. Wu, Angiotensin I Converting Enzyme inhibitory peptides from simulated in vitro gastrointestinal digestion of cooked eggs, J. Agric. Food Chem. 57 (2009) 471-477.

[42] Y. Yang, E. Marczak, M. Yokoo, H. Usui, M. Yoshikawa, Isolation and antihypertensive effect of angiotensin I-converting enzyme (ACE) inhibitory peptides from spinach Rubisco, J. Agric. Food Chem. 51 (2003) 4897-4902.

[43] J.A. Gómez-Ruiz, M. Ramos, I. Recio, Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques, Electrophoresis 28 (2007) 4202-4211.

[44] T. Matsui, A. Yukiyoshi, S. Doi, H. Sugimoto, H. Yamada, K. Matsumoto, Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR, J. Nutr. Biochem. 13 (2002) 80-86.

[45] C. Li, T. Matsui, K. Matsumoto, R. Yamasaki, T. Kawasaki, Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein, J. Pept. Sci. 8 (2002) 267-274.

[46] A. Quiros, R. Chichon, I. Recio, R. López-Fandiño, The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin, Food Chem. 104 (2007) 1734-1739.

[47] J. Jia, H. Ma, W. Zhao, Z. Wang, W. Tian, L. Luo, R. He, The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein, Food Chem. 119 (2010) 336-342.

[48] H. Tonouchi, M. Suzuki, M. Uchida, M. Oda, Antihypertensive effect of an angiotensin converting enzyme inhibitory peptide from enzyme modified cheese, J. Dairy Res. 75 (2008) 284-290.

[49] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, Application of high-performance liquid chromatography-tandem mass spectrometry to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion, J. Chromatogr. A 1049 (2004) 107-114.

[50] J. Chobert, K. El-Zahar, M. Sitohy, M. Dalgalarrondo, F. Metro, Y. Choiset, T. Haertle, Angiotensin I-converting-enzyme (ACE)-inhibitory activity of tryptic peptides of ovine beta-lactoglobulin and of milk yoghurts obtained by using different starters, Lait 85 (2005) 141-152.

[51] D. Hyoung Lee, J. Ho Kim, J. Sik Park, Y. Jun Choi, J. Soo Lee, Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom Tricholoma giganteum, Peptides 25 (2004) 621-627.

[52] K.C. Koo, D.H. Lee, J.H. Kim, H.E. Yu, J.S. Park, J.S. Lee, Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from Pholiota adiposa, J. Microbiol. Biotechn. 16 (2006) 757-763.

[53] Z. Shin, R. Yu, S. Park, D. Chung, C. Ahn, H. Nam, K. Kim, H. Lee, His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo, J. Agric. Food Chem. 49 (2001) 3004-3009.

[54] J. Gómez-Ruiz, M. Ramos, I. Recio, Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures, Int. Dairy J. 12 (2002) 697-706.

[55] J.A. Gómez-Ruiz, G. Taborda, L. Amigo, I. Recio, M. Ramos, Identification of ACE-inhibitory peptides in different Spanish cheeses by tandem mass spectrometry, Eur. Food Res. Technol. 223 (2006) 595-601.

[56] J. Meyer, U. Buetikofer, B. Walther, D. Wechsler, R. Sieber, Hot topic: Changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese varieties, J. Dairy Sci. 92 (2009) 826-836.

[57] T. Saito, T. Nakamura, H. Kitazawa, Y. Kawai, T. Itoh, Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese, J. Dairy Sci. 83 (2000) 1434-1440.

[58] M. Fujii, N. Matsumura, K. Mito, T. Shimizu, M. Kuwahara, S. Sugano, H. Karaki, Antihypertensive effects of peptides in autolysate of bonito bowels on spontaneously hypertensive rats, Biosci. Biotech. Bioch. 57 (1993) 2186-2188.

[59] N. Matsumura, M. Fujii, Y. Takeda, K. Sugita, T. Shimizu, Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate, Biosci. Biotech. Bioch. 57 (1993) 695-697.

[60] A.G.P. Samaranayaka, D.D. Kitts, E.C.Y. Li-Chan, Antioxidative and angiotensin-1-converting enzyme inhibitory potential of a Pacific Hake (Merluccius productus) fish protein hydrolysate subjected to simulated gastrointestinal digestion and Caco-2 cell permeation, J. Agric. Food Chem. 58 (2010) 1535-1542.

[61] Y. Nogata, T. Nagamine, M. Yanaka, H. Ohta, Angiotensin i converting enzyme inhibitory peptides produced by autolysis reactions from wheat bran, J. Agric. Food Chem. 57 (2009) 6618-6622.

[62] J. Je, J. Park, W. Jung, P. Park, S. Kim, Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce, Crassostrea gigas, Food Chem. 90 (2005) 809-814.

[63] J. Je, P. Park, H. Byun, W. Jung, S. Kim, Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, Mytilus edulis, Bioresour. Technol. 96 (2005) 1624-1629.

[64] Q. Meng, S. Oparil, Purification and assay methods for angiotensin-converting enzyme, J. Chromatogr. A 743 (1996) 105-122.

[65] B. Holmquist B, P. Bunning P, J.F. Riordan, A continuous spectrophotometric assay for angiotensin converting enzyme, Anal. Biochem. 95 (1979) 540-548.

[66] M. Alves, M. Araujo, M. Juliano, E. Oliveira, J. Krieger, D. Casarini, L. Juliano, A. Carmona, A continuous fluorescent assay for the determination of plasma and tissue angiotensin I-converting enzyme activity, Braz. J. Med. Biol. Res. 38 (2005) 861-868.

[67] J. Wu, R. Aluko, A. Muir, Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions, J. Chromatogr. A 950 (2002) 125-130.

[68] S. Van Dyck, S. Vissers, A. Van Schepdael, J. Hoogmartens, Kinetic study of angiotensin converting enzyme activity by capillary electrophoresis after in-line reaction at the capillary inlet, J. Chromatogr. A 986 (2003) 303-311.

[69] M.S. Rohrbach, [Glycine-1-14C]-hippuryl-L-histidyl-L-leucine: a substrate for the radiochemical assay of angiotensin converting enzyme, Anal. Biochem. 84 (1978) 272-276.

[70] D. Cushman, H. Cheung, Spectrophotometric assay and properties of angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637-1648.

[71] Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, T. Takano, Purification and characterization of angiotensin I-converting enzyme-inhibitors from sour milk, J. Dairy Sci. 78 (1995) 777-783.

[72] V. Vermeirssen, J. Van Camp, W. Verstraete, Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides, J. Biochem. Biophys. Methods. 51 (2002) 75-87.

[73] T. Kodera, N. Nio, Identification of an angiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive effects of hydrolysates in spontaneously hypertensive model rats, J. Food Sci. 71 (2006) C164-C173.

[74] L. Vercruysse, G. Smagghe, G. Herregods, J. Van Camp, ACE inhibitory activity in enzymatic hydrolysates of insect protein, J. Agric. Food Chem. 53 (2005) 5207-5211.

[75] M. Murakami, H. Tonouchi, R. Takahashi, H. Kitazawa, Y. Kawai, H. Negishi, T. Saito, Structural analysis of a new anti-hypertensive peptide (beta-lactosin B) isolated from a commercial whey product, J. Dairy Sci. 87 (2004) 1967-1974.

[76] H. Fujita, M. Yoshikawa, LKPNM: a prodrug-type ACE-inhibitory peptide derived from fish protein, Immunopharmacology 44 (1999) 123-127.

[77] N. Yamamoto, M. Ejiri, S. Mizuno, Biogenic peptides and their potential use, Curr. Pharm. Des. 9 (2003) 1345-1355.

[78] J. Jiang J, Chen ,F. Ren, Z. Luo Z, S.S. Zeng, Yak milk casein as a functional ingredient: preparation and identification of angiotensin-I-converting enzyme inhibitory peptides, J. Dairy Res. 74 (2007) 18-25.

[79] A. Jang, M. Lee, Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates, Meat Sci. 69 (2005) 653-661.

[80] X. Mao, J. Ni, W. Sun, P. Hao, L. Fan, Value-added utilization of yak milk casein for the production of angiotensin-I-converting enzyme inhibitory peptides, Food Chem. 103 (2007) 1282-1287.

[81] M.S. Nielsen, T. Martinussen, B. Flambard, K.I. Sorensen, J. Otte, Peptide profiles and angiotensin-I-converting enzyme inhibitory activity of fermented milk products: Effect of bacterial strain, fermentation pH, and storage time, Int. Dairy J. 19 (2009) 155-165.

[82] A. Quiros, M. Ramos, B. Muguerza, M.A. Delgado, P.J. Mártin-Alvarez, A. Aleixandre, I. Recio, Determination of the antihypertensive peptide LHLPLP in fermented milk by high-performance liquid chromatography-mass spectrometry, J. Dairy Sci. 89 (2006) 4527-4535.

[83] A. Quiros, M. Ramos, B. Muguerza, M.A. Delgado, M. Miguel, A. Aleixandre, I. Recio, Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis, Int. Dairy J. 17 (2007) 33-41.

[84] A. Geerlings, I.C. Villar, F. Hidalgo Zarco, M. Sánchez, R. Vera, A. Zafra Gómez, J. Boza, J. Duarte, Identification and characterization of novel angiotensin-converting enzyme inhibitors obtained from goat milk, J. Dairy Sci. 89 (2006) 3326-3335.

[85] M. Maeno, N. Yamamoto, T. Takano, Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from Lactobacillus helveticus CP790, J. Dairy Sci. 79 (1996) 1316-1321.

[86] D. Pan, Y. Luo, M. Tanokura, Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of Lactobacillus helveticus JCM1004, Food Chem. 91 (2005) 123-129.

[87] C.G. Papadimitriou, A. Vafopoulou-Mastrojiannaki, S.V. Silva, A. Gomes, F.X. Malcata, E. Alichanidis, Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity, Food Chem. 105 (2007) 647-656.

[88] O.N. Donkor, A. Henriksson, T.K. Singh, T. Vasiljevic, N.P. Shah, ACE-inhibitory activity of probiotic yoghurt, Int. Dairy J. 17 (2007) 1321-1331.

[89] A. Quiros, B. Hernández-Ledesma, M. Ramos, L. Amigo, I. Recio, Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir, J. Dairy Sci. 88 (2005) 3480-3487.

[90] J.A. Gómez-Ruiz, M. Ramos, I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1054 (2004) 269-277.

[91] S. Haileselassie, B. Lee, B. Gibbs, Purification and identification of potentially bioactive peptides from enzyme-modified cheese, J. Dairy Sci. 82 (1999) 1612-1617.

[92] A. Abubakar, T. Saito, H. Kitazawa, Y. Kawai, T. Itoh, Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion, J. Dairy Sci. 81 (1998) 3131-3138.

[93] M. Robert, A. Razaname, M. Mutter, M. Juillerat, Peptides derived from sodium caseinate Hydrolysates produced by Lactobacillus helveticus NCC 2765, J. Agric. Food Chem. 52 (2004) 6923-6931.

[94] J. Otte, S.M.A. Shalaby, M. Zakora, M.S. Nielsen, Fractionation and identification of ACE-inhibitory peptides from alpha-lactalbumin and beta-casein produced by thermolysin-catalysed hydrolysis, Int. Dairy J. 17 (2007) 1460-1472.

[95] J. Chen, T. Okada, K. Muramoto, K. Suetsuna, S. Yang, Identification of angiotensin I-converting enzyme inhibitory peptides derived from the peptic digest of soybean protein, J. Food Biochem. 26 (2002) 543-554.

[96] S.J. Rho, J. Lee, Y. Il Chung, Y. Kim, H.G. Lee, Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract, Process Biochem. 44 (2009) 490-493.

[97] K.G.M. Gouda, L.R. Gowda, A.G.A. Rao, V. Prakash, Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max), J. Agric. Food Chem. 54 (2006) 4568-4573.

[98] T. Nakahara, A. Sano, H. Yamaguchi, K. Sugimoto, H. Chikata, E. Kinoshita, R. Uchida, Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin i-converting enzyme inhibitory substances, J. Agric. Food Chem. 58 (2010) 821-827.

[99] H. Motoi, T. Kodama, Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides from wheat gliadin hydrolysate, Nahrung-Food. 47 (2003) 354-358.

[100] C.G. Rizzello, A. Cassone, R. Di Cagno, M. Gobbetti, Synthesis of angiotensin I-converting enzyme (ACE)-inhibitory peptides and gamma-aminobutyric acid (GABA) during sourdough fermentation by selected lactic acid bacteria, J. Agric. Food Chem. 56 (2008) 6936-6943.

[101] G. Li, M. Qu, J. Wan, J. You, Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats, Asia Pac. J. Clin. Nutr. 16 (2007) 275-280.

[102] M. Kuba, K. Tanaka, M. Sesoko, F. Inoue, M. Yasuda, Angiotensin I-converting enzyme inhibitory peptides in red-mold rice made by Monascus purpureus, Process Biochem. 44 (2009) 1139-1143.

[103] S. Miyoshi, H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka, S. Maruyama, Structures and activity of angiotensin-converting enzyme-inhibitors in an alpha-zein hydrolysate, Agric. Biol. Chem. 55 (1991) 1313-1318.

[104] S. Yano, K. Suzuki, G. Funatsu, Isolation from alpha-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity, Biosci. Biotech. Bioch. 60 (1996) 661-663.

[105] E.G. Tovar-Pérez, I. Guerrero-Legarreta, A. Farres-González, J. Soriano-Santos, Angiotensin I-converting enzyme-inhibitory peptide fractions from albumin 1 and globulin as obtained of amaranth grain, Food Chem. 116 (2009) 437-444.

[106] G. Li, J. Wan, G. Le, Y. Shi, Novel angiotensin I-converting enzyme inhibitory peptides isolated from Alcalase hydrolysate of mung bean protein, J. Pept. Sci. 12 (2006) 509-514.

[107] D. Nakano, K. Ogura, M. Miyakoshi, F. Ishii, H. Kawanishi, D. Kurumazuka, C. Kwak, K. Ikemura, M. Takaoka, S. Moriguchi, T. Iino , A. Kusumoto, S. Asami, H. Shibata, Y. Kiso, Y. Matsumura, Antihypertensive effect of angiotensin I-converting enzyme inhibitory peptides from a sesame protein hydrolysate in spontaneously hypertensive rats, Biosci. Biotechnol. Biochem. 70 (2006) 1118-1126.

[108] R. Kapel, E. Rahhou, D. Lecouturier, D. Guillochon, P. Dhulster, Characterization of an antihypertensive peptide from an Alfalfa white protein hydrolysate produced by a continuous enzymatic membrane reactor, Process Biochem. 41 (2006) 1961-1966.

[109] K. Nakade, R. Kamishima, Y. Inoue, A. Ahhmed, S. Kawahara, T. Nakayama, M. Maruyama, M. Numata, K. Ohta, T. Aoki, M. Muguruma, Identification of an antihypertensive peptide derived from chicken bone extract, Animal Science J. 79 (2008) 710-715.

[110] A. Saiga, K. Iwai, T. Hayakawa, Y. Takahata, S. Kitamura, T. Nishimura, F. Morimatsu, Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate, J. Agric. Food Chem. 56 (2008) 9586-9591.

[111] F. Cheng, T. Wan, Y. Liu, C. Chen, L. Lin, R. Sakata, Determination of angiotensin-I converting enzyme inhibitory peptides in chicken leg bone protein hydrolysate with alcalase, Animal Science J. 80 (2009) 91-97.

[112] H. Fujita, K. Yokoyama, M. Yoshikawa, Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins, J. Food Sci. 65 (2000) 564-569.

[113] K. Katayama, H.E. Anggraeni, T. Mori, A.A. Ahhmed, S. Kawahara, M. Sugiyama, T. Nakayama, M. Maruyama, M. Mugurumat, Porcine skeletal muscle troponin is a good source of peptides with angiotensin-I converting enzyme inhibitory activity and anti hypertensive effects in spontaneously hypertensive rats, J. Agric. Food Chem. 56 (2008) 355-360.

[114] Y. Yu, J. Hu, Y. Miyaguchi, X. Bai, Y. Du, B. Lin, Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin, Peptides 27 (2006) 2950-2956.

[115] M. Muguruma, A.M. Ahhmed, K. Katayama, S. Kawahara, M. Maruyama, T. Nakamura, Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects in vivo, Food Chem. 114 (2009) 516-522.

[116] K. Katayama, Jamhari, T. Mori, S. Kawahara, K. Miake, Y. Kodama, M. Sugiyama, Y. Kawamura, T. Nakayama, M. Muruyama, M. Muguruma, Angiotensin-I converting enzyme inhibitory peptide derived from porcine skeletal muscle myosin and its antihypertensive activity in spontaneously hypertensive rats, J. Food Sci. 72 (2007) S702-S706.

[117] J. Tauzin, L. Miclo, J. Gaillard, Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alpha(S2)-casein, FEBS Lett. 531 (2002) 369-374.

[118] N. Lee, J. Cheng, T. Enomoto, I. Nakamura, The antihypertensive activity of angiotensin-converting enzyme inhibitory peptide containing in bovine lactoferrin, Chin. J. Physiol. 49 (2006) 67-73.

[119] N. Lee, J. Cheng, T. Enomoto, I. Nakamura, Antihypertensive effect of angiotensin-converting enzyme inhibitory peptide obtained from hen ovotransferrin, J. Chin. Chem. Soc. 53 (2006) 495-501.

[120] A. Hatanaka, H. Miyahara, K.I. Suzuki, S. Sato, Isolation and identification of antihypertensive peptides from antarctic krill tail meat hydrolysate, J. Food Sci. 74 (2009) H116-H120.

[121] Y. Wang, H. He, X. Chen, C. Sun, Y. Zhang, B. Zhou, Production of novel angiotensin I-converting enzyme inhibitory peptides by fermentation of marine shrimp Acetes chinensis with Lactobacillus fermentum SM 605, Appl. Microbiol. Biotechnol. 79 (2008) 785-791.

[122] Y. Zhao, B. Li, Z. Liu, S. Dong, X. Zhao, M. Zeng, Antihypertensive effect and purification of an ACE inhibitory peptide from sea cucumber gelatin hydrolysate, Process Biochem. 42 (2007) 1586-1591.

[123] Y. Zhao, B. Li, S. Dong, Z. Liu, X. Zhao, J. Wang, M. Zeng, A novel ACE inhibitory peptide isolated from Acaudina molpadioidea hydrolysate, Peptides 30 (2009) 1028-1033.

[124] J. Wang, J. Hu, J. Cui, X. Bai, Y. Du, Y. Miyaguchi, B. Lin, Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the anti hypertensive effect of hydrolysate in spontaneously hypertensive rats, Food Chem. 111 (2008) 302-308.

[125] K. Suetsuna, T. Nakano, Identification of an antihypertensive peptide from peptic digest of wakame (Undaria pinnatifida), J. Nutr. Biochem. 11 (2000) 450-454.

[126] M. Sato, T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, T. Nakano, Angiotensin I-converting enzyme inhibitory peptides derived from wakame (Undaria pinnatifida) and their antihypertensive effect in spontaneously hypertensive rats, J. Agric. Food Chem. 50 (2002) 6245-6252.

[127] K. Suetsuna, K. Maekawa, J. Chen, Antihypertensive effects of Undaria pinnatifida (wakame) peptide on blood pressure in spontaneously hypertensive rats, J. Nutr. Biochem. 15 (2004) 267-272.

[128] S. Ono, M. Hosokawa, K. Miyashita, K. Takahashi, Isolation of peptides with angiotensin I-converting enzyme inhibitory effect derived from hydrolysate of upstream chum salmon muscle, J. Food Sci. 68 (2003) 1611-1614.

[129] H. Enari, Y. Takahashi, M. Kawarasaki, M. Tada, K. Tatsuta, Identification of angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their antihypertensive effect, Fisheries Sci. 74 (2008) 911-920.

[130] H. Byun, S. Kim, Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (Theragra chalcogramma) skin, Process Biochem. 36 (2001) 1155-1162.

[131] W. Jung, E. Mendis, J. Je, P. Park, B. Son, H. Kim, Y. Choi, S. Kim, Angiotensin I-converting enzyme inhibitory peptide from yellowfin sole (Limanda aspera) frame protein and its antihypertensive effect in spontaneously hypertensive rats, Food Chem. 94 (2006) 26-32.

[132] X. Zhu, K. Watanabe, K. Shiraishi, T. Ueki, Y. Noda, T. Matsui, K. Matsumoto, Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers, Peptides 29 (2008) 338-344.

[133] J. Zhang, E. Tatsumi, C. Ding, L. Li, Angiotensin I-converting enzyme inhibitory peptides in douchi, a Chinese traditional fermented soybean product, Food Chem. 98 (2006) 551-557.

[134] M. M. Contreras, R. Carron, M. J. Montero, M. Ramos, I. Recio, Novel casein-derived peptides with antihypertensive activity, Int. Dairy J. 19 (2009) 566-573.

[135] C. De Simone, G. Picariello, G. Mamone, P. Stiuso, A. Dicitore, D. Vanacore, L. Chianese, F. Addeo, P. Ferranti, Characterisation and cytomodulatory properties of peptides from Mozzarella di Bufala Campana cheese whey, J. Pept. Sci. 15 (2009) 251-258.

[136] P. Schratter, Purification and concentration by ultrafiltration, in: P. Cutler (Ed.), Protein Purification Protocols, Humana Press, 2004, pp. 101-116.

[137] M. Miguel, M.M. Contreras, I. Recio, A. Aleixandre, ACE-inhibitory and antihypertensive properties of a bovine casein hydrolysate, Food Chem. 112 (2009) 211-214.

[138] C. Zhang, W. Cao, P. Hong, H. Ji, X. Qin, J. He, Angiotensin I-converting enzyme inhibitory activity of Acetes chinensis peptic hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Int. J. Food Sci. Tech. 44 (2009) 2042-2048.

[139] N. Sewald, H. Jakubke, Fundamental chemical and structural principles, in: Peptides: Chemistry and Biology, Wiley-VCH Verlag GmbH & Co. KGaA, 2009; 2009, pp. 5-61.

[140] C. Selkirk, Ion-Exchange Chromatography, in: P. Cutler (Ed.), Protein purification protocols, Humana Press, 2004, pp. 125-131.

[141] T. Herraiz, Sample preparation and reversed phase-high performance liquid chromatography analysis of food-derived peptides, Anal. Chim. Acta. 352 (1997) 119-139.

[142] W.A. Neville, Reversed-phase chromatography of proteins, in: P. Cutler (Ed.), Protein purification protocols, Humana Press, 2003, pp. 259-281.

[143] A. Quiros, A. Davalos, M.A. Lasuncion, M. Ramos, I. Recio, Bioavailability of the antihypertensive peptide LHLPLP: Transepithelial flux of HLPLP, Int. Dairy J. 18 (2008) 279-286.

[144] V. Vermeirssen, J. Van Camp, W. Verstraete, Bioavailability of angiotensin I converting enzyme inhibitory peptides, Br. J. Nutr. 92 (2004) 357-366.

[145] A. Pihlanto-Leppala, Bioactive peptides derived from bovine whey proteins: opioid and ace-inhibitory peptides, Trends Food Sci. Technol. 11 (2000) 347-356.

[146] V. Vermeirssen, P. Augustijns, N. Morel, J. Van Camp, A. Opsomer, W. Verstraete, In vitro intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests, Int. J. Food Sci. Nutr. 56 (2005) 415-430.

[147] G. Li, G. Le, Y. Shi, S. Shrestha, Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, Nutr. Res. 24 (2004) 469-486.

[148] N. Lee, J. Cheng, T. Enomoto, Y. Nakano, One peptide derived from hen ovotransferrin as pro-drug to inhibit angiotensin converting enzyme, J. Food Drug Anal. 14 (2006) 31-35.

[149] M. M. Contreras, I. López-Expósito, B. Hernández-Ledesma, M. Ramos, I. Recio, Application of mass spectrometry to the characterization and quantification of food-derived bioactive peptides, J. AOAC Int. 91 (2008) 981-94.

[150] C.S. Gilani, C. Xiao, N. Lee, Need for accurate and standardized determination of amino acids and bioactive peptides for evaluating protein quality and potential health effects of foods and dietary supplements, J. AOAC Int. 91 (2008) 894-900.

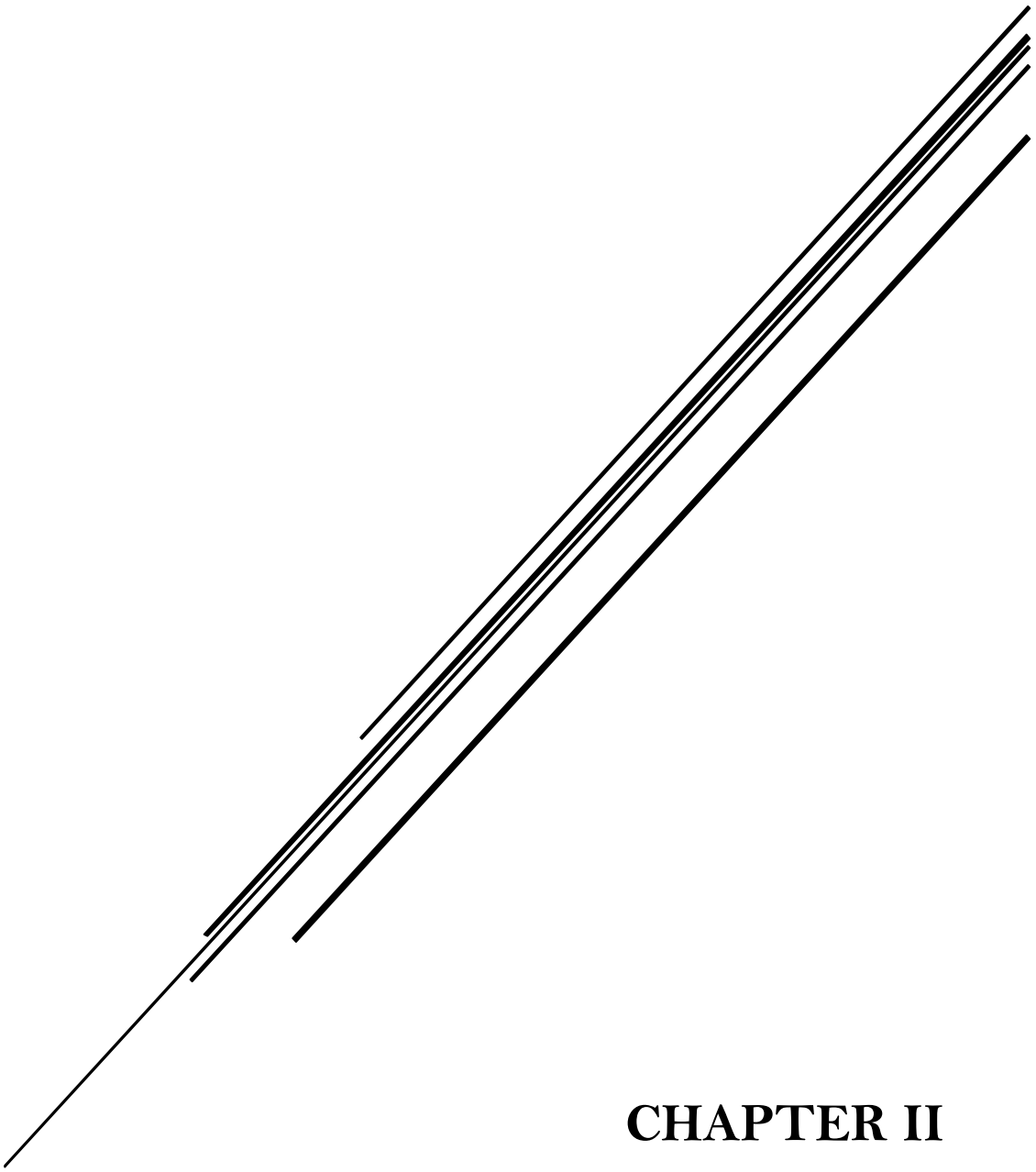
[151] J.M. Curtis, D. Dennis, D.S. Waddell, T. MacGillivray, H.S. Ewart, Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS, J. Agric. Food Chem. 50 (2002) 3919-3925.

[152] U. Buetikofer, J. Meyer, R. Sieber, D. Wechsler, Quantification of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses, Int. Dairy J. 17 (2007) 968-975.

[153] K. Inoue, T. Gotou, H. Kitajima, S. Mizuno, T. Nakazawa, N. Yamamoto, Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein, J. Biosci. Bioeng. 108 (2009) 111-115.

[154] K.H. Tokunaga, C. Yoshida, K.M. Suzuki, H. Maruyama, Y. Futamura, Y. Araki, S. Mishima, Antihypertensive effect of peptides from Royal Jelly in spontaneously hypertensive rats, Biol. Pharm. Bull. 27 (2004) 189-192.

[155] E.M.N. Nakashima, A. Kudo, Y. Iwaihara, M. Tanaka, K. Matsumoto, T. Matsui, Application of C-13 stable isotope labeling liquid chromatography-multiple reaction monitoring-tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats, Anal. Biochem. 414 (2011) 109-116.



CHAPTER II

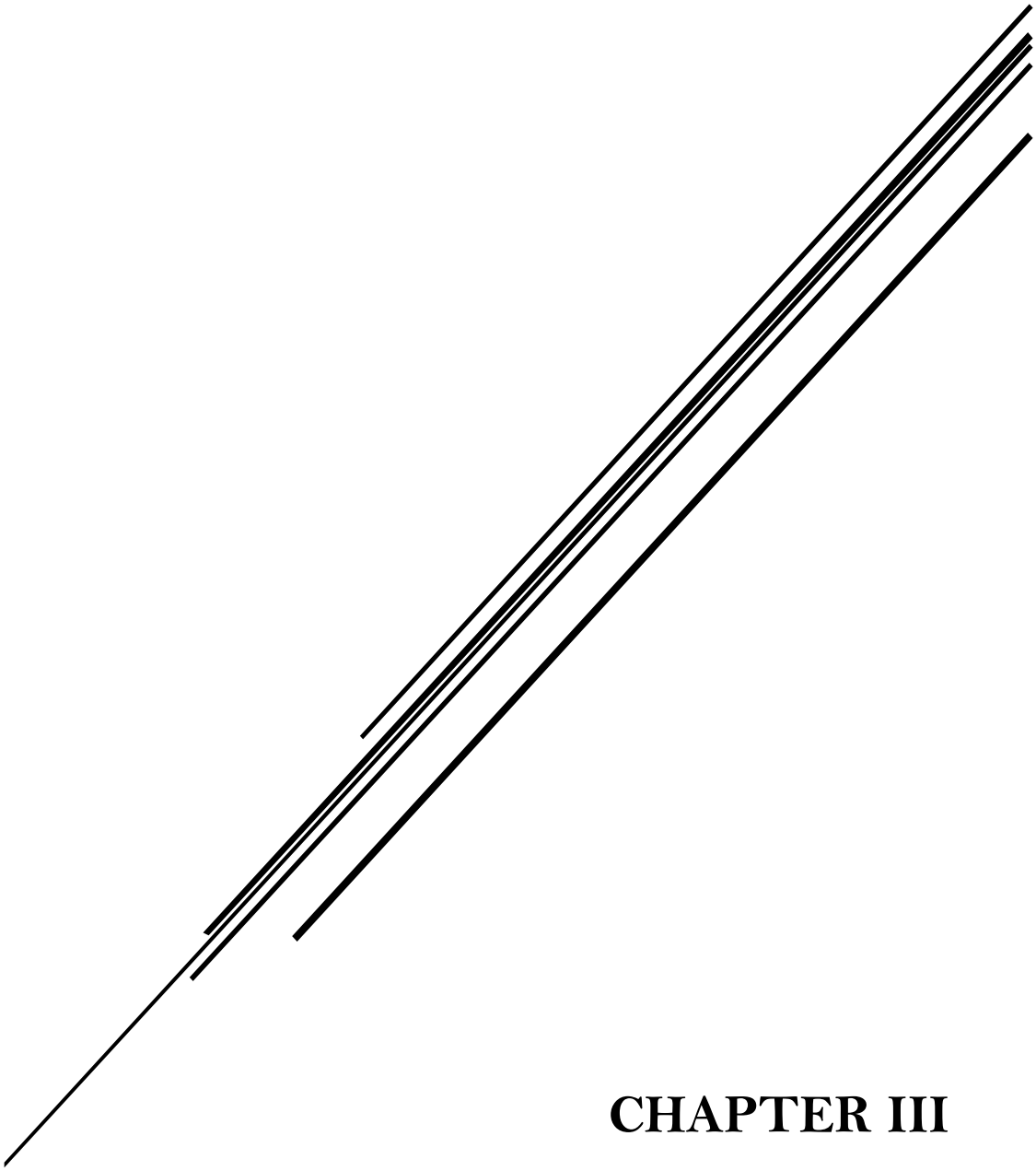
OBJECTIVES

II. OBJECTIVES

The main goal of the present research work has been the characterization, identification, and quantification of peptides and proteins having a significant influence on the prevention and understanding of hypertension. For this purpose, our efforts were aimed to develop new analytical methodologies for the determination of proteins and peptides from different plant and animal origin tissues using the most recent technological achievements in the separation and detection areas.

In order to achieve this general goal, the following specific objectives were proposed:

- To investigate the presence of native antihypertensive and antioxidant peptides in commercial soybean based infant formulas.
- To identify potential antihypertensive and antioxidant peptides from soybean based infant formulas using mass spectrometry.
- To study the influence of simulated gastrointestinal digestion on the bioactivity of soybean based infant formulas.
- To develop analytical methodologies based on mass spectrometry enabling the sensitive and selective quantification of highly potent antihypertensive peptides in soybean and maize crops.
- To evaluate the content of highly potent antihypertensive peptides in different soybean and maize crops.
- To develop an analytical methodology for the quantification of isoforms of important proteins involved in the regulation of cardiovascular function (PKA, PKG, and CaMKII) using mass spectrometry and proteomics tools.
- To apply the developed analytical method for the analysis of healthy and pathological heart rat tissues.



CHAPTER III

RESULTS AND DISCUSSION

III.1.
Results

III.1.1.

Characterization and identification of antioxidant and antihypertensive peptides in commercial soybean infant formulas

III.1.1. Characterization and identification of antioxidant and antihypertensive peptides in commercial soybean infant formulas.

Preface

The most frequently studied bioactive peptides of cardiovascular interest from foodstuffs are antihypertensive and antioxidant peptides. From the vast list of bioactive peptide sources, peptides from an animal origin have received a special attention. Nevertheless, as already mentioned, plant origin bioactive peptides have proved several times to provide peptides with much more potent bioactivities. Among the most promising plant sources, soybean and its derived products have shown to be an attractive source of bioactive peptides. Despite milk and dairy products and soybean and its derived products are the most investigated sources of both antihypertensive and antioxidant peptides, there is no work devoted to the determination of these peptides in soybean based infant formulas.

Soybean based infant formulas are widely used to feed children suffering from allergy to cow's milk and when breast milk is not possible. They are inexpensive and nutritionally adequate as substitutive of milk based formulas and rarely elicit allergic reactions [246]. Soybean based infant formulas are based on soybean protein isolate which contains around 90% of proteins [247]. Soybean infant formulas are submitted to heating and/or partial protein hydrolysis during their manufacture preparation. This procedure can result in the release of various peptides from parent proteins, differing in length, sequence, and, thus, properties. Therefore, soybean based infant formulas can contain native peptides that can have potential to offer specific health effects (antihypertensive and/or antioxidant) in addition to nutritional benefits. Due to the different manufacturing conditions, soybean based infant formulas can present different protein and peptide contents [248]. Peptides exhibiting one or two of these bioactivities are of particular interest as food components, since they could improve cardiovascular health and control related diseases [249]. Finally, in order to exert effects at a molecular level in the organism, ingested peptides must preserve their sequence and bioactivity after passing the gastrointestinal track. Native antihypertensive and antioxidant peptides resistant to the action of gastrointestinal enzymes show the real nutritional value of the product and their identification is of very high interest.

Objectives

The specific objectives of this work were:

- To propose and evaluate various extraction methods to obtain antihypertensive and antioxidant peptides from different commercial soybean based infant formulas.
- To fractionate complex peptide extracts from soybean based infant formulas using chromatographic, electrophoretic, and membrane separation techniques.
- To investigate the antioxidant and antihypertensive activities of peptides present in soybean based infant formulas using various *in vitro* assays.
- To identify potential antioxidant and antihypertensive peptides from soybean based infant formulas using highly potent mass spectrometrics and bioinformatics tools.
- To study the resistance of the identified antioxidant and antihypertensive peptides to the action of gastrointestinal enzymes.
- To characterize and investigate the bioactivity of the identified and selected peptides.

Results

The results obtained in this research work are included in the following scientific articles:

- **Article 2:** *Isolation and identification of antioxidant peptides from commercial soybean based infant formulas.*
  P. Puchalska, M. L. Marina, M. C. García.
  Food Chem., 2014, 148, 147-154.

- **Article 3:** *Identification of native angiotensin I converting enzyme inhibitory peptides in commercial soybean based infant formulas using HPLC-Q-TOF-MS.*
  P. Puchalska, M. C. García, M. L. Marina.
  Food Chem., in press.

Article 2

Isolation and identification of antioxidant peptides from commercial soybean based infant formulas

P. Puchalska, M. L. Marina, M. C. García

Food Chem., 2014, 148, 147-154

Abstract

Soybean based infant formulas (SBIFs) based on soybean protein isolate (90% of proteins) are an interesting alternative to cow's milk infant formulas. Different works have demonstrated the presence of bioactive peptides in different soybean based foodstuffs. The aim of this work was the evaluation, for the first time, of antioxidant peptides in five different commercially available SBIFs. Ultrafiltration through 10 kDa molecular weight cut-off filters was the most suitable extraction method. Despite peptide concentrations ranging between 1.19 and 2.27 mg/mL, similar antioxidant capacities were detected in all SBIF extracts. Extracts were further fractionated according to their molecular weight by ultrafiltration, and fractions from 5 to10 kDa, 3 to 5 kDa, and below 3 kDa were obtained. The most active fraction was further fractionated by off-gel isoelectrofocusing and reversed-phase chromatography. Antioxidant fractions were also submitted to simulated gastrointestinal digestion with pepsin and pancreatin to evaluate their antioxidant capacity after digestion. Peptides were identified by HPLC-ESI-Q-ToF-MS/MS. At least 120 peptides were identified in every antioxidant fraction, with 42 peptides common to all SBIFs.

Keywords:

Bioactive peptides; Antioxidant capacity; Soybean based infant formula; Q-TOF; Food analysis

1. Introduction

Cows' milk and soybean are the most widely used sources of proteins in infant formulas. Soybean based infant formulas (SBIFs) were introduced onto the market more than 60 years ago. At the moment, SBIFs accounts for 25% of total infant formulas sold in the USA [1]. Initially, SBIFs were developed for infants with immunoglobulin E-mediated milk allergy and post infectious diarrhoea (lactose intolerance) [1]. Nowadays, they are also targeted at infants with galactosaemia, for infants from families who are strict vegans, and for the treatment of common feeding problems [2]. Early SBIFs were based on soybean flour while modern SBIFs are based on soybean protein isolate (SPI) and possess higher protein digestibility. SPI contains around 90% protein based on a dry weight basis and a highly balanced concentration of essential amino acids [2]. Moreover, these formulas are enriched in amino acids, such as methionine [3], carnitine [4], taurine [5], and choline [6], and other nutrients, such as lipids, vitamins and minerals [7].

Proteins are the precursors of many bioactive peptides, released by the action of gastrointestinal proteolysis or food processing [8]. Biologically active peptides can be absorbed through the intestine and can produce several biological effects at tissue level. Indeed, peptides released in the gastrointestinal tract are of high importance, since they can exert a beneficial effect on the body when they are adsorbed in the intestine. This fact is especially important in infants whose gastrointestinal tract is still not yet completely developed [9]. Soybean based infant formulas are submitted to heating and partial protein hydrolysis during manufacture, which can result in the release of peptides. The antioxidant capacity of peptides has been widely reported [10-14]. Antioxidant peptides inhibit reactive oxygen species (ROS) and other free radicals, formed as a consequence of cellular metabolism in aerobic organisms. ROS and free radicals can cause extensive damage in DNA, lipids, and proteins [15, 16]. Under normal conditions, they are removed by enzymatic or non-enzymatic antioxidants constituting the body antioxidant defence system [10]. However, environmental conditions, way of life or pathological situations may cause an imbalance between free radicals and antioxidants. This process, known as oxidative stress, has been associated with cell apoptosis and several diseases, including diabetes, atherosclerosis and cancer [17].

Antioxidant peptides are rich in histidine and other aromatic and/or hydrophobic amino acids [10]. It has been also suggested that peptide linkage and peptide conformation could also affect the antioxidant capacity of peptides [18, 19].

The aim of this work is to evaluate the presence of peptides with antioxidant capacity in commercial SBIFs. The isolation and fractionation of peptides from five different SBIFs was performed. Antioxidant peptides obtained after *in vitro* gastrointestinal digestion have been identified.

2. Materials and methods

2.1. Chemicals and samples

Acetonitrile (ACN) was purchased from Fisher Scientific (Pittsburgh, PA). Water was freshly taken daily from a Milli-Q system (Millipore, Bedford, MA). Methanol, ethanol, trichloroacetic acid (TCA), and acetic acid (AA) were from Scharlau Chemie (Barcelona, Spain). Sodium dodecyl sulfate (SDS), hydrochloric acid, 2-mercaptoethanol, sodium bicarbonate, and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Sodium tetraborate, *o*-phthalaldehyde (OPA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), glutathione (GSH), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, potassium phosphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,10-phenantroline, ferrous sulfate, hydrogen peroxide, pepsin from porcine gastric mucosa (P7012), and pancreatin from porcine pancreas (P-7545) were purchased from Sigma (St. Louis, MO). All chemicals were of analytical grade purity.

Thiamine (B_1), riboflavin (B_2), nicotinic acid (B_3), pyridoxine (B_6), biotin (B_7), folic acid (B_9), and cobalamin (B_{12}) were from Sigma, and pantothenic acid (B_5) and ascorbic acid (C) were from Fluka (Buchs, Switzerland). Vitamins B_1, B_3, B_5, B_6, B_9, C (all at 1 mg/ mL) and B_{12} (4 mg/mL) were prepared in water. Vitamins B2 (1 mg/mL) and B7 (0.5 mg/mL) were dissolved in 20 mM $NaHCO_3$ due to their lower solubility.

The five different soybean based infant formulas (SBIF 1- SBIF 5) were purchased at a local pharmacy and stored at room temperature. SBIF 1- 4 were indicated for newborn infants (stage 1 infant formulas), while SBIF 5 was for infants older than 6 months (stage 2 infant formula).

2.2. Peptide isolation from infant formulas

Infant formula samples were prepared at the concentration suggested by manufacturers for infant ingestion (1 g of SBIF powder in 6 mL of solution). In order to separate peptides from proteins and other components in infant formulas, different extraction methods were proposed. *Method 1* consisted of mixing infant formula with 12% TCA as previously described [20] using

ultrasonic energy for 5 min followed by centrifugation (10 min, 4000g, 4°C). *Method 2* was also performed as previously described with modifications [21]. Sample was mixed with water (previously boiled) at 40°C, boiled for 15 min, mixed with TCA to attain a final concentration of 5% TCA, boiled for another 15 min, left in the fridge for 1 h, and centrifuged (10 min, 4000 x g,4°C). *Method 3* consisted of ultrafiltering extracts obtained from Method 2 using Millipore filters with 10 kDa molecular weight cut-off (Mwco) (1 h, 4000g, 25°C). *Method 4* consisted of mixing SBIF with water at 40°C (water was boiled before addition) and utrafiltering through Millipore filters with 10 kDa Mwco (1 h, 4000g, 25°C). In all cases, extracts were filtered through 0.45 μm regenerated cellulose filters and stored at -20°C until use.

2.3. Separation of peptide extracts/fractions and vitamins

Peptide extracts, their fractions, and vitamin solutions were separated using an Agilent Technologies liquid chromatograph (Santa Clara, CA) with an Ascentis Express Peptide ES-C18 column (100 mm x 2.1 mm I.D., 2.7 μm particle size) and an Ascentis Express Guard column (5 mm x 2.1 mm I.D., 2.7 μm particle size), both from Supelco (Bellefonte, PA). Chromatographic conditions were: mobile phase A, Milli-Q water/0.1% (v/v) TFA; mobile phase B, ACN/0.1% (v/v) TFA; binary gradient: 5–95% B in 30 min and 95–5% in 5 min; temperature, 25°C; flow rate, 0.3 mL/min; injection volume, 2 μL; UV detection at 210, 254, and 280 nm for peptides and 210, 245, 265, and 280 nm for vitamins.

2.4. Fractionation of peptide extracts

2.4.1. Ultrafiltration (UF)

Peptide extracts from each SBIF were ultrafiltered sequentially using Amicon filters with 10 kDa Mwco (Millipore), Vivaspin 500 PES filters with 5 kDa Mwco (Sartorious Stedim Biotech, Goettingen, Germany), and Amicon filters with 3 kDa Mwco (Millipore). All recovered fractions (fractions from 5 to 10 kDa, 3 to 5 kDa, and below 3 kDa) were dissolved to the initial volume in water.

2.4.2. Separation by off-gel isoelectrofocusing (IEF)

Most active peptide fractions from every SBIF obtained after UF were further separated based on their isoelectric points (pI). For that purpose, a 3100 OFFGEL fractionator (Agilent Technologies) and immobilised pH gradient (IPG) gel strips (General Electric Healthcare, Freiburg, Germany) from pH 3 to 10 and 24-wells were used. IPG gel strips were rehydrated in

the assembled device by adding 40 μL of focusing buffer (12% (v/v) glycerol with ampholytes (pH 3–10) to every well. Peptide fractions (0.72 mL) were mixed with 2.88 mL of focusing buffer and 150 μL of this mixture were loaded in the device. In order to obtain suitable peptide focusing, a maximum current of 50 μA was applied and separation was continued until 50 kV/h was reached.

2.4.3. Separation of ampholytes from peptides

Separation of peptides fractionated by IEF from ampholytes was tried with OMIX C18 pipette tips (Varian Inc., Cary, NC), peptide clean-up C18 spin tubes (Agilent Technologies), and RP-LC. Fractionation by RP-LC was performed in a Chromolith Performance column (100 mm x 4.6 mm I.D.) from Merck and peptides were collected with an automatic fraction collector from Agilent Technologies. The optimized conditions were: mobile phase A, Milli-Q water/0.1% (v/v) TFA; mobile phase B, ACN/0.1% (v/v) TFA; flow rate, 0.5 mL/min; temperature, 25°C; injection volume, 10 μL; gradient: 5–95% in 10 min and 95–5% in 2 min. The detection was performed at 210 and 280 nm. During the fractionation step, samples of interest were collected between 6.8 and 10 min. Collected fractions were evaporated and resuspended in 100 μL of water.

2.5. OPA assay

The determination of peptide concentration in extracts was performed by the OPA assay with modifications [22] using a spectrophotometer Lambda 35 (Perkin-Elmer, Waltham, MA) and cuvettes designed for small volumes (UVetter® , Eppendorf, Hamburg, Germany). The procedure involved mixing 2.5 μL of sample with 100 μL of OPA mixture (2.5 mL of sodium tetraborate, 1 mL of 5% (w/v) SDS, 100 μL of 40 mg/mL OPA in methanol, 10 μL of 2-mercaptoethanol, and 1.39 mL of water). Afterwards, the mixture was left for 8 min at room temperature and signal was measured at 340 nm. The peptide content was calculated by interpolation in a GSH standard calibration curve in the range from 0 to 5 mg/mL. GSH, a tripeptide with one primary amine was used as a standard in the OPA assay.

The degree of hydrolysis (%DH) of sample was calculated by dividing the concentration of peptide obtained for sample extract (peptide content), by the concentration of proteins provided on the sample label, and multiplied by 100.

2.6. Antioxidant capacity assays

The estimation of antioxidant capacity was performed using DPPH, ABTS, and hydroxyl radical-scavenging assays. Solvent blanks were measured for every assay. Three replicates were prepared for every sample or fraction and all measurements were done at least three times.

2.6.1. DPPH radical-scavenging capacity assay

The assay was carried out using a previously developed method [23] with some modifications. Sample (50 μL) was mixed with 50 μL of 0.1 mM DPPH in 95% ethanol and kept for 30 min (at room temperature) in the dark. The absorbance at 517 nm of the resulting solution was measured. A calibration curve of GSH (0–5 mg/mL) was performed, before measurements, as positive control. The DPPH radical-scavenging capacity was calculated as follows:

$$\text{DPPH radical scavenging capacity (\%)} = \left(1 - \frac{Abs_{sample} - Abs_{samplecontrol}}{Abs_{blank}}\right) x100$$

where Abs_{sample} is the absorbance of the sample with DPPH solution; $Abs_{samplecontrol}$ is the absorbance of the sample without DPPH solution; Abs_{blank} is the absorbance of the sample solvent (without peptides) with the DPPH solution.

2.6.2. ABTS radical-scavenging assay

ABTS assay was performed according to a previously developed method [24]. ABTS stock solution was obtained by mixing 7.4 mM ABTS with 2.6 mM potassium persulfate in 10 mM phosphate buffer (PB) (pH 7.4) and by its incubation in the dark for 16 h. Before analysis, the ABTS radical stock solution was dissolved in 10 mM PB (pH 7.4) to attain an absorbance of 0.7 ± 0.1 AU at 734 nm (ABTS radical working solution). Prepared ABTS radical working solution (100 μL) was mixed with 1 μL of sample, incubated for 6 min, and signal was measured at 734 nm. A calibration curve of Trolox (0–5 mg/mL) was performed before measurements as positive control. The ABTS radical-scavenging capacity was calculated using the following equation:

$$\text{ABTS radical scavenging capacity (\%)} = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) x\ 100$$

Where Abs_{sample} is the absorbance of the sample with the ABTS radical working solution and Abs_{blank} is the absorbance of the sample background solution with the ABTS radical working solution.

2.6.3. Hydroxyl radical-scavenging assay

The hydroxyl radical-scavenging capacity was measured using a previously developed method [25] with some modifications. Sample (25 µL) was mixed with 25 µL of ferrous sulfate (3 mM) and 25 µL of 1,10-phenanthroline (3 mM, dissolved in 0.1 M PB (pH 7.4)). To initiate the reaction, 25 µL of 0.01% (v/v) hydrogen peroxide were added. Mixture was incubated for 1 h at 37°C and signal was measured at 536 nm in the spectrophotometer. A calibration curve of GSH (0–5 mg/mL) was performed before measurements as positive control. Hydroxyl radical-scavenging capacity was calculated according to the following equation:

$$\text{Hydroxyl radical scavenging capacity (\%)} = \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control} - Abs_{blank}}\right) x\ 100$$

Where Abs_{sample} is the absorbance of the sample; Abs_{blank} is the absorbance of a blank solution containing water; $Abs_{control}$ is the absorbance of a control solution in absence of hydrogen peroxide.

2.7. Gastrointestinal digestion

Peptide fractions were digested according to the method described by Garrett *et al.* [26], with some modifications. Briefly, sample was adjusted to pH 2 with 1 M HCl and mixed with pepsin at an enzyme to substrate ratio of 1:35. Reaction mixture was incubated for 1 h at 37°C with shaking. Afterwards, pH of sample was first adjusted with 0.1 M $NaHCO_3$ to pH 5 and then with 0.1 M NaOH to pH 7–8. Next, pancreatin enzyme dissolved in 0.1 M PB (pH 8) was added at an enzyme to sample ratio of 1:25. Reaction mixture was incubated for 2 h at 37°C with shaking. Digestion was stopped by boiling for 10 min.

2.8. HPLC-MS/MS

Identification of peptides was performed using a Quadrupole-Time-of-Flight (Q-ToF) MS (instrument series 6530) from Agilent Technologies coupled to a 1100 Series liquid chromatograph also from Agilent Technologies. HPLC separation was carried out on the Ascentis Express column previously employed, using as mobile phases Milli-Q water/0.3%

(v:v) AA (mobile phase A) and ACN/0.3% (v:v) AA (mobile phase B). The elution gradient was from 3% to 95% B in 30 min and from 95 to 3% B in 5 min. The flow rate was 0.3 mL/min, the column temperature was 25°C, and the injected volume was 15 μL. Simultaneous UV (210, 254, 280 nm) and MS signals were registered. The mass spectrometer operated in positive ion mode and a mass range from *m/z* 100 to 3200 was selected. ESI conditions were: fragmentator voltage, 200 V; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350°C; gas flow, 12 L/min; skimmer, 60 V. The Jet Stream sheath gas flow and temperature were 12 L/min and 400°C, respectively. MS/MS was performed using the auto mode, 1 precursor per cycle, dynamic exclusion after three spectra, and collision energy of 35 V. MS/MS spectra were analyzed using PEAKS Studio Version 6 (Bioinformatics Solutions Inc., Waterloo, Canada). Analysis of data with PEAKS software was performed using the PEAKS proteome database tool. Results were always refined using a false discovery rate (FDR) of 1%. Proteome of soybean (*Glycine max*) was downloaded from the UniProt protein database and contained just reviewed proteins (380 proteins).

2.9. Statistical analysis

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warrenton, VA). In order to find statistically significant differences among results, analysis of variance (ANOVA) was applied when three replicates of every sample were analysed in triplicate.

3. Results and discussion

3.1. Selection of the extraction method

In order to study the peptidome of SBIFs, two different extraction methods (Method 1 and 2) enabling the removal of proteins were tested. Both methods were applied to the extraction of five SBIFs and peptide concentrations, determined by OPA assay, are shown in Fig. 1A. Method 1 resulted in a very poor peptide yield in all SBIFs, giving peptide concentrations in the range 0.84– 1.34 mg/mL. Method 2 yielded almost double the concentration of peptides (1.83–2.90 mg/mL). This fact could be explained by co-precipitation of peptides with proteins when Method 1 is used, or by an incomplete removal of proteins when Method 2 is employed. In order to assure that Method 2 completely precipitated proteins and that signal observed by OPA assay did not correspond to proteins, a further clean-up by ultrafiltration through 10 kDa Mwco filters (Method 3) was carried out. Peptide content using *Method 3* was significantly

lower than for Method 2 (ANOVA, $P < 0.05$). Peptide content obtained by Method 3 was significantly higher than that obtained by Method 1 (ANOVA, $P < 0.05$) demonstrating that some peptides co-precipitated with proteins when Method 1 was employed. Method 4, consisting of the direct ultrafiltration of peptides through 10 kDa Mwco filters, was tried and results were compared with those obtained by Method 3. Peptide contents when using Method 4 (1.19–2.27 mg/mL) (direct UF) were statistically similar to those obtained by Method 3 (1.09–2.13 mg/mL) (combining precipitation with TCA + UF).

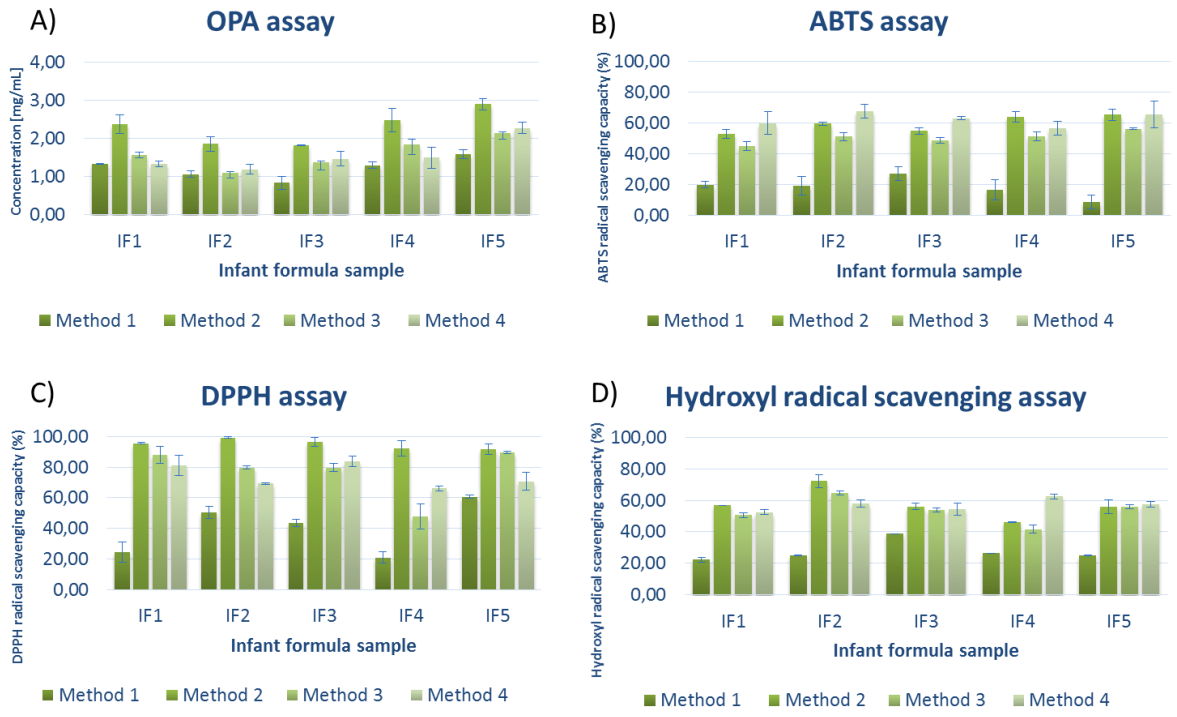


Fig. 1. Summary of results for OPA (A), ABTS (B), DPPH (C), and hydroxyl radical-scavenging capacity (D) assays for five SBIF extracts obtained using four different extraction methods.

A comparison of the different SBIF revealed higher degree of hydrolysis for SBIF 5 (11.4%) while no statistical differences were observed (ANOVA, $P < 0.05$) among the other four SBIF. Interestingly, SBIF 1–4 were designed for neonates while SBIF 5 was designed for older infants. The antioxidant capacities of SBIF extracts obtained by all four extraction methods were measured using three different antioxidant assays (see Fig. 1B–D). In most cases, there were statistically significant differences among antioxidant capacities (ANOVA, $P < 0.05$). In general, all extracts showed a high antioxidant capacity and differences observed between them were based on different peptide concentrations obtained with every extraction method. As expected, the extract obtained by Method 1 always showed the lowest antioxidant capacity, while the extract obtained by Method 4 resulted in one of the highest antioxidant capacities.

There were some exceptions, especially when using DPPH assay, where Method 2 showed very high antioxidant capacities. These higher antioxidant capacities could come from the proteins extracted with the peptides when this extracting method was employed. Taking into account these results and the simplicity of Method 4, this was the method selected for further studies. It is important to highlight the differences between SBIFs designed for newborn infants (stage 1 infant formula, SBIFs 1–4) and SBIF 5 that was designed for infants older than 6 months (stage 2 infant formula). Although, the peptide content was higher for SBIF 5, the antioxidant capacity was not significantly different.

Extracts obtained by UF with 10 kDa Mwco filters can also contain small molecules (*e.g.*, vitamins, minerals, and amino acids), which could also contribute to antioxidant capacity. Therefore, in order to assure these small molecules were absent, a further investigation was needed. According to the manufacturer's label, the most active antioxidant compounds added to the SBIFs recipe were vitamins. SBIF extracts were injected into a chromatographic system and chromatograms were compared with those obtained when injecting water-soluble vitamins present in SBIF (vitamin C, B_1, B_2, B_3, B_5, B_6, B_7, B_9, and B_{12}). Chromatograms obtained for all SBIF were very similar and peptide signals were detected from 5 to 17 min. Vitamins B_1, B_3, B_5, B_6, and C eluted in the dead volume, while vitamins B_2, B_7, B_9, and B_{12} eluted from 6.6. to 8.2 min. The presence of vitamins which eluted within the elution time of peptides was checked by spiking SBIFs with vitamin standard solutions. Results revealed that these vitamins were not present in the peptide extracts.

3.2. Fractionation of peptide extracts

Peptide extracts were firstly fractionated by ultrafiltration through different Mwco filters, in order to obtain peptide fractions from 5 to 10 kDa, 3 to 5 kDa, and below 3 kDa. Peptide concentration and antioxidant capacity of these fractions were next measured, observing in most cases statistically significant differences among them ($P < 0.05$). The highest peptide content was always observed in fractions containing 5 to 10 kDa peptides or in fractions with peptides below 3 kDa (see Fig. 2A). ABTS radical scavenging assay (see Fig. 2B) showed the highest antioxidant capacity for the fraction containing 5 to 10 kDa peptides, followed by the fraction with peptides below 3 kDa. The results obtained with the DPPH assay (see Fig. 2C) also showed that peptide fractions containing 5 to 10 kDa peptides or peptides below 3 kDa yielded the highest antioxidant capacity. The hydroxyl radical scavenging assay (see Fig. 2D) revealed that

5 to 10 kDa peptide fractions provided higher or similar activity (range 16.8–27.6%) to peptide fractions below 3 kDa (19.6–20.8%).

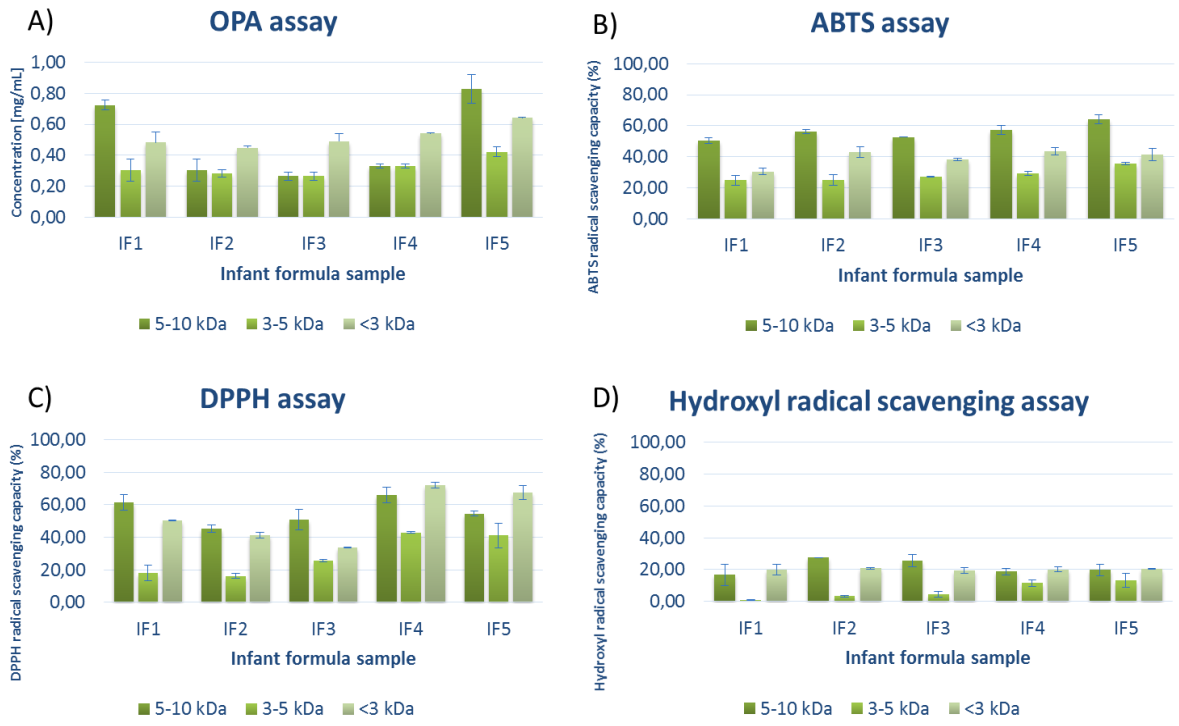


Fig. 2. Summary of results for OPA (A), and ABTS (B), DPPH (C), and hydroxyl radical-scavenging capacity (D) assays corresponding to the 5 to 10 kDa, 3–5 kDa, and below 3 kDa peptide fractions from five SBIF extracts.

The fraction containing peptides from 5 to 10 kDa was selected for further studies, since it yielded, in most cases, the highest antioxidant capacity. This higher antioxidant capacity might be connected to the higher number of hydrophobic and aromatic amino acids within the peptides obtained in these fractions. Moreover, the use of this fraction assured the absence of other SBIF ingredients, like vitamins, minerals, and amino acids that could exert antioxidant capacity. Furthermore, comparison among SBIFs revealed no significant difference among them ($P < 0.05$).

Further fractionation of bioactive peptides from the peptide fraction from 5 to 10 kDa was proposed using OFFGEL isoelectrofocusing. OFFGEL system separates peptides based on their pI and requires the addition of ampholytes to the peptide solution to obtain the desired pH gradient. In this case, the OFFGEL separation was carried out using a gradient from pH 3 to 10 into 24 wells to obtain a high resolution among peptides. Nevertheless, the assays employed for the determination of peptide concentration (OPA assay) and antioxidant capacity (ABTS and DPPH radical-scavenging assays) of the obtained fractions resulted were seriously interfered

by ampholytes used in the OFFGEL separation. This could be explained taking into account that ampholytes are zwitterionic low molecular weights polypeptides. Moreover, although the exact structure of commercially available ampholytes is unknown, their influence on other bioactive assays has already been proven [27]. Thus, blank separations (separations just with focusing buffer (12% (v/v) glycerol and ampholytes (pH 3–10)) were performed and blank solutions corresponding to the 24 wells were obtained. The blanks corresponding to every well were analyzed by OPA, ABTS, and DPPH assays (see Fig. 3).

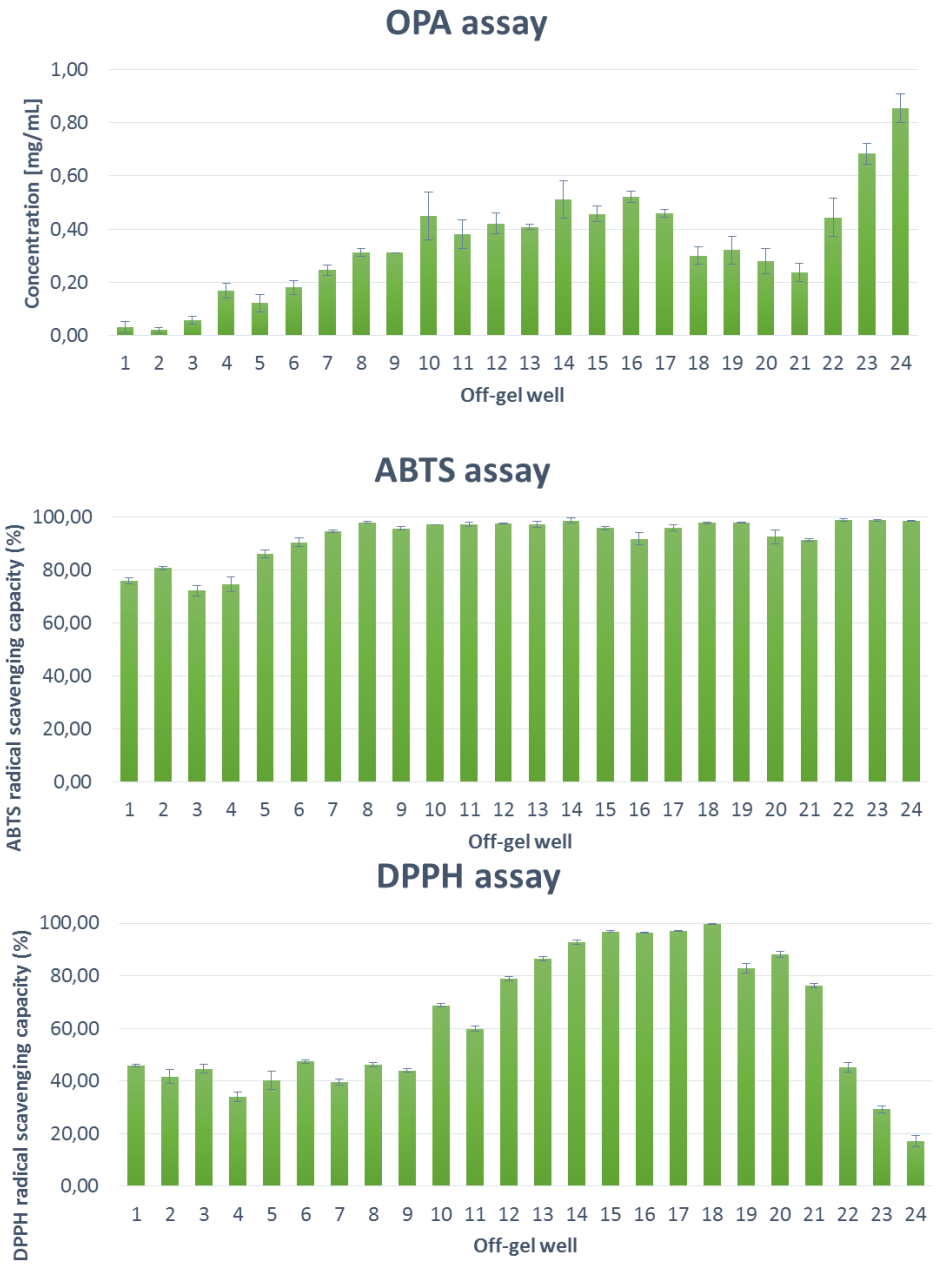


Fig. 3. Summary of results for OPA (A), and ABTS (B), and DPPH (C) radical-scavenging assays corresponding to the off-gel blanks obtained for the 24 OFFGEL wells.

Results obtained by OPA assay showed detectable signals in most wells, especially at more alkaline pHs. Moreover, false positive signals were also observed when OFFGEL blanks were analysed by the ABTS and DPPH radical-scavenging assays. Therefore, a method for the removal of ampholytes was required. Since ampholytes are small polypeptide molecules while investigated peptides were much larger (from 5 to 10 kDa), UF through 5 kDa Mwco filters was firstly proposed to separate them. Nevertheless, results showed that UF did not enable a complete removal of ampholytes. Zip-tips and spin columns, recently introduced on the market to easily remove interferences in peptide mixtures, were the second choice. Zip-tips and spin columns removed a high amount of ampholytes, but a significant number of peptides still could not be recovered. Another alternative was the use of RP-LC. After optimization, it was possible to separate peptide fractions from ampholytes using a monolithic column. Peptides were observed just in the first five wells which demonstrated the presence of mainly acidic peptides in SBIF. Collected fractions were evaporated, redissolved, and assayed using OPA and ABTS methods (see Fig. 4).

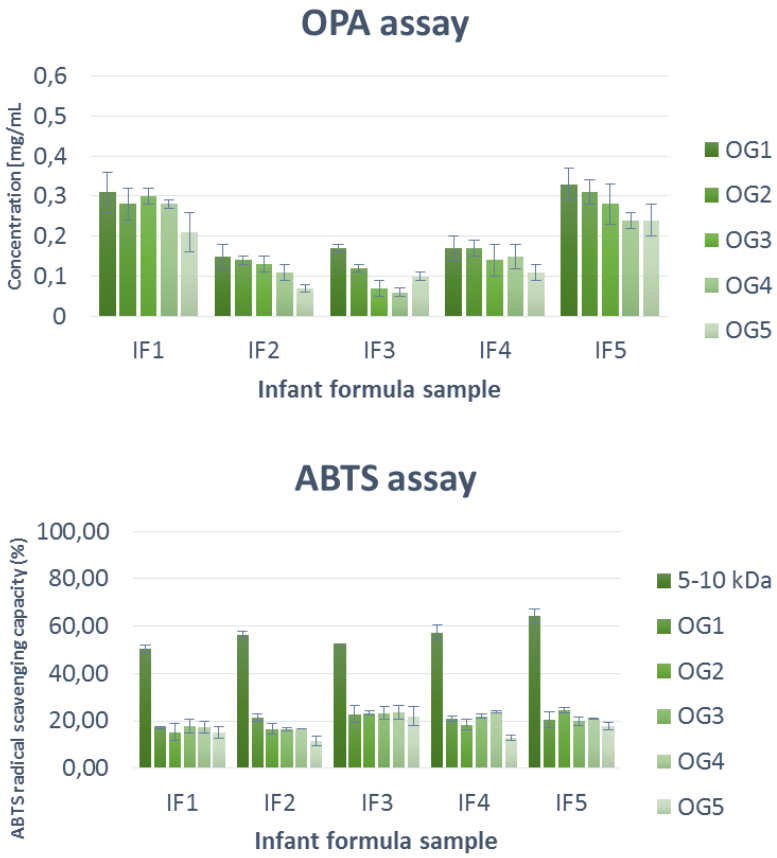


Fig. 4. Summary of results for OPA (A) and ABTS (B) radical-scavenging assays corresponding to the whole 5 to 10 kDa fraction and the OFFGEL subfractions containing peptides (OG1–OG5).

Results showed that the most abundant fraction was always the most acidic one (well 1). The lowest amount of peptides was measured in SBIF 3 (0.06–0.17 mg/mL) and the highest in SBIF 5 (0.24–0.33 mg/mL). These results were in accordance with previous results (Figs. 1 and 2). The ABTS radical-scavenging assay revealed that every well contained peptides with antioxidant capacity. Nevertheless, antioxidant capacity of the whole 5 to 10 kDa extract was much higher than that obtained with the individual OFFGEL fractions. This synergistic effect has already been described among antioxidant compounds [28]. Therefore, the OFFGEL separation was rejected and the whole 5 to 10 kDa fraction was employed for further investigations.

3.3. Evaluation of the antioxidant capacity of fraction from 5 to 10 kDa after gastrointestinal digestion

Whole fractions from 5 to 10 kDa were next digested sequentially with pepsin and pancreatin according to a previous method [26]. Every SBIF extract was digested twice in order to confirm the reproducibility of the simulated gastrointestinal digestion. The antioxidant capacity of these digested fractions was measured using the ABTS assay. The comparison of the ABTS radical-scavenging capacity of samples (see supplemental material 1) showed that antioxidant capacity of extracts after gastrointestinal digestion was lower than the observed for the extract before gastrointestinal digestion for SBIFs 1, 2, and 4 while there was no significant difference between antioxidant capacities for SBIFs 3 and 5 (t-test, $P < 0.05$). In order to clarify this fact, the peptide content before and after gastrointestinal digestion were evaluated and compared. Results (see supplemental material 1) showed a higher peptide content after gastrointestinal digestion for all SBIFs, which meant that some of the original peptides have been degraded. This degradation could be responsible for the slight decrease in the antioxidant capacity that was observed in some SBIFs when they were submitted to gastrointestinal digestion (SBIFs 1, 2, and 4). On the other hand, the similar antioxidant capacity before and after gastrointestinal digestion showed by SBIFs 3 and 5 could be because the resulting peptides also exerted antioxidant properties.

3.4. Identification of peptides from 5 to 10 kDa fractions

Peptide fractions after simulated gastrointestinal digestion were analyzed by RP-LC coupled to ESI-Q-ToF-MS/MS. The identification of peptides after gastrointestinal digestion shows the real nutritional value of SBIFs. At least 120 peptides were identified in every SBIF and a total

of 278 different peptides were identified among the five SBIFs. The analysis of the amino acid composition of SBIFs showed a high amount of highly hydrophobic (V, I, and L) and aromatic (H, F, W, Y) amino acid residues, typical features of antioxidant peptides. The percentage of highly hydrophobic and aromatic amino acids ranged from 26.75% for SBIF 4 to 30.11% and 30.66% for SBIF 3 and 5, respectively.

Within all identified peptides, 42 were common to all samples. The list of these peptides with their experimental molecular masses and protein origin (accession as in the Uniprot database) is presented in Table 1. Most of these peptides came from the glycinin protein subunits (from GLYG1 to GLYG5; where GLY means glycinin protein, and G1–G5 specify its subunit); glycinin is a major seed storage protein of soybean (around 40% of soybean storage proteins). A second major group of peptides came from the alpha chain (GLCA) of b-conglycinin, another seed storage protein (around 28% of soybean storage proteins). The presence of already identified peptides was checked using the bioactive peptide database BIOPEP. This study enabled the detection of part of the sequence of soystatin (VAWWMY), a soybean peptide previously reported as strongly cholesterol absorption inhibiting and bile acid binding [29]. The antioxidant activity of this peptide (VAWWM) could be justified taking into account that it contained two aromatic amino acid residues (W) and most of the amino acids in the sequence were hydrophobic (V, W, M).

The infant formula with the highest antioxidant capacity (SBIF 5) showed thirty unique peptides. The presence of these peptides in the BIOPEP database was checked. Among them, peptide SGDAL had previously been reported within two longer sequences of antioxidant peptides, LQ*SGDAL*RVPSGTTYY and LN*SGDAL*RVPSGTTYY, both from soybeanb-conglycinin [30].

This work has evaluated, for the first time, the presence of peptides with antioxidant capacity in infant formulas based on soybean. The direct ultrafiltration of the sample through 10 kDa Mwco filters enabled the removal of proteins. The fractionation of the extracts using different Mwco filters and the measurement of antioxidant capacity showed the highest antioxidant capacity in the peptide fraction from 5 to 10 kDa. Fractions with peptides from 5 to 10 kDa were digested using a simulated gastrointestinal digestion observing that antioxidant capacity was maintained. A total of 278 different peptides were identified among the five SBIFs of which 42 were common to all. Many of these peptides possessed typical features of antioxidant peptides.

Table 1. List of common peptides identified in the five SBIFs after simulated gastrointestinal digestion.

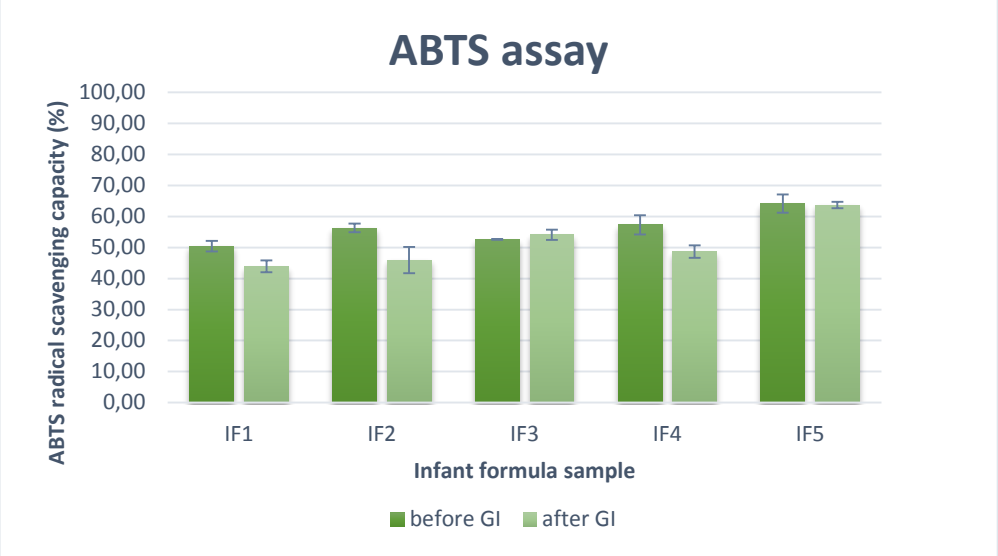
Protein accession[a]	Mass	Peptide
P04405|GLYG2_SOYBN	557.2697	ALPEE
P04405|GLYG2_SOYBN	645.2904	DQMPR
P04347|GLYG5_SOYBN	727.3613	DQNPRV
P11827|GLCAP_SOYBN	730.3861	EEINKV
P11827|GLCAP_SOYBN	667.3329	FAFGIN
P04405|GLYG2_SOYBN	609.2798	FAPEF
P29531|OLEO2_SOYBN	618.3125	FEAPR
P02858|GLYG4_SOYBN	905.3766	FEEPQEQ
P04776|GLYG1_SOYBN	594.3013	FSVDK
P13916|GLCA_SOYBN	803.4177	FVDAQPK
P11827|GLCAP_SOYBN	803.3813	FVDAQPQ
P11827|GLCAP_SOYBN	1488.6844	FVDAQPQQKEEGN
P25974|GLCB_SOYBN	616.3584	FVIPAA
P04405|GLYG2_SOYBN	666.3013	GFAPEF
P04405|GLYG2_SOYBN	556.322	IAVPTG
P04405|GLYG2_SOYBN	1486.7205	IETWNPNNKPFQ
P04347|GLYG5_SOYBN	543.2904	IPSEV
P04776|GLYG1_SOYBN	558.3741	IVTVK
P02858|GLYG4_SOYBN	587.3643	KQIVT
P02858|GLYG4_SOYBN	1062.5134	KYEGNWGPL
P13916|GLCA_SOYBN	789.4021	LAFPGSAQ
P04776|GLYG1_SOYBN	681.3697	LVPPQE
P04347|GLYG5_SOYBN	656.3605	NIARPS
P13916|GLCA_SOYBN	693.3486	NPFLFG
P04347|GLYG5_SOYBN	796.4079	NSGPLVNP
P04776|GLYG1_SOYBN	703.3137	NSLENQ
P02858|GLYG4_SOYBN	900.4188	NTGDEPVVA
P04776|GLYG1_SOYBN	622.3074	RPSYT
P02858|GLYG4_SOYBN	661.3282	SQVSEL
P02858|GLYG4_SOYBN	629.3748	SVISPK
P13916|GLCA_SOYBN	1022.492	TISSEDKPF
P04776|GLYG1_SOYBN	691.3152	VAWWM
P13916|GLCA_SOYBN	656.3493	VDAQPK
P04776|GLYG1_SOYBN	596.3282	VIQHT
P02858|GLYG4_SOYBN	542.3428	VISPK
P04776|GLYG1_SOYBN	646.3538	VSIIDT
P04776|GLYG1_SOYBN	760.3967	VSIIDTN
P11828|GLYG3_SOYBN	646.3538	VSLIDT
P11828|GLYG3_SOYBN	760.3967	VSLIDTN
P04776|GLYG1_SOYBN	798.3159	WWMYN
P02858|GLYG4_SOYBN	934.4185	YEGNWGPL
P13916|GLCA_SOYBN	575.3319	YPVVV

[a] From UniProt protein database.

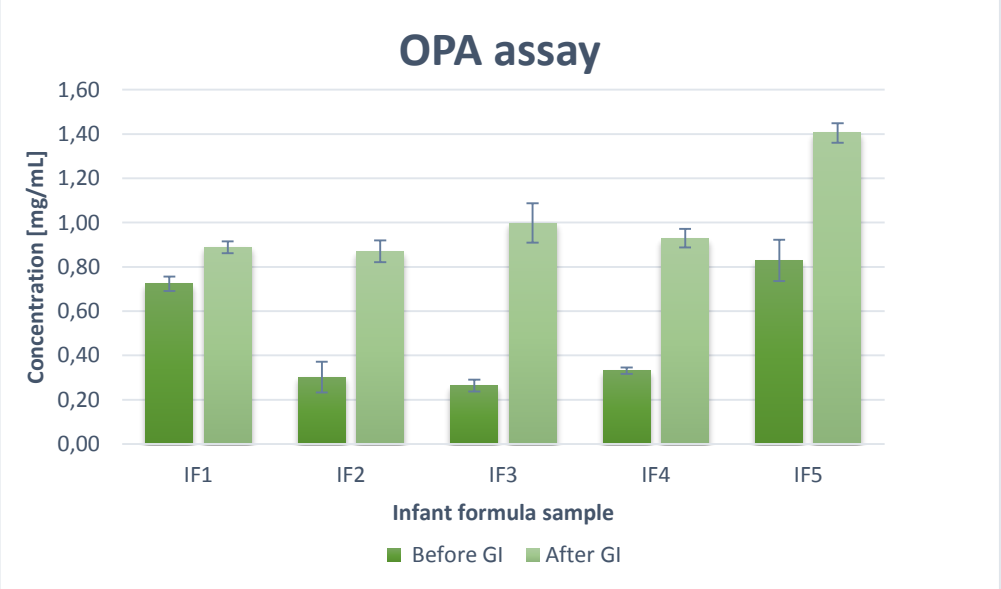
Appendix A. Supplementary data

Supplemental material 1. Comparison of the antioxidant capacity (A) and peptide content (B) before and after simulated gastrointestinal digestion (GI) of the 5 to 10 kDa peptide fraction of five SBIFs.

A)



B)



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References for Article 2:

[1] M. Méndez, M. Anthony, L. Arab, Soy-based formulae and infant growth and development: A review, J. Nutr. 132 (2002) 2127-2130.

[2] R. Merritt, B. Jenks, Safety of soy-based infant formulas containing isoflavones: The clinical evidence, J. Nutr. 134 (2004) 1220S-1224S.

[3] P. Johns, R. Phillips, L. Dowlati, Direct determination of free methionine in soy-based infant formula, J. AOAC Int. 87 (2004) 123-128.

[4] M. Castro-Puyana, C. García-Ruiz, A.L. Crego, M. L. Marina, Development of a CE-MS2 method for the enantiomeric separation of L/D-carnitine: Application to the analysis of infant formulas, Electrophoresis 30 (2009) 337-348.

[5] C. Agostoni, B. Carratu, C. Boniglia, E. Riva, E. Sanzini, Free amino acid content in standard infant formulas: Comparison with human milk, J. Am. Coll. Nutr. 19 (2000) 434-438.

[6] M.H. McNary, W. Cheng, M. Mar, S. Fussell, S. Zeisel, Choline and choline esters in human and rat milk and in infant formulas, Am. J. Clin. Nutr. 64 (1996) 572-576.

[7] M.C. García, M. Torre, M.L. Marina, A perfusion reversed-phase chromatographic method for ultrarapid determination of soybean proteins in soybean infant formulas and soybean milks: Method development and validation, J. Chromatogr. Sci. 36 (1998) 527-534.

[8] H. Korhonen, A. Pihlanto, Food-derived bioactive peptides - opportunities for designing future foods, Curr. Pharm. Des. 9 (2003) 1297-1308.

[9] F. De Leo, S. Panarese, R. Gallerani, L.R. Ceci, Angiotensin Converting Enzyme (ACE) inhibitory peptides: production and implementation of functional food, Curr. Pharm. Des. 15 (2009) 3622-3643.

[10] B.H. Sarmadi, A. Ismail, Antioxidative peptides from food proteins: A review, Peptides 31 (2010) 1949-1956.

[11] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[12] N.P. Moller, K.E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, Bioactive peptides and proteins from foods: indication for health effects, Eur. J. Nutr. 47 (2008) 171-182.

[13] F. Shahidi, Y. Zhong, Bioactive peptides, J. AOAC Int. 91 (2008) 914-931.

[14] A. Pihlanto, Antioxidative peptides derived from milk proteins, Int. Dairy J. 16 (2006) 1306-1314.

[15] B. Ames, M. Shigenaga, T. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 7915-7922.

[16] R.J. Elias, S.S. Kellerby, E.A. Decker, Antioxidant activity of proteins and peptides, Crit. Rev. Food Sci. Nutr. 48 (2008) 430-441.

[17] M.C. García, P. Puchalska, C. Esteve, M.L. Marina, Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities, Talanta 106 (2013) 328-349.

[18] H. Chen, K. Muramoto, F. Yamauchi, K. Fujimoto, K. Nokihara, Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein, J. Agric. Food Chem. 46 (1998) 49-53.

[19] K. Erdmann, W.Y.C. Belinda, H. Schroder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease, J. Nutr. Biochem. 19 (2008) 643-654.

[20] M.M. Calvo, R. Gómez, Peptidic profile, molecular mass distribution and immunological properties of commercial hypoallergenic infant formulas, Milchwissenschaft 57 (2002) 187-190.

[21] F. Di Girolamo, A. D'Amato, P.G. Righetti, Assessment of the floral origin of honey via proteomic tools, J. Proteomics 75 (2012) 3688-3693.

[22] D. Wang, L. Wang, F. Zhu, J. Zhu, X.D. Chen, L. Zou, M. Saito, L. Li, In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food), Food Chem. 107 (2008) 1421-1428.

[23] L. You, M. Zhao, J.M. Regenstein, J. Ren, In vitro antioxidant activity and in vivo anti-fatigue effect of loach (Misgurnus anguillicaudatus) peptides prepared by papain digestion, Food Chem. 124 (2011) 188-194.

[24] C. Wiriyaphan, B. Chitsomboon, J. Yongsawadigul, Antioxidant activity of protein hydrolysates derived from threadfin bream surimi by-products, Food Chem. 132 (2012) 104-111.

[25] C.F. Ajibola, J.B. Fashakin, T.N. Fagbemi, R.E. Aluko, Effect of peptide size on antioxidant properties of african yam bean seed (Sphenostylis stenocarpa) protein hydrolysate fractions, Int. J. Mol. Sci. 12 (2011) 6685-6702.

[26] D. Garrett, M. Failla, R. Sarama, Development of an in vitro digestion method to assess carotenoid bioavailability from meals, J. Agric. Food Chem. 47 (1999) 4301-4309.

[27] S. Riazi, S. Dover, Y. Turovskiy, M.L. Chikindas, Commercial ampholytes used for isoelectric focusing may interfere with bioactivity based purification of antimicrobial peptides, J. Microbiol. Methods. 71 (2007) 87-89.

[28] A. Ghiselli, M. Serafini, F. Natella, C. Scaccini, Total antioxidant capacity as a tool to assess redox status: critical view and experimental data, Free Radic. Biol. Med. 29 (2000) 1106-1114.

[29] S. Nagaoka, A. Nakamura, H. Shibata, Y. Kanamaru, Soystatin (VAWWMY), a novel bile acid-binding peptide, decreased micellar solubility and inhibited cholesterol absorption in rats, Biosci. Biotechnol. Biochem. 74 (2010) 1738-1741.

[30] H. Chen, K. Muramoto, F. Yamauchi, Structural-analysis of antioxidative peptides from soybean beta-conglycinin, J. Agric. Food Chem. 43 (1995) 574-578.

Article 3

Identification of native angiotensin I converting enzyme inhibitory peptides in commercial soybean based infant formulas using HPLC-Q-ToF-MS

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Food Chem., in press

Abstract

This work evaluates, the presence of native antihypertensive peptides in five soybean based infant formulas (SBIFs). SBIFs peptide extracts (< 10 kDa) and their subfractions (5 to 10 kDa, 3 to 5 kDa, and <3 kDa) from a variety of samples were obtained by ultrafiltration and ACE inhibitory activity was determined. The highest activities were observed in the smaller (<5 kDa) peptide fractions (IC_{50} values of 1.20 ± 0.05 and 0.57 ± 0.04 μg/mL). A set of peptides presented in various SBIFs were studied, and identified using HPLC-Q-ToF-MS. Despite ACE inhibitory activity decreased after *in vitro* gastrointestinal digestion, it still remained at a high value (IC_{50} values of 18.2 ± 0.1 and 4.9 ± 0.1 μg/mL). Peptides resisting the action of gastrointestinal enzymes were identified and compared to previously identified peptides highlighting the presence of peptide RPSYT. Discovered peptide was synthesized, its antihypertensive and antioxidant activity were evaluated, and its resistance to *in vitro* gastrointestinal digestion and to high processing temperatures were studied.

Keywords:

Soybean based infant formulas; Bioactive peptides; Antihypertensive activity; HPLC; Q-ToF-MS

1. Introduction

Soybean based infant formulas (SBIFs) are based on soybean protein isolate (SPI) and are enriched with non-protein amino acids [1] and several nutrients like lipids, vitamins or minerals [2]. SBIFs are suitable for infants with intolerance or allergy to milk (lactose intolerance, galactose intolerance, milk protein intolerance/allergy, *etc.*), for infants from families that are strict vegans, and for the treatment of common feeding problems [3]. Its presence in the market is significant since they are consumed by a 25% of infants fed with infant formulas in the USA [4]. Despite this data, SBIFs have not been much explored in comparison with milk and infant formulas from animal origin.

Indeed, milk and dairy products have been widely studied for its content in bioactive peptides [5-7]. The characterization and/or identification of antihypertensive and antioxidant peptides in human milk and bovine protein based infant formulas [8-11] have especially been reported. Among milk bioactive peptides, it is remarkable the presence of peptides VPP and IPP exerting high ACE inhibitory activity (IC_{50} values, 9.13 μM (2.80 μg/mL) and 5.15 μM (1.67 μg/mL), respectively). Antihypertensive peptides enable the reduction of blood pressure acting mainly on the renin-angiotensin system by inhibiting angiotensin I converting enzyme (ACE). This enzyme catalyzes the conversion of angiotensin I to angiotensin II, which is a potent vasoconstrictor [12,13]. Moreover, ACE also removes a dipeptide from C-terminus of bradykinin (RPPGFSPFR) resulting in the inactivation of this vasodilator. Therefore, ACE inhibitors decrease blood pressure by lowering the level of angiotensin II and increasing the level of bradykinin. Despite different bioactive peptides have been identified in soybean [14-15] the studies concerning the evaluation of the SBIFs bioactive peptide are scare [16]. In fact, there is only one work, carried out by our research group, dealing with the evaluation of the presence of a group of antioxidant peptides in SBIFs [17]. Nevertheless, there is still none work devoted to the complex study of ACE inhibitory peptides in various SBIFs.

ACE inhibitory peptides are small peptides with high level of hydrophobic amino acids and, commonly, with proline at C-terminal [18]. ACE inhibitory peptides can be found as independent entities or can be in a latent state as part of the sequence of a protein from which they are released during the course of gastrointestinal digestion or processing. SBIFs are submitted to intense heating and/or partial protein hydrolysis during processing. Thus, this processing could result in the release of peptides that could have potential to offer specific health effects in addition to nutritional benefits.

The aim of this work has been the evaluation of the presence of native peptides with ACE inhibitory activity in commercial SBIFs. The extracts and fractions from various SBIFs were obtained and its ACE inhibitory capacity was evaluated. Antihypertensive activities were also explored after a simulated gastrointestinal digestion and antihypertensive peptides were identified by HPLC-tandem mass spectrometry.

2. Materials and methods

2.1. Chemicals and samples

All chemicals were of analytical grade purity. Water was taken every day from a Milli-Q System (Millipore, Bedfore, MA, USA). Acetonitrile (ACN) was from Fisher Scientific (Pittsburgh, PA, USA). Acetic acid (AA) was purchased from Scharlau Chemie (Barcelona, Spain). Sodium dodecyl sulfate (SDS), hydrochloric acid, 2-mercaptoethanol, sodium bicarbonate, hydrochloric acid, and sodium hydroxide were from Merck (Darmstadt, Germany). Angiotensin-I converting enzyme (ACE) from rabbit lung, hipuryl-His-Leu (HLL), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), trifluoroacetic acid (TFA), sodium chloride, sodium tetraborate, o-phthalaldehyde (OPA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), glutathione (GSH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, potassium phosphate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,10-phenantroline, ferrous sulfate, hydrogen peroxide, pepsin from porcine gastric mucosa (P7012), and pancreatin from porcine pancreas (P-7545) were supplied by Sigma (St. Luis, MO, USA). Five different soybean based infant formulas (SBIFs) were purchased at a local pharmacy and stored at room temperature when not in use. SBIFs 1-4 were indicated for newborn infants (stage 1 infant formulas), while SBIF 5 was designed for infants after completing 6 months (stage 2 infant formula).In order to avoid degradation, prepared samples, when not in use, were stored at -20°C. Peptide (RPSYT) was synthesized by Genescript (Genescript Corp., Piscataway, NJ, USA).

2.2. Extraction and fractionation of peptides

Infant formulas were prepared according to the manufactures' label.1 g of SBIF powder was mixed with 6 mL of previously boiled water at 40˚C and shaken till an homogenous sample was obtained. In order to obtain whole peptide extract, sample was ultrafiltrated through Millipore filters with 10 kDa Mwco (1 h, 4000 x g, 25 ºC). Fractionation of whole peptide extracts was performed by their further sequential utrafiltration using Vivaspin 500 PES filters

with 5 kDa Mwco (Sartorious Stedim Biotech, Goettingen, Germany) and Amicon filters with 3 kDa Mwco (Millipore). Each recovered fraction (fraction from 5 to10 kDa, 3 to 5 kDa, and below 3 kDa) was dissolved to the initial volume in water. Extracts and fractions were stored at -20°C until use**.**

2.3. OPA assay

Peptide concentration was determined in fractions by the OPA (o-phthalaldehyde) assay with modifications [19] using a spectrophotometer Lambda 35 (Perkin-Elmer, Wathman, MA, USA) and cuvettes designed for small volumes (UVetter ®, Eppendorf, Hamburg, Germany). The procedure was as follows: 2.5 µL of sample was mixed with 100 µL of OPA mixture (2.5 mL of sodium tetraborate, 1 mL of 5% (m/v) SDS, 100 µL of 40 mg/mL OPA in methanol, 10 µL of 2-mercaptoethanol, and 1.39 mL of water). Solution was left for 8 min at room temperature and signal was measured at 340 nm. Peptide content was calculated by interpolation in a GSH standard calibration curve in the range from 0 to 5 mg/mL. Three replicates were prepared for every sample or fraction and all measurements were done at least three times.

2.4. ACE inhibitory activity assay

The measurement of ACE inhibition was performed according to a previous method [20] with modifications. Reaction solution contained 20 µL of ACE (0.05 U/mL, dissolved in water), 10 µL HHL (1.25 mg/mL in 50 mM HEPES buffer pH 8.3/ 300 mM NaCl), 35 µL HEPES buffer/NaCl, and 5 µL sample solution. Control sample contained peptide solvent instead of sample solution. Prepared mixtures were incubated at 37°C in a hot air oven (Memmert, model 300, Schwabach, Germany). Reaction was stopped with 100 µL of cold ACN. In order to calculate the IC_{50} value for ACE inhibition, investigated peptide solution was prepared in series of concentrations (at least five dilutions for each peptide sample). Prepared samples were injected into the HPLC-UV system. Measurements were performed in a modular capillary chromatographic system (Agilent Technologies, Pittsburgh, PA). HPLC assembly consisted of a micro vacuum degasser (model 1100), a capillary LC pump (model 1100), a thermostatized autosampler (model 1100), a thermostatized column compartment (model 1200), and a multiple wavelength detector (model 1200). HP Chemstation software was used to control HPLC instrument. Reaction mixture was separated in a C18 Zorbax 300 SB (1500 mm x 0.5 mm I.D., 5µm particle size, and 80 Å pore size) (Agilent Technologies, Pittsburgh, PA) using a flow rate of 20 µL/min, a temperature of 25°C, a binary gradient from 5 to 100% B in 7 min, 100% B in

2 min, 100-5% in 2 min, and mobile phases consisting of Milli-Q water/0.025% (v:v) TFA (mobile phase A) and ACN/0.025% (v:v) TFA (mobile phase B). The injected volume was 1 µL and the detection was performed at a wavelength of 228 nm. For each of five dilutions the percentage of inhibition of the activity of ACE enzyme was estimated calculating the percentage of decreasing the HA signal in comparison to control sample were the inhibition did not occur. Percentages of ACE inhibition have been plotted against the peptide concentrations in the studied sample, and IC_{50} was calculated. Three replicates were prepared for every sample or fraction and all measurements were done at least three times.

2.5. Gastrointestinal digestion

Peptide fractions were digested according to Garrett *et al.* method [21] with modifications. Briefly, the pH of sample was adjusted to 2 with 1 M HCl and pepsin (enzyme: substrate ratio 1:35) was added. Reaction mixture was incubated in a thermomixer for 1 h at 37°C with shaking. Afterwards, pH of sample was adjusted with 0.1 M $NaHCO_3$ to pH 5 and further with 0.1 M NaOH to pH 7-8. Pancreatin (enzyme: substrate ratio 1:25) was added and reaction mixture was incubated for 2 h at 37°C with shaking. Digestion was stopped by boiling for 10 min.

2.6. Identification of peptides

A Quadrupole-Time-of-Flight (Q-ToF) MS (instrument series 6530) from Agilent Technologies coupled to a liquid chromatograph 1100 series also from Agilent Technologies were used for the identification of peptides prior and after the gastrointestinal digestion of infant formula fractions. HPLC separation was obtained using an Ascentis Express Peptide ES-C18 column (100 mm× 2.1 mm I.D., with 2.7 µm particle size) with an Ascentis Express Guard column (5 mm×2.1 mm I.D., with 2.7 µm particle size) both from Supelco (Bellefonte, PA,USA). Mobile phases were: A, Milli-Q water/0.3% (v:v) AA and B, ACN/0.3% (v:v) AA; the elution gradient was: 3-95% B in 30 min and 95-3% B in 5 min. Flow rate was 0.3 mL/min, the column temperature was 25°C, and the injection volume was 15 µL. The mass spectrometer operated in the positive ion mode with a mass range from 100 to 3200 m/z. ESI conditions were as follows: fragmentator voltage, 200 V; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 350˚C; gas flow, 12 L/min; skimmer, 60 V; Jet Stream sheath gas flow and temperature, 12 L/min and 400˚C, respectively. Auto MS/MS mode with fixed collision energy 35 V was employed where 1 precursor per cycle was selected and dynamically excluded after three spectra. To identify the list of present in the sample peptides,

the analysis of MS/MS spectra (mgf files) was performed using PEAKS Studio Version 6 (Bioinformatics Solutions Inc., Waterloo, Canada). Search was performed using database search tool (against soybean proteome) with improved algorithm that validate and assist the database search with the *de novo* sequencing results. Proteome of soybean (*Glycine max*) was downloaded from the UniProt protein database and contained just reviewed proteins (380 proteins). In order to select a proper -10lgP value threshold for identified peptides, obtained results have been refined using a False Discovery Rate (FDR) of 1%. Peptides identified above appropriate -10lgP threshold have been treated as true positive identification and used in further data analysis. Venn Diagrams were created using the GenoToul bioinformatics facility.

2.7. Characterization of synthetic peptide RPSYT

In vitro gastrointestinal digestion of 0.25 mg/mL of peptide in water was performed following the protocol described above. Peptide (0.25 mg/mL) was also incubated at 100°C for 10 min to study its resistance to high processing temperature. To investigate whenever peptide structure changed, samples were injected into the HPLC-Q-ToF-MS system using the same parameters previously employed for the identification of peptides but using just MS mode. ACE inhibitory activity of RPSYT peptide was estimated using method previously described (see section 2.4). The estimation of antioxidant capacity of peptide RPSYT was performed using ABTS and DPPH assays. Solvent blanks were measured for every assay. Three replicates were performed. *DPPH radical scavenging capacity assay.* The assay was carried out following a previously developed method [22] with some modifications. A calibration curve of GSH (0-5 mg/mL) was performed before measurements as positive control. Peptide at a concentration of 0.5 mg/mL (50 µL) was mixed with 50 µL of 0.1 mM DPPH in 95% ethanol and kept for 30 min (at room temperature) in the dark. The absorbance at 517 nm of the resulting solution was measured in the spectrophotometer. The DPPH radical scavenging capacity was calculated as follow:

$$\text{DPPH radical scavenging capacity (\%)} = \left(1 - \frac{Abs_{sample} - Abs_{samplecontrol}}{Abs_{blank}}\right) x100$$

where Abs_{sample} is the absorbance of the sample with DPPH solution; $Abs_{samplecontrol}$ is the absorbance of the sample without DPPH solution; Abs_{blank} is the absorbance of the solvent (without peptides) with the DPPH solution.

ABTS radical scavenging assay. ABTS assay was carried out using a previously developed method [23]. ABTS stock solution was obtained by mixing 7.4 mM ABTS with 2.6 mM potassium persulfate in 10 mM phosphate buffer (PB) (pH 7.4). This stock solution was incubated in the dark for 16 h. Before analysis, the ABTS stock solution was dissolved in 10 mM PB (pH 7.4) to attain an absorbance of 0.7 ± 0.1 AU at 734 nm (ABTS working solution). ABTS working solution (100 µL) was mixed with 1 µL of peptide (0.5 mg/mL), incubated for 6 min, and signal was measured at 734 nm. The ABTS radical scavenging capacity was calculated using the following equation:

$$\text{ABTS radical scavenging capacity (\%)} = \left(\frac{Abs_{blank} - Abs_{sample}}{Abs_{blank}}\right) x\ 100$$

where Abs_{sample} is the absorbance of the sample with the ABTS working solution and Abs_{blank} is the absorbance of the solution in which peptides were dissolved with the ABTS working solution.

2.8. Statistical analysis

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc.,Warrention, VA, USA). In order to find statistically significant differences among results, the analysis of variance (ANOVA) was applied when three replicates of every sample were analyzed by triplicate.

3. Results and discussion

3.1. ACE inhibitory activity of infant formula extracts and fractions

Peptides from five different commercially available SBIFs were obtained by the dilution of powder in water according to manufactures' suggestions and their ultrafiltration through 10 kDa Mwco filters [17]. These extracts with molecular weights below 10 kDa were next fractionated by ultrafiltration to obtain fractions from 5 to 10 kDa, from 3 to 5 kDa, and below 3 kDa. ACE inhibitory activity of SBIF extracts and their fractions are presented in Table 1.

Table 1. Antihypertensive capacity corresponding to peptide fractions below 10 kDa (whole extract), from 5 to 10 kDa, from 3 to 5 kDa, and below 3 kDa of five commercial SBIFs.

	IC_{50} [µg/mL]				
Fractions	**1IF**	**2IF**	**3IF**	**4IF**	**5IF**
below 10 kDa	41.85±1.27	50.70 ±0.78	63.90 ±1.70	5.88±0.12	2.45±0.07
5-10 kDa	71.71±1.14	26.21±0.39	17.99±0.03	17.26±0.59	40.19±1.01
3-5 kDa	14.20 ±0.08	11.67±0.37	11.61±0.13	17.52±0.22	15.15±0.21
<3 kDa	10.48±0.21	10.86±0.36	18.03±0.25	1.20 ±0.05	0.57±0.04

In all cases, whole peptide extracts presented certain antihypertensive bioactivity being 4IF and 5IF the most active ones (IC_{50} values of 5.88 ± 0.12 and 2.45 ± 0.07µg/mL, respectively). Regarding fractions, in general, the lower the molecular weight, the higher the ACE inhibitory activity. In fact, fractions from 5 to 10 kDa yielded the lowest ACE inhibitory activity (with the exception of 3IF) while fractions from 3 to 5 kDa and below 3 kDa were more active. This behavior is in agreement with the fact that antihypertensive peptides are small peptides. The highest activity was shown in fractions below 3 kDa from 4IF and 5IF with IC_{50} values of 1.20 ± 0.05 and 0.57 ± 0.04µg/ mL, respectively. This antihypertensive activities were even higher than the potent and extensively studied milk peptides VPP (2.80 µg/mL) and IPP (1.67 µg/mL) [24].

3.2. Identification of antihypertensive peptides in infant formulas fractions

Fractions exerting the highest ACE inhibitory activity (fractions of 3 to 5 kDa and below 3 kDa) in all SBIFs were injected into the HPLC-Q-ToF-MS in order to identify potential antihypertensive peptides. During the analysis of these fractions, tandem MS/MS spectra were obtained for most abundant molecular ions. A table with all identified peptides is available as supplemental material (S1). In some cases, two peptide isoforms were identified and included into the table (for example KGAIG and KGALG). Since the equipment is not able to differentiate I from L due to their equal molecular masses, both sequences have been included into the table. Interestingly, those peptides have been assigned to different parent proteins, which can be explained by high protein sequence homologies among some soybean proteins. The summary of peptides identified that were in common among SBIFs or specific of every SBIFs is presented by a Venn diagram (see Figure 1A). A total of 141 individual peptides were

identified in peptide fractions between 3 to 5 kDa. Among them, 12 peptides were common in all SBIFs. Since, the inhibition of ACE was identified in all SBIFs fractions, these 12 peptides may be listed as potential ACE inhibitors. Interestingly, most identified peptides in fraction from 3 to 5kDa had much lower molecular masses than expected. Indeed, the averaged molecular mass of peptides in this fraction was 1 kDa. This result could be explained by the poor selectivity of ultrafiltration, especially at very low molecular weights, and could explain the high ACE inhibitory activity reported in these fractions. Then, all identified peptides were checked against BIOPEP database observing that peptides QSGDALR and SGDALR had previously been reported within two longer sequences (L*QSGDAL*RVPSGTTYY and LNS*GDAL*RVPSGTTYY) from the soybean beta-conglycinin and that both peptides possessed antioxidant activity [25]. Regarding fractions below 3 kDa, the summary of common peptides (7 peptides) and peptides identified just in every SBIF fraction is depicted in Figure 1B. Indicated peptides may be added to the list of potential ACE inhibitors. 94 individual peptides were detected (see S1). Surprisingly, some of these peptides were detected previously in fraction 3 to 5 kDa. In fact, two peptides APAMR and RPSYT were determined in all five SBIFs in both 3 to 5 kDa and below 3 kDa fractions. The averaged molecular mass of identified peptides in this fraction was around 0.7 kDa. Also in this case, QSGDALR and SGDALR peptides were found when checking against BIOPEP database. Special relevance could have the 29 peptides identified just in the fraction below 3 kDa in 4IF and 5IF since these fractions yielded the highest ACE inhibitory activity (see Table 1). However, it must be added that not just one but several peptides may contribute to the exceptional ACE inhibitory activities of 4IF, 5IF peptide fractions below 3 kDa.

3.3. Simulated gastrointestinal digestion of infant formula fractions

One of the most important properties of a bioactive peptide in order to exert a certain biological effect is its ability to reach targeted organs in an active form. The most important step in bioavailability studies is gastrointestinal digestion. Those peptides resisting this physiological process could reach the circulatory system and exert antihypertensive effects. Therefore, an study evaluating the ACE inhibitory activity of most antihypertensive fractions after a simulated gastrointestinal digestion was next carried out. For that purpose, sequential digestion by pepsin/pancreatin have been reported to provide a proper model to estimate the release of peptides in the digestive system [26].

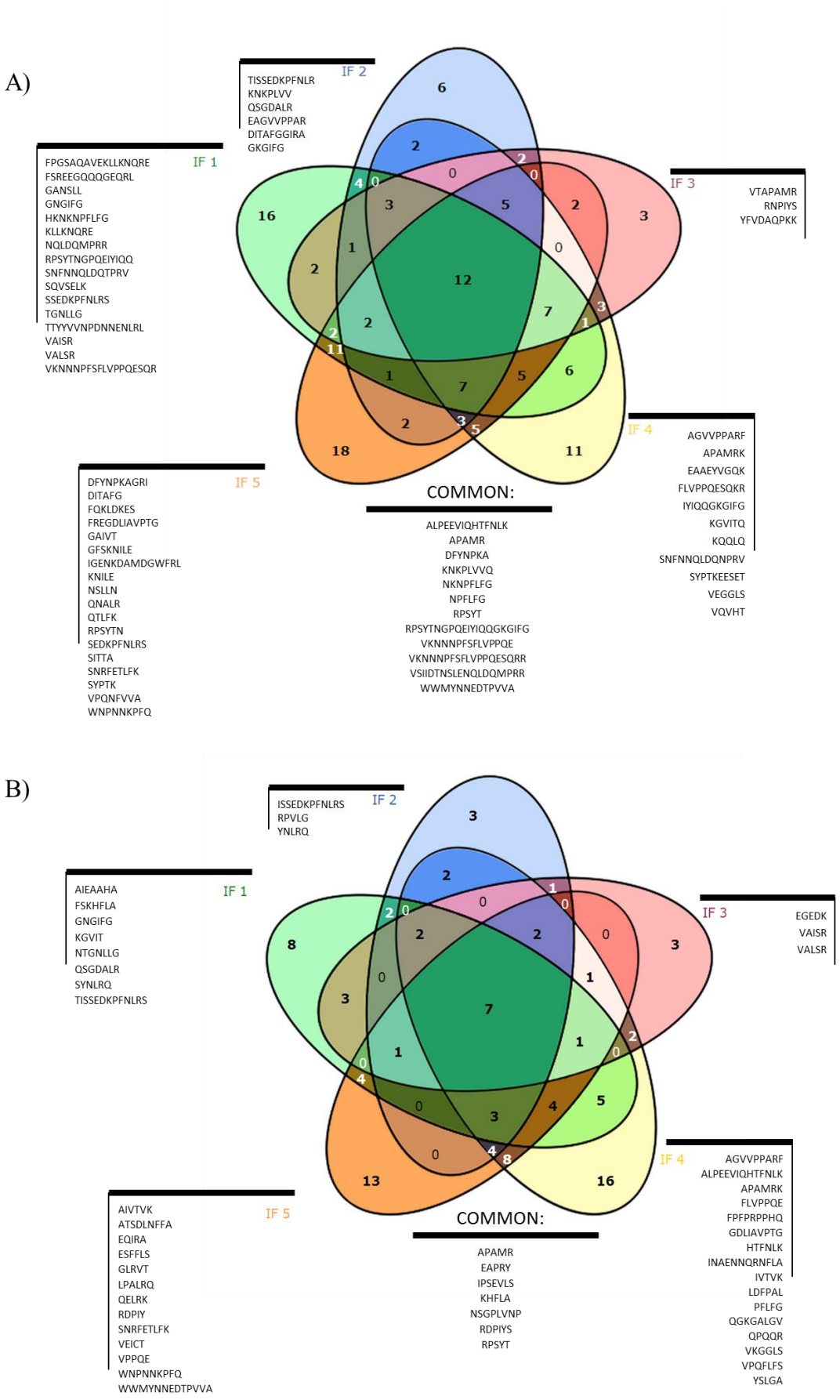

Fig. 1. Venn diagrams representing peptides identified by HPLC-Q-ToF-MS in five SBIFs for the fraction from 3 to 5 kDa (A) and the fraction below 3 kDa (B). Peptides exclusively identified in every SBIF or common to all SBIFs have been included.

Digestion with pepsin endopeptidase offers more accessible sites for subsequent pancreatin hydrolysis. Following, the hydrolysis with pancreatin, a mixture of different peptidases (trypsin, α-chymotrypsin, elastase, and carboxypeptidases A and B), is normally performed. Fractions from 3 to 5 kDa and below 3 kDa were submitted to simulated gastrointestinal digestion. Figure 2 compares peptide concentration before and after gastrointestinal digestion for fractions from 3 to 5 kDa and below 3 kDa.

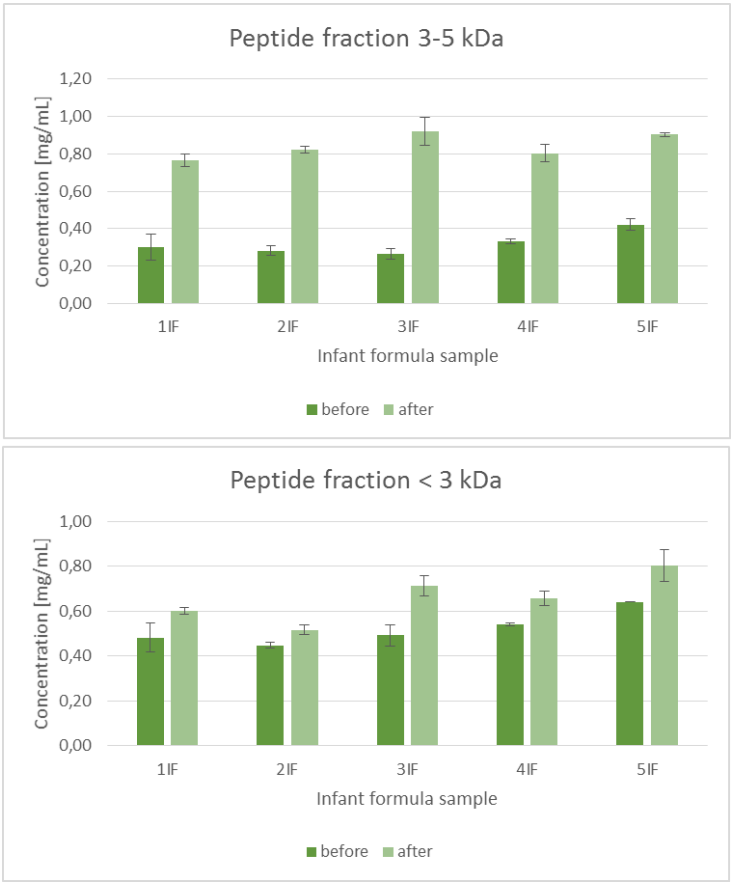

Fig. 2. Comparison of peptide contents in fractions from 3 to 5 kDa (A) and below 3 kDa (B) of five commercial soybeans based infant formulas before and after simulated gastrointestinal digestion.

For all fractions, from 3 to 5 kDa and also below 3 kDa, peptide contents before and after the gastrointestinal digestion were significantly different (test-t, $P < 0.05$). An extensive proteolysis was observed after simulated gastrointestinal digestion, especially, in the case of fractions from 3 to 5 kDa. ACE inhibitory activity of digested fractions was also measured (see Table 2) observing, in all cases, a decrease in ACE inhibitory activity in comparison with results shown in Table 1. By the action of gastrointestinal enzymes, peptides can break down into smaller peptides exhibiting different sequence, size, and, as a consequence, bioactivity. However, antihypertensive activities were still maintained at high level highlighting, again, the

fraction below 3 kDa in 4IF and 5IF with IC_{50} values of 18.2 ± 0.1 and 4.9 ± 0.1 μg/ mL, respectively.

Table 2. Antihypertensive capacity corresponding to peptide fractions from 3 to 5 kDa and below 3 kDa of five commercial SBIFs after *in vitro* gastrointestinal digestion.

	IC_{50} [μg/mL] after gastrointestinal digestion				
Fractions	**1IF**	**2IF**	**3IF**	**4IF**	**5IF**
3-5 kDa	34.71±0.70	20.25±0.68	17.79±1.53	31.75±2.05	36.94±1.18
<3 kDa	33.27±0.62	26.63±0.44	59.03±0.22	18.18±0.10	4.87±0.13

3.4. Identification of peptides after in vitro gastrointestinal digestion of infant formulas fractions

In order to identify those antihypertensive peptides standing gastrointestinal digestion, 3 to 5 kDa and below 3 kDa fractions that had been submitted to simulated gastrointestinal digestion were analyzed by HPLC-Q-ToF-MS. Total of 133 and 127 peptides were identified for fractions 3 to 5 kDa and below 3 kDa, respectively. Peptides identified in every hydrolyzed infant formula fraction are listed in the supplemental material (S1). The averaged peptide mass in the fraction from 3 to 5 kDa decreased after gastrointestinal digestion from 1 kDa to 0.7 kDa, while in fraction below 3 kDa it was maintained at 0.7 kDa. This fact explains partly the number of detected peptides after the gastrointestinal digestion. Although, apparently shorter peptides were created (see S1), some parts of cleaved peptides due to their small molecular weights (amino acids, di- or tri- peptides) are impossible to be identified in digested samples. In addition, some digested part might be the copy of already identified peptides. Peptides identified in each fraction prior and after gastrointestinal digestion were compared and those resisting gastrointestinal digestion have been summarized in Table 3. Fractions from 3 to 5 kDa showed 13 peptides resisting gastrointestinal digestion being 5IF that with the highest number of peptides standing this digestion. Regarding fractions below 3 kDa, 20 peptides were able to resist gastrointestinal digestion being most of them in 2IF. It is also important to highlight peptide NQLDQ that only appeared in the fraction < 3 kDa of 5IF since this fraction yielded the highest ACE inhibitory activity after gastrointestinal digestion (see Table 2). Furthermore, peptide with sequence RPSYT was identified in all fractions from 3 to 5 kDa and below 3 kDa while peptides NSGPLVNP and RDPIYS were present in all fractions below 3 kDa.

Table 3. Peptides from fractions 3 to 5kDa and below 3 kDa identified in the five SBIFs resisting *in vitro* gastrointestinal digestion.

Peptide sequence	(-10lgP)	Mass	m/z	RT [min]	Protein assesion[a]	Infant formula
Fractions 3-5 kDa						
DFYNPKA	72.33	853.397	854.4159	8.75	P04347|GLYG5_SOYBN	2, 5 IF
FLVPPQE	38.87	828.4381	829.4523	10.13	P04776|GLYG1_SOYBN	5 IF
FPFPRPPHQ	52.81	1121.577	561.8013	10.38	P13916|GLCA_SOYBN	5 IF
GKGIFG	27.57	577.3224	578.3302	9.49	P04776|GLYG1_SOYBN	2 IF
KGAIG	27.51	444.2696	445.2782	2.55	P04347|GLYG5_SOYBN	1IF
KGALG	27.51	444.2696	445.2782	2.55	P02858|GLYG4_SOYBN	1IF
KGIFG	29.68	520.3009	521.3115	9.74	P04776|GLYG1_SOYBN	2, 4, 5 IF
KGLFG	29.69	520.3009	521.3115	9.74	Q02920|NO70_SOYBN	2, 4, 5 IF
KNILE	26.77	615.3591	616.3695	7.91	P11827|GLCAP_SOYBN	5 IF
NSGPLVNP	100.12	796.4079	797.4203	8.81	P04347|GLYG5_SOYBN	1, 3, 4, 5 IF
RDPIYS	47.37	749.3708	750.3798	7.47	P13916|GLCA_SOYBN	2, 3 IF
RPSYT	51.00	622.3074	623.3178	4.03	P04776|GLYG1_SOYBN	1-5 IF
YNLRQ	31.71	692.3605	693.3647	7.04	P02858|GLYG4_SOYBN	1 IF
Fractions < 3kDa						
ANSLLN	35.63	630.3337	631.3411	8.46	P04776|GLYG1_SOYBN	1-3 IF
EEGGSV	27.2	576.2391	577.2692	6.99	P04347|GLYG5_SOYBN	1 IF
FLVPPQE	38.87	828.4381	829.4487	10.14	P04776|GLYG1_SOYBN	4 IF
FPFPRPPHQ	52.81	1121.577	561.7978	10.34	P13916|GLCA_SOYBN	4 IF
GANSLLN	31.54	687.3552	688.371	8.76	P04776|GLYG1_SOYBN	2 IF
GKGIFG	27.57	577.3224	578.3334	9.5	P04776|GLYG1_SOYBN	2 IF
IPSEVLA	43.24	727.4116	728.4222	9.9	P02858|GLYG4_SOYBN	2 IF
IPSEVLS	36.53	743.4065	744.4169	9.35	P04347|GLYG5_SOYBN	1-3 IF, 5IF
KGAIG	27.51	444.2696	445.2787	2.51	P04347|GLYG5_SOYBN	1 IF
KGALG	27.51	444.2696	445.2787	2.51	P02858|GLYG4_SOYBN	1 IF
KGVITQ	44.93	644.3857	645.3962	5.71	P22895|P34_SOYBN	2, 3 IF
KHFLA	34.47	614.354	615.3593	7.33	P04347|GLYG5_SOYBN	2, 5 IF
NQLDQ	33.71	616.2816	617.2919	3.54	P04347|GLYG5_SOYBN	5 IF
NSGPLVNP	100.12	796.4079	797.4205	8.86	P04347|GLYG5_SOYBN	1-5 IF
NTGNLLG	42.11	687.3552	688.3624	9.15	P22895|P34_SOYBN	1 IF
RDPIYS	47.37	749.3708	750.3771	7.43	P13916|GLCA_SOYBN	1-5 IF
RNPIYS	39.67	748.3868	749.3976	7.57	P25974|GLCB_SOYBN	2, 3 IF
RPSYT	51.00	622.3074	623.3182	3.98	P04776|GLYG1_SOYBN	1-5 IF
VEGGLS	28.10	560.2806	561.2917	6.09	P04347|GLYG5_SOYBN	1 IF
YNLRQ	31.71	692.3605	693.3718	7.04	P02858|GLYG4_SOYBN	2 IF

[a] From UniProt protein database.

These three peptides resistant to the gastrointestinal digestion, might present a potent ACE inhibitory activities. Due to the fact that peptide RPSYT could resist gastrointestinal digestion, it was presented in a high amount in all SBIFs, and it was also identified in previous studies as a potential antioxidant peptide [17], it has been selected for its further characterization. Therefore, a synthetic counterpart was synthetized and studied.

3.5. Characteristics of RPSYT peptide

Synthetic RPSYT was characterized by the study of its, bioactivity and resistance to simulated gastrointestinal digestion and high processing temperatures. *Resistance to gastrointestinal digestion and high processing temperatures.* In order to clearly confirm that RPSYT peptide could stand gastrointestinal digestion, it was subjected to the action of pepsin and pancreatin enzymes. Comparison of MS spectra prior and after this digestion did not show significant changes concluding that this peptide could resist it. Furthermore, the application of high temperatures (up to 100 ºC) for 10 min did not influence peptide signal which is an interesting data when submitting to heat processing. *ACE inhibitory and antioxidant activity studies.* ACE inhibitory activity of RPSYT peptide was evaluated. RPSYT yielded a moderate ACE inhibitor activity with an IC_{50} value of 245 ± 3 μg/mL (393 μmol/L). According to the literature, this IC_{50} value is within the range of IC_{50}values shown by most milk protein derived ACE inhibitors (100-500 μmol/L) [27]. Additionally, the antioxidant activity of this peptides was also measured. Peptides yielding both antihypertensive and antioxidant activities are of great interest due to the relationship between hypertension and oxidative stress. Indeed, it has been proved that angiotensin II amplifies oxidative stress and, therefore, ACE inhibitors amplify antioxidant defense system in animals and humans by inhibiting angiotensin II formation [28]. Antioxidant activity was measured by DPPH assay and ABTS assay observing a 33 ± 4 % of scavenging capacity in the case of radical DPPH and 79 ± 2 % in the case of radical $ABTS^{\cdot+}$. These results demonstrate that RPYST peptide had both antioxidant and antihypertensive properties. The ACE inhibitory and antioxidant activity of discovered peptide RPSYT can be explained by its moderate hydrophobicity. On the other hand, intensified antioxidant activity could be caused by the presence of aromatic tyrosine within the peptide sequence.

4. Conclusions

Five commercial soybean infant formulas have been fractionated according to their molecular weights observing the highest ACE inhibitory activity in fractions from 3 to 5 kDa and below 3 kDa. It was remarkable the ACE inhibitory activity of fraction below 3 kDa in two different infant formulas exerting IC_{50} values (1.20 ± 0.05 and 0.57 ± 0.04µg/ mL) lower than those corresponding to antihypertensive milk peptides VPP (2.80 µg/mL) and IPP (1.67 µg/mL). Identification of peptides in most active fractions by HPLC-Q-ToF-MS revealed the presence of common peptides among all infant formulas. Despite *in vitro* gastrointestinal digestion decreased ACE inhibitory activity in all fractions, it still remained at a high level. HPLC-Q-ToF-MS enabled the identification of 13 peptides in fractions from 3 to 5 kDa and 20 peptides in fraction below 3 kDa after simulated gastrointestinal digestion. Among them, RPSYT peptide was highlighted since it appeared in all infant formulas. The characterization studies of this peptide confirmed its resistance to gastrointestinal enzymes and high processing temperatures. Moreover, RPSYT peptide possessed, beyond moderate antihypertensive activity, a potent antioxidant activity.

Appendix A. Supplementary data

Supplemental material 1.

Peptides in fractions 3-5 kDa 1IF 81 peptides Peptide	-lg10P	Mass	m/z	RT [min]	Protein accession
AGVTVSK	23,41	660,3806	661,3337	6,33	P02858|GLYG4_SOYBN
AGVVPPAR	50,87	765,4497	766,4664	7,12	P29531|OLEO2_SOYBN
ALPEEVIQ	38,48	897,4807	898,4965	10,08	P04405|GLYG2_SOYBN
ALPEEVIQHTFNLK	101,68	1637,8777	819,9628	12,54	P04405|GLYG2_SOYBN
ALPEEVIQHTFNLKS	86,34	1724,9097	863,4651	12,4	P04405|GLYG2_SOYBN
ANSLLN	35,63	630,3337	631,3474	8,5	P04405|GLYG2_SOYBN
APAMR	41,96	544,2791	545,2877	2.84, 2.89, 2.93	P04405|GLYG2_SOYBN
DFYNPKA	72,33	853,397	854,4198	8,65	P02858|GLYG4_SOYBN
EAPRY	22,95	634,3074	635,3185	6,04	P29531|OLEO2_SOYBN
EEPRE	38,22	658,2922	659,3045	1,56	P02858|GLYG4_SOYBN
EQIRA	22,53	615,334	616,3441	5,35	P13916|GLCA_SOYBN
EQIRQ	27,12	672,3555	673,3738	2.34, 6.29	P25974|GLCB_SOYBN
ESFFLS	31,1	728,3381	729,3566	12	P13916|GLCA_SOYBN
ESVIVEISKEQIRA	38,64	1599,8832	800,9573	11,16	P13916|GLCA_SOYBN
FAPEFLK	45,43	850,4589	851,5073	10,92	P04405|GLYG2_SOYBN
FGINAENNQRNFLA	70,73	1606,7852	804,4113	11,21	P11827|GLCAP_SOYBN
FPFPRPPHQ	38,89	1121,577	1122,5906	10.13, 10.23, 10.28	P13916|GLCA_SOYBN
FPGSAQAVEKLLK	29,57	1386,787	1387,8062	11,26	P13916|GLCA_SOYBN
FPGSAQAVEKLLKNQRE	79,56	1914,0322	958,0334	10,97	P13916|GLCA_SOYBN
FSKHFLA	50,42	848,4545	849,4709	9,09	P02858|GLYG4_SOYBN
FSREEGQQQGEQRL	26,83	1690,8022	846,4271	7,67	P13916|GLCA_SOYBN
FSRNILE	30,71	877,4658	878,4778	9,64	P13916|GLCA_SOYBN
GANSLL	26,06	573,3122	574,3313	9,74	P04405|GLYG2_SOYBN
GANSLLN	31,54	687,3552	688,3776	8,7	P04405|GLYG2_SOYBN
GFAPEFLK	49,97	907,4803	908,5094	11,41	P04405|GLYG2_SOYBN
GFSKHFLA	55,18	905,4759	906,4973	9,49	P02858|GLYG4_SOYBN
GNGIFG	22,4	563,2703	564,2919	10,03	P04405|GLYG2_SOYBN
HENIARPSRA	40,04	1149,6002	575,8027	5,79	P02858|GLYG4_SOYBN
HGGIATDDDYPYRA	129,13	1549,6797	775,8544	8,9	P22895|P34_SOYBN
HKNKNPFLFG	40,28	1200,6404	1201,7444	10,87	P13916|GLCA_SOYBN
HVRVLQ	30,74	750,4501	751,4633	6,78	P11827|GLCAP_SOYBN
IGINAENNQRNFLA	62,22	1572,8008	787,4176	10,67	P13916|GLCA_SOYBN
IIDTNSLENQLDQMPRR	27,54	2042,0215	681,6924	11,02	P04405|GLYG2_SOYBN
IIIAQGKGALG	66,35	1039,6389	1040,6549	9,54	P02858|GLYG4_SOYBN
IKNNNPFKF	48,67	1120,6029	1121,6466	10,42	P04776|GLYG1_SOYBN
IKNNNPFKFLVPPQE	36,06	1783,962	892,99	12,45	P04776|GLYG1_SOYBN
IPNSISI	36,01	742,4225	743,4518	10,72	P09186|LOX3_SOYBN
IPSEVLA	28,65	727,4116	728,4366	9,83	P02858|GLYG4_SOYBN
ISKEQIRA	21,71	943,545	944,5586	6,93	P13916|GLCA_SOYBN
ISLLDTSNFNNQLDQTPRV	124,46	2174,0967	1088,0536	12,3	P02858|GLYG4_SOYBN
KGAIG	25,62	444,2696	445,2795	2,49	P04347|GLYG5_SOYBN
KGALG	25,62	444,2696	445,2795	2,49	P02858|GLYG4_SOYBN
KHFLA	34,47	614,354	615,3644	7,27	P02858|GLYG4_SOYBN
KLLKNQRE	22,5	1027,6138	514,8127	2,54	P13916|GLCA_SOYBN
KNKNPFLFG	54,37	1063,5814	1064,6049	11,36	P13916|GLCA_SOYBN
KNKPLVVQ	50,89	924,5756	925,5945	6.98, 7.02	P01071|ITRB_SOYBN
KNPFLFG	51,68	821,4435	822,4576	12,15	P13916|GLCA_SOYBN
KPLVVQ	36,54	682,4377	683,4514	7,52	P01071|ITRB_SOYBN
LHENIARPSRA	34,47	1262,6843	632,35	6,83	P02858|GLYG4_SOYBN
LSKEQIRQ	34,25	1000,5665	501,2951	6,24	P25974|GLCB_SOYBN
NKNPFLFG	61,06	935,4865	936,5142	12.05, 12.1	P13916|GLCA_SOYBN
NPFLFG	35,9	693,3486	694,3635	13,04	P13916|GLCA_SOYBN
NQLDQMPRR	27,14	1156,5771	579,3108	7,71	P04405|GLYG2_SOYBN
NSGPLVNP	68,11	796,4079	797,4257	8,85	P04347|GLYG5_SOYBN
QRSPQLQ	39,49	855,4562	856,47	6,19	P13916|GLCA_SOYBN
RPSYT	50,91	622,3074	623,3126	4.02, 4.07, 4.12	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQ	28,48	1792,8744	897,4635	9,78	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGKGIFG	88,59	2352,1863	1177,1188	11,51	P04776|GLYG1_SOYBN
SFEWVLE	40,53	908,428	909,4421	13,48	P22895|P34_SOYBN
SGDAIR	43,61	617,3132	618,3238	3,08	P07135|RR7_SOYBN
SGDALR	43,61	617,3132	618,3238	3,08	P13916|GLCA_SOYBN
SNFNNQLDQTPRV	74,81	1531,7379	766,884	9,69	P02858|GLYG4_SOYBN
SQVSELK	51	789,4232	790,436	6,73	P02858|GLYG4_SOYBN
SSEDKPFNLRS	50,11	1278,6204	640,3274	9,24	P13916|GLCA_SOYBN
TGNLLG	39,65	573,3122	574,3234	8,95	P22895|P34_SOYBN

TISSEDEPFNLRS	56,02	1493,6997	747,876	9.34, 9.39, 9.44, 10.38	P25974|GLCB_SOYBN
TTYYVVNPDNNENLRL	77,31	1923,9326	962,9849	11,31	P13916|GLCA_SOYBN
VAISR	29,33	544,3333	545,3419	6,09	C6T1G0|CSPL1_SOYBN
VALSR	29,33	544,3333	545,3419	6,09	P04405|GLYG2_SOYBN
VAWWMYNNEDTPVVA	55,34	1793,8083	897,9208	13,33	P04405|GLYG2_SOYBN
VKGGLR	26,43	628,402	629,4133	2.15, 2.2	P04405|GLYG2_SOYBN
VKGGLRV	36,52	727,4705	728,4863	7,22	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQE	32,15	1728,8834	865,4514	13.63, 13.68	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQESQR	25,66	2100,0752	701,0344	12,79	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQESQRR	68,8	2256,1763	753,0692	12.2, 12.25	P04405|GLYG2_SOYBN
VNMQIVRN	33,81	972,5175	973,5349	8,75	P04405|GLYG2_SOYBN
VPTGV	26,08	471,2693	472,2809	4.56, 4.61	P04405|GLYG2_SOYBN
VSIIDTNSLENQLDQMPRR	67,19	2228,1218	743,7213	11.76, 11.8	P04405|GLYG2_SOYBN
WWMYNNEDTPVVA	41,27	1623,7028	812,8675	13,14	P04405|GLYG2_SOYBN
YNLRQ	22,67	692,3605	693,3788	7,07	P02858|GLYG4_SOYBN
2IF 50 peptides					
ALPEEVIQHTFNLK	96,84	1637,8777	819,9534	12,59	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQESQRR	80,44	2256,1763	753,0779	12.2, 12.3	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGNGIFG	75,26	2338,1343	1170,0901	11,9	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGKGIFG	75,2	2352,1863	1177,113	11,56	P04776|GLYG1_SOYBN
TISSEDKPFNLRS	72,66	1492,7521	747,3875	9,44	P13916|GLCA_SOYBN
FPGSAQAVEKLLK	71,32	1386,787	694,4092	11.26, 11.31	P13916|GLCA_SOYBN
ALPEEVIQHTFNLKS	68,28	1724,9097	863,4677	12,45	P04405|GLYG2_SOYBN
VSIIDTNSLENQLDQMPRR	64,68	2228,1218	743,7189	11.76, 11.8	P04405|GLYG2_SOYBN
DFYNPKA	64,33	853,397	854,4124	8,7	P02858|GLYG4_SOYBN
IIIAQGKGALG	60,37	1039,6389	1040,6611	9,59	P02858|GLYG4_SOYBN
KNKPLVVQ	58,58	924,5756	925,5938	7,02	P01071|ITRB_SOYBN
NKNPFLFG	54,53	935,4865	936,4923	12,1	P13916|GLCA_SOYBN
NGPQEIYIQQGKGIFG	51,38	1747,8893	874,9647	11,71	P04776|GLYG1_SOYBN
ISSEDKPFNLRS	50,36	1391,7045	696,863	9,29	P13916|GLCA_SOYBN
KNKNPFLFG	48,11	1063,5814	1064,594	11.41, 11.46	P13916|GLCA_SOYBN
TISSEDKPFNLR	47,37	1405,7201	703,8729	9,54	P13916|GLCA_SOYBN
RDPIYSNKLG	46,67	1161,6141	581,8229	8,45	P13916|GLCA_SOYBN
KNKPLVV	46,17	796,517	797,5298	7,57	P01071|ITRB_SOYBN
RDPIYS	45,93	749,3708	750,3824	7,42	P13916|GLCA_SOYBN
RPSYT	45,45	622,3074	623,3107	4,17	P04405|GLYG2_SOYBN
FPFPRPPHQ	44,49	1121,577	1122,5918	10.23, 10.28	P13916|GLCA_SOYBN
HENIARPSRA	42,73	1149,6002	575,8065	5,84	P02858|GLYG4_SOYBN
APAMR	42,41	544,2791	545,2814	2.93, 2.98, 3.03	P04405|GLYG2_SOYBN
SGDALR	42,16	617,3132	618,3146	3,13	P13916|GLCA_SOYBN
SGDAIR	42,16	617,3132	618,3146	3,13	P07135|RR7_SOYBN
FAPEFLK	38,91	850,4589	851,4797	10,97	P04405|GLYG2_SOYBN
WWMYNNEDTPVVA	38,11	1623,7028	812,8593	13,18	P04405|GLYG2_SOYBN
NPFLFG	37,57	693,3486	694,3572	12,99	P13916|GLCA_SOYBN
GNDTFPYPRR	36,44	1221,589	611,8087	8,85	P09186|LOX3_SOYBN
VNMQIVR	35,52	858,4745	859,4877	9,04	P04405|GLYG2_SOYBN
RDPIY	33,94	662,3387	663,3502	7,91	P13916|GLCA_SOYBN
ANSLLN	32,93	630,3337	631,3534	8,5	P04405|GLYG2_SOYBN
IKNNNPFK	31,49	973,5345	974,5465	7,62	P04776|GLYG1_SOYBN
IKNNNPFKFLVPPQE	31,01	1783,962	892,9921	12,49	P04776|GLYG1_SOYBN
QSGDALR	30,79	745,3718	746,3782	3,87	P13916|GLCA_SOYBN
VKNNNPFSFLVPPQE	30,1	1728,8834	865,4716	12.84, 13.68	P04405|GLYG2_SOYBN
KGIFG	29,69	520,3009	521,3137	9.69, 9.74	P04776|GLYG1_SOYBN
KGLFG	29,69	520,3009	521,3137	9.69, 9.75	Q02920|NO70_SOYBN
VNMQIVRN	29,64	972,5175	973,5325	8,75	P04405|GLYG2_SOYBN
EAPRY	29,48	634,3074	635,3126	6,04	P29531|OLEO2_SOYBN
SREWRS	28,68	819,3987	820,4072	5,15	P02858|GLYG4_SOYBN
EAGVVPPAR	28	894,4922	895,5049	7,37	P29531|OLEO2_SOYBN
DITAFGGIRA	27,89	1019,54	1020,5509	11,21	P01071|ITRB_SOYBN
PHSVQVHTTTTH	27,72	1343,6582	672,8555	8,6	P29530|OLEO1_SOYBN
VKGGLRV	26,53	727,4705	728,4834	7,27	P04405|GLYG2_SOYBN
YNLRQ	26,45	692,3605	693,371	7,07	P02858|GLYG4_SOYBN
ISKEQIRA	26,2	943,545	944,5534	6,97	P13916|GLCA_SOYBN
LSKEQIRQ	26,08	1000,5665	501,2938	6,29	P25974|GLCB_SOYBN
IPSEVLA	25,79	727,4116	728,4312	9,83	P02858|GLYG4_SOYBN
GKGIFG	25,4	577,3224	578,3409	9,49	P04776|GLYG1_SOYBN
3IF 45 peptides					
APAMR	39	544,2791	545,2906	2.8, 2.85, 2.9	P04405|GLYG2_SOYBN
RDPIYSNKLG	34,58	1161,6141	581,8254	8,43	P13916|GLCA_SOYBN
GNDTFPYPRR	33,78	1221,589	611,8144	8,82	P09186|LOX3_SOYBN
KHFLA	31,94	614,354	615,3666	7,25	P02858|GLYG4_SOYBN

RPSYT	46,88	622,3074	623,3142	3.98, 4.03, 4.08	P04405|GLYG2_SOYBN
EAPRY	29,88	634,3074	635,3167	6,05	P29531|OLEO2_SOYBN
EEPRE	30,54	658,2922	659,3047	1,57	P02858|GLYG4_SOYBN
AGVTVSK	43,95	660,3806	661,3822	4,87	P02858|GLYG4_SOYBN
KPLVVQ	36,49	682,4377	683,4502	7,49	P01071|ITRB_SOYBN
NPFLFG	32,56	693,3486	694,3663	12,96	P13916|GLCA_SOYBN
FPGSAQAVEKLLK	74	1386,787	694,4165	11.24, 11.29	P13916|GLCA_SOYBN
ISSEDKPFNLRS	60,08	1391,7045	696,8757	9,27	P13916|GLCA_SOYBN
INAENNQRNFLA	34,35	1402,6953	702,3701	9,61	P13916|GLCA_SOYBN
IPNSISI	35,4	742,4225	743,4434	10.7, 10.75	P09186|LOX3_SOYBN
VSIIDTNSLENQLDQMPRR	48,69	2228,1218	743,7332	11,78	P04405|GLYG2_SOYBN
VTAPAMR	32,84	744,3953	745,4089	7,4	P04405|GLYG2_SOYBN
TISSEDKPFNLRS	61,96	1492,7521	747,4	9,42	P13916|GLCA_SOYBN
RNPIYS	28,25	748,3868	749,4011	7,54	P25974|GLCB_SOYBN
RDPIYS	30,24	749,3708	750,3849	7,44	P13916|GLCA_SOYBN
VKNNNPFSFLVPPKESQRR	40,11	2256,2126	753,0833	12,27	P11828|GLYG3_SOYBN
VKNNNPFSFLVPPQESQRR	60,02	2256,1763	753,0833	12,22	P04405|GLYG2_SOYBN
HTFNLK	51,71	758,4075	759,4429	7,69	P04405|GLYG2_SOYBN
HGGIATDDDYPYRA	86,47	1549,6797	775,8627	8,92	P22895|P34_SOYBN
NSGPLVNP	67,81	796,4079	797,4299	8.78, 8.87	P04347|GLYG5_SOYBN
WWMYNNEDTPVVA	32,01	1623,7028	812,8699	13,16	P04405|GLYG2_SOYBN
ALPEEVIQHTFNLK	88,57	1637,8777	819,9622	12,57	P04405|GLYG2_SOYBN
KNPFLFG	53,51	821,4435	822,4667	12,17	P13916|GLCA_SOYBN
FSKHFLA	45,9	848,4545	849,4764	9,12	P02858|GLYG4_SOYBN
DFYNPKA	65,07	853,397	854,4199	8,68	P02858|GLYG4_SOYBN
VKNNNPFSFLVPPQE	32,07	1728,8834	865,4623	13,6	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPKE	37,3	1728,9198	865,4814	12,77	P11828|GLYG3_SOYBN
IKNNNPFKFLVPPQE	31,38	1783,962	893,0041	12.42, 12.47	P04776|GLYG1_SOYBN
VAWWMYNNEDTPVVA	62,26	1793,8083	897,9249	13.31, 13.36	P04405|GLYG2_SOYBN
ALPEEVIQ	35,1	897,4807	898,4957	10,11	P04405|GLYG2_SOYBN
GFSKHFLA	51,89	905,4759	906,501	9,51	P02858|GLYG4_SOYBN
KNKPLVVQ	48,82	924,5756	925,5905	7	P01071|ITRB_SOYBN
NKNPFLFG	54,92	935,4865	936,5145	12,08	P13916|GLCA_SOYBN
VNMQIVRN	34,44	972,5175	973,5425	8,73	P04405|GLYG2_SOYBN
IKNNNPFK	38,4	973,5345	974,5481	7,59	P04776|GLYG1_SOYBN
IIIAQGKGALG	54,85	1039,6389	1040,6641	9,56	P02858|GLYG4_SOYBN
YFVDAQPKK	48,09	1094,576	1095,592	8,09	P13916|GLCA_SOYBN
FPFPRPPHQ	41,44	1121,577	1122,6021	10.25, 10.3	P13916|GLCA_SOYBN
ALPEEVIQHTFNLKSQQARQ	71,32	2336,2236	1169,1401	11,58	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGNGIFG	61,97	2338,1343	1170,0962	11,88	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGKGIFG	66,83	2352,1863	1177,1235	11,53	P04776|GLYG1_SOYBN
4IF 70 peptides					
AGVVPPAR	53,27	765,4497	766,4664	7,17	P29531|OLEO2_SOYBN
AGVVPPARF	38,56	912,5181	913,5347	9.88, 9.93	P29531|OLEO2_SOYBN
ALPEEVIQHTFNLK	105,06	1637,8777	819,9598	12.54, 12.59, 12.64	P04405|GLYG2_SOYBN
ALPEEVIQHTFNLKSQQARQ	74,33	2336,2236	1169,126	11,6	P04405|GLYG2_SOYBN
APAMR	37,26	544,2791	545,2881	2.93, 2.98, 3.03	P04405|GLYG2_SOYBN
APAMRK	25,78	672,3741	673,3484	3,62	P04405|GLYG2_SOYBN
DFYNPKA	65,49	853,397	854,4153	8,7	P04347|GLYG5_SOYBN
EAAEYVGQK	45,28	993,4767	994,5111	6,78	P29531|OLEO2_SOYBN
EAPRY	31,6	634,3074	635,3193	6,04	P29531|OLEO2_SOYBN
EEPRE	23,84	658,2922	659,3053	1,8	P02858|GLYG4_SOYBN
ESVIVEISKEQIRA	32,04	1599,8832	800,9568	11,16	P13916|GLCA_SOYBN
FAPEFLK	48,05	850,4589	851,4778	10.91, 10.96	P04405|GLYG2_SOYBN
FEAPRY	31,29	781,3759	782,3841	8,99	P29531|OLEO2_SOYBN
FGINAENNQRNFLA	48,79	1606,7852	804,3985	11,21	P25974|GLCB_SOYBN
FLVPPQE	35,37	828,4381	829,4644	10,13	P04405|GLYG2_SOYBN
FLVPPQESQKR	49,31	1327,7247	664,8831	8,8	P04776|GLYG1_SOYBN
GDLIAVPTG	34,33	841,4545	842,4684	10.57, 10.62	P04405|GLYG2_SOYBN
GFAPEFLK	48,74	907,4803	908,5035	11,41	P04405|GLYG2_SOYBN
GFSKHFLA	58,6	905,4759	906,4879	9,49	P04347|GLYG5_SOYBN
HGGIATDDDYPYRA	89,66	1549,6797	775,8615	8,94	P22895|P34_SOYBN
IGINAENNQRNFLA	66,84	1572,8008	787,42	10.67, 10.72	P13916|GLCA_SOYBN
IIDTNSLENQLDQMPRR	25,48	2042,0215	681,7013	11,01	P04405|GLYG2_SOYBN
IKNNNPFK	39,85	973,5345	974,5474	7,61	P04776|GLYG1_SOYBN
IKNNNPFKFLVPPQE	36,82	1783,962	893,0034	12.39, 12.49	P04776|GLYG1_SOYBN
IPNSISI	27,6	742,4225	743,4401	10,77	P08170|LOX1_SOYBN
IPSEVLA	34,09	727,4116	728,432	9.78, 9.83	P02858|GLYG4_SOYBN
ISSEDKPFNLRS	72,17	1391,7045	696,8748	9.29, 9.24	P13916|GLCA_SOYBN
IYIQQGKGIFG	33,89	1222,671	1223,6768	11,31	P04776|GLYG1_SOYBN
KGIFG	29,68	520,3009	521,3167	9.68, 9.68	P04776|GLYG1_SOYBN

KGLFG	29,68	520,3009	521,3167	9.68, 9.69	Q02920|NO70_SOYBN
KGVITQ	44,96	644,3857	645,3937	5,74	P22895|P34_SOYBN
KHFLA	31,94	614,354	615,366	7,32	P04347|GLYG5_SOYBN
KNKNPFLFG	52,1	1063,5814	1064,6108	11,36	P13916|GLCA_SOYBN
KNKPLVVQ	52,54	924,5756	925,5872	7,02	P01071|ITRB_SOYBN
KPLVVQ	35,61	682,4377	683,4479	7,51	P01071|ITRB_SOYBN
KQQLQ	33,36	643,3653	644,3751	1,9	P02858|GLYG4_SOYBN
LHENIARPSRA	38,81	1262,6843	632,3531	6,83	P04347|GLYG5_SOYBN
LSKEQIRQ	27,11	1000,5665	501,2939	6,28	P25974|GLCB_SOYBN
NALPEEVIQHTFNLK	65,99	1751,9207	876,9775	12,69	P04405|GLYG2_SOYBN
NKNPFLFG	60,95	935,4865	936,5137	12.1, 12.05	P13916|GLCA_SOYBN
NPFLFG	42,25	693,3486	694,3571	12,98	P13916|GLCA_SOYBN
NQLDQ	33,71	616,2816	617,2952	3,67	P04405|GLYG2_SOYBN
NSGPLVNP	57,91	796,4079	797,4156	8,89	P04347|GLYG5_SOYBN
PHSVQVHTTTTH	23,51	1343,6582	672,8621	8,6	P29530|OLEO1_SOYBN
RDPIY	35,53	662,3387	663,3584	7,86	P13916|GLCA_SOYBN
RDPIYS	46,41	749,3708	750,3907	7.37, 7.42	P13916|GLCA_SOYBN
RDPIYSNKLG	51,32	1161,6141	581,8258	8,45	P13916|GLCA_SOYBN
RPSYT	47,57	622,3074	623,3134	4.11, 4.16, 4.21	P04405|GLYG2_SOYBN
RPSYTNGPQEIYIQQGKGIFG	77,23	2352,1863	1177,1071	11,51	P04776|GLYG1_SOYBN
SFEWVLE	35,63	908,428	909,4366	13,48	P22895|P34_SOYBN
SGDAIR	44,25	617,3132	618,3231	3,13	P07135|RR7_SOYBN
SGDALR	44,25	617,3132	618,3231	3,13	P13916|GLCA_SOYBN
SNFNNQLDQNPRV	61,36	1544,7332	773,388	9,19	P04347|GLYG5_SOYBN
SREWRS	31,28	819,3987	820,4037	5,1	P02858|GLYG4_SOYBN
SYPTKEESET	50,93	1169,5088	1170,5261	6,38	P22895|P34_SOYBN
TISSEDEPFNLRS	58,83	1493,6997	747,8771	10.37, 10.42	P25974|GLCB_SOYBN
TISSEDKPFNLRS	72,46	1492,7521	747,3993	9.39, 9.44	P13916|GLCA_SOYBN
VAWWMYNNEDTPVVA	61,42	1793,8083	1794,8508	13.33, 13.38	P04405|GLYG2_SOYBN
VEGGLS	24,45	560,2806	561,2895	6,18	P04347|GLYG5_SOYBN
VKGGLR	28,01	628,402	629,4109	2.29, 2.34	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPKE	42,21	1728,9198	865,4757	12.74, 12.79	P11828|GLYG3_SOYBN
VKNNNPFSFLVPPKESQRR	72,3	2256,2126	753,0776	12,2	P11828|GLYG3_SOYBN
VKNNNPFSFLVPPQE	25,72	1728,8834	865,45	13.53, 13.67	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQESQRR	79,07	2256,1763	753,0776	12.15, 12.25	P04405|GLYG2_SOYBN
VNMQIVRN	35,1	972,5175	973,5398	8,75	P04405|GLYG2_SOYBN
VPTGV	24,13	471,2693	472,2904	4,66	P04405|GLYG2_SOYBN
VQVHT	28,27	582,3126	583,3218	5,3	P29531|OLEO2_SOYBN
VSIIDTNSLENQLDQMPRR	72,05	2228,1218	743,7304	11.7, 11.75, 11.8	P04405|GLYG2_SOYBN
WWMYNNEDTPVVA	55,07	1623,7028	1624,7466	13.13, 13.18	P04405|GLYG2_SOYBN
YNLRQ	24,81	692,3605	693,3714	7,07	P02858|GLYG4_SOYBN
5IF 82 peptides					
AGVVPPAR	46,68	765,4497	766,4603	7.16, 7.21	P29531|OLEO2_SOYBN
ALPEEVIQHTFNLK	104,85	1637,8777	819,9572	12.54, 12.58,12.63	P04776|GLYG1_SOYBN
ANSLLN	31,05	630,3337	631,352	8,49	P04776|GLYG1_SOYBN
APAMR	41,28	544,2791	545,2913	2.87, 2.97	P04405|GLYG2_SOYBN
DFYNPKA	68,28	853,397	854,4231	8,69	P02858|GLYG4_SOYBN
DFYNPKAGRI	33,97	1179,6036	590,8165	9,48	P02858|GLYG4_SOYBN
DITAFG	23,21	622,2962	623,3112	10,02	P01070|ITRA_SOYBN
EQIRA	22,28	615,334	616,3437	5.34, 5.39	P13916|GLCA_SOYBN
EQIRQ	25,62	672,3555	673,3682	2,33	P25974|GLCB_SOYBN
ESFFLS	21,82	728,3381	729,3515	11,94	P13916|GLCA_SOYBN
FAPEFLK	46,37	850,4589	851,5252	10.91, 10.96	P04405|GLYG2_SOYBN
FEAPRY	21,47	781,3759	782,3947	8,99	P29531|OLEO2_SOYBN
FLVPPQE	34,32	828,4381	829,4628	10,12	P04776|GLYG1_SOYBN
FPFPRPPHQ	38,67	1121,577	1122,6074	10.22, 10.27, 10.32	P13916|GLCA_SOYBN
FPGSAQAVEKLLK	75,51	1386,787	694,403	11,25	P13916|GLCA_SOYBN
FQKLDKES	33,11	993,5131	994,5262	6,67	P01070|ITRA_SOYBN
FREGDLIAVPTG	19,41	1273,6666	637,8542	11,11	P04776|GLYG1_SOYBN
FSKHFLA	48,75	848,4545	849,4788	9.13, 9.18	P02858|GLYG4_SOYBN
FSRNILE	27,36	877,4658	878,4806	9,63	P13916|GLCA_SOYBN
GAIVT	20,97	459,2693	460,2806	6,37	P04776|GLYG1_SOYBN
GANSLLN	28,38	687,3552	688,3746	8,74	P04776|GLYG1_SOYBN
GDLIAVPTG	32,77	841,4545	842,4683	10.61, 10.56	P04776|GLYG1_SOYBN
GFSKHFLA	41,25	905,4759	906,5023	9,53	P02858|GLYG4_SOYBN
GFSKNILE	41,35	906,4811	907,5018	9,87	P11827|GLCAP_SOYBN
HGGIATDDDYPYRA	123,19	1549,6797	775,8525	8,94	P22895|P34_SOYBN
HTFNLK	55,93	758,4075	759,4222	7,7	P04776|GLYG1_SOYBN
HVRVLQ	37,53	750,4501	751,465	6,77	P11827|GLCAP_SOYBN
IGENKDAMDGWFRL	66,01	1650,7823	826,4037	13.42, 13.47	P01070|ITRA_SOYBN
IIDTNSLENQLDQMPRR	41,7	2042,0215	681,6876	11,01	P04776|GLYG1_SOYBN

IKNNNPFK	40,92	973,5345	974,5466	7.56, 7.61	P04776|GLYG1_SOYBN
IKNNNPFKF	27,54	1120,6029	1121,6221	10,47	P04776|GLYG1_SOYBN
INAENNQRNFLA	46,86	1402,6953	702,3677	9,58	P13916|GLCA_SOYBN
IPNSISI	27,6	742,4225	743,4495	10,76	P24095|LOXX_SOYBN
IPSEVLA	30,53	727,4116	728,4305	9.78, 9.82	P02858|GLYG4_SOYBN
ISLLDTSNFNNQLDQTPRV	101,62	2174,0967	1088,0457	12,34	P02858|GLYG4_SOYBN
ISSEDKPFNLRS	59,83	1391,7045	696,8665	9,28	P13916|GLCA_SOYBN
KGAIG	22,9	444,2696	445,2786	2.53, 2.59	P04347|GLYG5_SOYBN
KGALG	22,9	444,2696	445,2786	2.53, 2.58	P02858|GLYG4_SOYBN
KGIFG	26,62	520,3009	521,3171	9,68	P04776|GLYG1_SOYBN
KGLFG	26,62	520,3009	521,3171	9,68	Q02920|NO70_SOYBN
KHFLA	32,68	614,354	615,3702	7,26	P02858|GLYG4_SOYBN
KNILE	22,74	615,3591	616,3737	7,95	P11827|GLCAP_SOYBN
KNKNPFLFG	53,38	1063,5814	1064,5978	11.35, 11.4, 11.45	P13916|GLCA_SOYBN
KNKPLVVQ	57,86	924,5756	925,5957	7,01	P01070|ITRA_SOYBN
KNPFLFG	53,94	821,4435	822,4618	12,19	P13916|GLCA_SOYBN
KPLVVQ	44,65	682,4377	683,452	7,51	P01070|ITRA_SOYBN
LHENIARPSRA	38,01	1262,6843	632,3513	6,82	P02858|GLYG4_SOYBN
LSKEQIRQ	19,64	1000,5665	501,2986	6,28	P25974|GLCB_SOYBN
NALPEEVIQHTFNLK	59,21	1751,9207	876,9781	12,73	P04776|GLYG1_SOYBN
NGPQEIYIQQGKGIFG	103,94	1747,8893	874,9542	11,65	P04776|GLYG1_SOYBN
NKNPFLFG	56,35	935,4865	936,4989	12.09, 12.14	P13916|GLCA_SOYBN
NPFLFG	33	693,3486	694,3583	12.98, 13.03	P13916|GLCA_SOYBN
NQLDQ	28,81	616,2816	617,2911	3.56, 3.61	P04776|GLYG1_SOYBN
NSGPLVNP	72,71	796,4079	797,4264	8.79, 8.89	P04347|GLYG5_SOYBN
NSLLN	25,56	559,2966	560,3074	8,3	P04776|GLYG1_SOYBN
QNALR	19,56	600,3344	601,342	2,63	Q9FZL4|MGDG_SOYBN
QRSPQLQ	37,58	855,4562	856,4615	6,18	P13916|GLCA_SOYBN
QTLFK	38,09	635,3643	636,3813	8,54	P11827|GLCAP_SOYBN
RDPIY	28,15	662,3387	663,3546	7,9	P13916|GLCA_SOYBN
RDPIYS	45,23	749,3708	750,3831	7.36, 7.41, 7.46	P13916|GLCA_SOYBN
RDPIYSNKLG	58,22	1161,6141	581,8245	8,44	P13916|GLCA_SOYBN
RPSYT	47,67	622,3074	623,3112	4.01, 4.11, 4.16	P04776|GLYG1_SOYBN
RPSYTN	35,9	736,3504	737,3555	3,37	P04776|GLYG1_SOYBN
RPSYTNGPQEIYIQQGKGIFG	79,06	2352,1863	1177,1293	11.5, 11.55	P04776|GLYG1_SOYBN
SEDKPFNLRS	30,18	1191,5884	596,8152	8,84	P13916|GLCA_SOYBN
SGDAIR	43,11	617,3132	618,3224	3.02, 3.08	P07135|RR7_SOYBN
SGDALR	43,11	617,3132	618,3224	3.02, 3.07	P13916|GLCA_SOYBN
SITTA	19,29	491,2591	492,268	4,06	P04405|GLYG2_SOYBN
SNRFETLFK	31,02	1140,5928	571,3143	10,71	P13916|GLCA_SOYBN
SYPTK	30,34	594,3013	595,3127	2,68	P22895|P34_SOYBN
TISSEDEPFNLRS	39,79	1493,6997	747,8678	10,42	P25974|GLCB_SOYBN
TISSEDKPFNLRS	72,7	1492,7521	747,3875	9,38	P13916|GLCA_SOYBN
VAWWMYNNEDTPVVA	59,6	1793,8083	897,9229	13.32,13.37	P04776|GLYG1_SOYBN
VKNNNPFSFLVPPQE	25,85	1728,8834	865,4539	13.57, 13.62	P04405|GLYG2_SOYBN
VKNNNPFSFLVPPQESQRR	19,61	2256,1763	1129,0918	12,24	P04405|GLYG2_SOYBN
VNMQIVR	34,29	858,4745	859,4933	9,04	P04405|GLYG2_SOYBN
VPQNFVVA	44,43	872,4756	873,4901	10,37	P04776|GLYG1_SOYBN
VPTGV	24,37	471,2693	472,2818	4.6, 4.65, 4.7	P04776|GLYG1_SOYBN
VSIIDTNSLENQLDQMPRR	49,27	2228,1218	1115,0854	11.75, 11.8	P04776|GLYG1_SOYBN
WNPNNKPFQ	58,75	1143,5461	1144,5516	9,33	P04776|GLYG1_SOYBN
WWMYNNEDTPVVA	35,3	1623,7028	812,8617	13,18	P04776|GLYG1_SOYBN
YNLRQ	20,58	692,3605	693,3715	7,06	P02858|GLYG4_SOYBN
Peptides in fractions <3 kDa					
1IF 40 peptides					
AIEAAHA	50,89	681,3445	682,3574	5,79	P22895|P34_SOYBN
ANSII	23,64	516,2908	517,3023	9.59, 9.64	P04405|GLYG2_SOYBN
ANSLLN	32,03	630,3337	631,3474	8.5, 8.55	P04405|GLYG2_SOYBN
APAMR	37,95	544,2791	545,291	2.98, 3.08, 3.13	P04405|GLYG2_SOYBN
DFYNPKA	72,27	853,397	854,4037	8.7 and 87.5	P04347|GLYG5_SOYBN
EAPRY	19,54	634,3074	635,3176	6,09	P29531|OLEO2_SOYBN
EEGGSV	23,28	576,2391	577,2771	7,03	P04347|GLYG5_SOYBN
EQIRQ	19,56	672,3555	673,3647	2,54	P25974|GLCB_SOYBN
FAPEFLK	48,07	850,4589	851,4752	10.97, 11.02	P04405|GLYG2_SOYBN
FSKHFLA	50,38	848,4545	849,4622	9,19	P04347|GLYG5_SOYBN
GNGIFG	21,69	563,2703	564,2834	10,13	P04405|GLYG2_SOYBN
IPNSISI	43,63	742,4225	743,4359	10,77	P38417|LOX4_SOYBN
IPSEVLA	43,24	727,4116	728,4208	9.83, 9.88	P02858|GLYG4_SOYBN
IPSEVLS	22,35	743,4065	744,4182	9,39	P04347|GLYG5_SOYBN
KGAIG	21,34	444,2696	445,2798	2,64	P04347|GLYG5_SOYBN
KGALG	21,34	444,2696	445,2798	2,64	P02858|GLYG4_SOYBN

KGVIT	33,33	516,3271	517,3336	5,35	P22895|P34_SOYBN
KHFLA	36,81	614,354	615,3675	7.32, 7.37	P04347|GLYG5_SOYBN
KNPFLFG	54,18	821,4435	822,4477	12,25	P13916|GLCA_SOYBN
KPLVVQ	36,49	682,4377	683,4493	7,57	P01071|ITRB_SOYBN
NKNPFLFG	43,81	935,4865	936,4952	12,2	P13916|GLCA_SOYBN
NPFLFG	38,49	693,3486	694,3556	12.99, 13.04, 13.09	P13916|GLCA_SOYBN
NSGPLVNP	77,14	796,4079	797,4247	8,8	P04347|GLYG5_SOYBN
NSLAR	28,89	559,3078	560,3038	8,36	P38417|LOX4_SOYBN
NTGNLLG	42,11	687,3552	688,3668	9,14	P22895|P34_SOYBN
QSGDALR	41,11	745,3718	746,3859	4,02	P13916|GLCA_SOYBN
RDPIYS	47,37	749,3708	750,3837	7.42, 7.47	P13916|GLCA_SOYBN
RPSYT	51	622,3074	623,3136	4.22, 4.27, 4.36	P04405|GLYG2_SOYBN
SGDAIR	47,31	617,3132	618,3253	3,23	P07135|RR7_SOYBN
SGDALR	47,31	617,3132	618,3253	3,23	P13916|GLCA_SOYBN
SREWRS	38,05	819,3987	820,4088	5,25	P02858|GLYG4_SOYBN
SYNLRQ	22,49	779,3926	780,3976	7,27	P02858|GLYG4_SOYBN
TGNLLG	34,3	573,3122	574,321	8,95	P22895|P34_SOYBN
TISSEDKPFNLRS	73,7	1492,7521	747,3859	9,44	P13916|GLCA_SOYBN
TPVVA	22,97	485,2849	486,2937	7,12	P04405|GLYG2_SOYBN
TVTATTATA	45,74	835,4287	836,4349	6,68	P29531|OLEO2_SOYBN
VEGGLS	20,44	560,2806	561,2879	6,19	P04347|GLYG5_SOYBN
VNMQIVR	30,49	858,4745	859,4855	9,09	P04405|GLYG2_SOYBN
VPTGV	23,81	471,2693	472,2916	4.71, 4.81, 4.86	P04405|GLYG2_SOYBN
VVPPAR	19,61	637,3911	638,3992	5,94	P29531|OLEO2_SOYBN
2IF 27 peptides					
AGVVPPAR	52,86	765,4497	766,4609	7.27, 7.32	P29531|OLEO2_SOYBN
ANSLLN	24,9	630,3337	631,3416	8,55	P04405|GLYG2_SOYBN
APAMR	30,04	544,2791	545,2881	3.38, 3.48, 3.53	P04405|GLYG2_SOYBN
DFYNPKAGRI	24,11	1179,6036	590,8104	9,59	P04347|GLYG5_SOYBN
EAPRY	24,12	634,3074	635,3184	6,29	P29531|OLEO2_SOYBN
FAPEFLK	25,63	850,4589	851,4645	11,02	P04405|GLYG2_SOYBN
FEAPRY	20,55	781,3759	782,3884	9,1	P29531|OLEO2_SOYBN
GANSLLN	21,37	687,3552	688,3698	8,8	P04405|GLYG2_SOYBN
GKGIFG	22,01	577,3224	578,3355	9,54	P04776|GLYG1_SOYBN
IPNSISI	45,58	742,4225	743,4329	10,82	P09186|LOX3_SOYBN
IPSEVLA	34,27	727,4116	728,4174	9,88	P02858|GLYG4_SOYBN
IPSEVLS	28,03	743,4065	744,417	9,39	P04347|GLYG5_SOYBN
ISSEDKPFNLRS	76,78	1391,7045	696,8602	9,34	P13916|GLCA_SOYBN
KGVITQ	27,54	644,3857	645,4028	5,99	P22895|P34_SOYBN
KHFLA	28,83	614,354	615,3632	7,42	P04347|GLYG5_SOYBN
KPLVVQ	35,24	682,4377	683,4519	7,62	P01071|ITRB_SOYBN
LSKEQIRQ	20,67	1000,5665	501,295	6,39	P25974|GLCB_SOYBN
NKNPFLFG	46,32	935,4865	936,4965	12,2	P13916|GLCA_SOYBN
NQLDQ	27,18	616,2816	617,2889	4,12	P04347|GLYG5_SOYBN
NSGPLVNP	77,57	796,4079	797,4174	8,9	P04347|GLYG5_SOYBN
NSLAR	26,45	559,3078	560,3116	8,41	P38417|LOX4_SOYBN
RDPIYS	42,07	749,3708	750,3882	7.52, 7.57	P13916|GLCA_SOYBN
RNPIYS	25,62	748,3868	749,3975	7,67	P25974|GLCB_SOYBN
RPSYT	40,32	622,3074	623,32	4.56, 4.61	P04405|GLYG2_SOYBN
RPVLG	21,56	540,3384	541,35	7,37	P09186|LOX3_SOYBN
VEGGLS	20,98	560,2806	561,2922	6,34	P04347|GLYG5_SOYBN
YNLRQ	22,07	692,3605	693,3702	7,17	P02858|GLYG4_SOYBN
3IF 23 peptides					
AGVVPPAR	22,22	765,4497	766,4638	7,28	P29531|OLEO2_SOYBN
ANSLLN	25,42	630,3337	631,3508	8,51	P04776|GLYG1_SOYBN
APAMR	31,54	544,2791	545,2882	2.85, 2.9, 2.95	P04405|GLYG2_SOYBN
DITAFG	22,99	622,2962	623,308	10,04	P01071|ITRB_SOYBN
EAPRY	24,5	634,3074	635,3214	6,1	P29531|OLEO2_SOYBN
EGEDK	23,34	576,2391	577,2753	7,04	P04776|GLYG1_SOYBN
GIENFRL	23,03	847,4552	848,463	11,52	Q04672|SBP_SOYBN
IPNSISI	43,54	742,4225	743,4355	10.73, 10.88	P09186|LOX3_SOYBN
IPSEVLA	34,81	727,4116	728,4221	9.84, 9.89	P02858|GLYG4_SOYBN
IPSEVLS	36,38	743,4065	744,4166	9,35	P04347|GLYG5_SOYBN
KGAIG	22,21	444,2696	445,2794	2,55	P04347|GLYG5_SOYBN
KGALG	22,21	444,2696	445,2794	2,55	P02858|GLYG4_SOYBN
KGVITQ	30,12	644,3857	645,4016	5,75	P22895|P34_SOYBN
KHFLA	32,27	614,354	615,3674	7.33, 7.38	P04347|GLYG5_SOYBN
KNKPLVVQ	47,57	924,5756	925,5882	7,09	P01071|ITRB_SOYBN
NPFLFG	24,12	693,3486	694,3553	13,05	P13916|GLCA_SOYBN
NSGPLVNP	71,12	796,4079	797,4171	8,91	P04347|GLYG5_SOYBN
RDPIYS	33,1	749,3708	750,3822	7,43	P13916|GLCA_SOYBN

RNPIYS	25,72	748,3868	749,3976	7,58	P25974|GLCB_SOYBN
RPSYT	37,5	622,3074	623,3181	4.03, 4.08, 4.18	P04776|GLYG1_SOYBN
TGNLLG	23,32	573,3122	574,3218	8,96	P22895|P34_SOYBN
VAISR	28,28	544,3333	545,3372	6,15	C6T1G0|CSPL1_SOYBN
VALSR	28,28	544,3333	545,3372	6,15	P04776|GLYG1_SOYBN
4IF 57 peptides					
AGVVPPAR	46,81	765,4497	766,4635	7,24	P29531|OLEO2_SOYBN
AGVVPPARF	18,01	912,5181	913,53	10	P29531|OLEO2_SOYBN
ALPEEVIQHTFNLK	73,39	1637,8777	819,9507	12,61	P04405|GLYG2_SOYBN
ANSLLN	28,56	630,3337	631,352	8,47	P04405|GLYG2_SOYBN
APAMR	31,33	544,2791	545,2869	2,9	P04405|GLYG2_SOYBN
APAMRK	17,62	672,3741	673,3467	3,59	P04405|GLYG2_SOYBN
DFYNPKA	63,4	853,397	854,4196	8,71	P04347|GLYG5_SOYBN
DFYNPKAGRI	17,1	1179,6036	590,8082	9,55	P04347|GLYG5_SOYBN
DITAFG	18,53	622,2962	623,3163	10,04	P01070|ITRA_SOYBN
EAPRY	23,64	634,3074	635,3179	6.05, 6.1	P29531|OLEO2_SOYBN
EEGGSV	18,86	576,2391	577,2775	6.94, 6.99	P04347|GLYG5_SOYBN
EQIRQ	23,62	672,3555	673,3666	2.35, 2.4	P25974|GLCB_SOYBN
FAPEFLK	40,97	850,4589	851,4727	10.93, 10.98, 11.03	P04405|GLYG2_SOYBN
FEAPRY	17,67	781,3759	782,3851	9,06	P29531|OLEO2_SOYBN
FGGIRA	14,55	619,3442	620,3546	8,62	P01070|ITRA_SOYBN
FLVPPQE	32,42	828,4381	829,448	10,14	P04405|GLYG2_SOYBN
FPFPRPPHQ	28,46	1121,577	1122,5988	10,29	P13916|GLCA_SOYBN
GANSLL	20,18	573,3122	574,3213	9.75, 9.8	P04405|GLYG2_SOYBN
GANSLLN	22,99	687,3552	688,3773	8,76	P04405|GLYG2_SOYBN
GDLIAVPTG	25,96	841,4545	842,4692	10,64	P04405|GLYG2_SOYBN
GIENFRL	15,47	847,4552	848,4659	11,52	Q04672|SBP_SOYBN
GKGIFG	23,34	577,3224	578,3397	9.45, 9.5	P04776|GLYG1_SOYBN
HSYNLRQ	34,76	916,4515	917,4611	6,6	P02858|GLYG4_SOYBN
HTFNLK	47,18	758,4075	759,4224	7,73	P04405|GLYG2_SOYBN
INAENNQRNFLA	44,94	1402,6953	702,368	9,65	P13916|GLCA_SOYBN
IPSEVLA	30,21	727,4116	728,423	9,9	P02858|GLYG4_SOYBN
IPSEVLS	24,8	743,4065	744,4184	9.35, 9.4	P04347|GLYG5_SOYBN
IVTVK	24,32	558,3741	559,3825	6,25	P04405|GLYG2_SOYBN
KGIFG	20,94	520,3009	521,3137	9,7	P04776|GLYG1_SOYBN
KGLFG	20,94	520,3009	521,3137	9,7	Q02920|NO70_SOYBN
KGVITQ	20,82	644,3857	645,3961	5,71	P22895|P34_SOYBN
KHFLA	27,82	614,354	615,368	7,28	P04347|GLYG5_SOYBN
KNKPLVVQ	49,56	924,5756	925,5909	7,04	P01070|ITRA_SOYBN
KNPFLFG	46,08	821,4435	822,4434	12,21	P13916|GLCA_SOYBN
KPLVVQ	34,74	682,4377	683,4454	7,53	P01070|ITRA_SOYBN
LDFPAL	21,3	674,3639	675,3725	13,05	P04405|GLYG2_SOYBN
LSKEQIRQ	19,99	1000,5665	501,2962	6,3	P25974|GLCB_SOYBN
NKNPFLFG	47,84	935,4865	936,5017	12,16	P13916|GLCA_SOYBN
NPFLFG	33,9	693,3486	694,3557	13, 13.1	P13916|GLCA_SOYBN
NQLDQ	25,06	616,2816	617,2943	3,64	P04405|GLYG2_SOYBN
NSGPLVNP	67	796,4079	797,4295	8.81, 8.86	P04347|GLYG5_SOYBN
NSLLN	20,33	559,2966	560,3123	8,32	P04405|GLYG2_SOYBN
PFLFG	16,8	579,3057	580,315	13,25	P13916|GLCA_SOYBN
QGKGALGV	14,72	728,4181	729,3614	6,89	P02858|GLYG4_SOYBN
QPQQR	19,89	655,3401	656,3431	6,5	P04776|GLYG1_SOYBN
RDPIYS	37,37	749,3708	750,3871	7.38, 7.43	P13916|GLCA_SOYBN
RPSYT	41,19	622,3074	623,3163	4.03, 4.08, 4.13	P04405|GLYG2_SOYBN
SNLNFFA	26,79	811,3864	812,3961	12,31	P13916|GLCA_SOYBN
SREWRS	22,54	819,3987	820,4049	5.02, 5.12	P02858|GLYG4_SOYBN
TPVVA	24,66	485,2849	486,298	7,09	P04405|GLYG2_SOYBN
TVTATTATA	50,42	835,4287	836,4424	6,64	P29531|OLEO2_SOYBN
VKGGLS	18,25	559,3329	560,3455	3,05	P04776|GLYG1_SOYBN
VNMQIVR	20,88	858,4745	859,4823	9,11	P04405|GLYG2_SOYBN
VPQFLFS	20,29	836,4432	837,4514	12,9	P13917|7SB1_SOYBN
VPTGV	19,9	471,2693	472,2908	4.53, 4.57	P04405|GLYG2_SOYBN
VQVHT	16,03	582,3126	583,3232	5.22, 5.31	P29531|OLEO2_SOYBN
YSLGA	15,59	509,2485	510,2624	7,58	Q2PMT9|PSBC_SOYBN
YSLGA		509,2485	510,2624	7,58	Q2PMT9|PSBC_SOYBN
5IF 48 peptides					
AGVVPPAR	53,16	765,4497	766,4645	7.17, 7.22	P29531|OLEO2_SOYBN
AIVTVK	41,34	629,4112	630,4255	7,67	P04405|GLYG2_SOYBN
ANSII	22,05	516,2908	517,3065	9,59	P04405|GLYG2_SOYBN
APAMR	35,57	544,2791	545,2895	2.79, 2.84, 2.89	P04405|GLYG2_SOYBN
ATSDLNFFA	50,28	984,4552	985,4573	12,45	P11827|GLCAP_SOYBN
EAPRY	22,09	634,3074	635,3198	6,09	P29531|OLEO2_SOYBN

EQIRA	21,52	615,334	616,3412	5,35	P13916|GLCA_SOYBN
ESFFLS	23,33	728,3381	729,3497	12, 12.05	P13916|GLCA_SOYBN
FAPEFLK	44,38	850,4589	851,4774	10.97, 11.02	P04405|GLYG2_SOYBN
FGGIRA	24,89	619,3442	620,3546	8,6	P01070|ITRA_SOYBN
GANSLL	21,25	573,3122	574,3224	9,78	P04405|GLYG2_SOYBN
GANSLLN	31,4	687,3552	688,3698	8,75	P04405|GLYG2_SOYBN
GKGIFG	27,57	577,3224	578,3353	9.44, 9.49	P04776|GLYG1_SOYBN
GLRVT	20,23	544,3333	545,3429	6,78	P04405|GLYG2_SOYBN
HSYNLRQ	51,07	916,4515	917,4591	6,58	P02858|GLYG4_SOYBN
IPNSISI	41,22	742,4225	743,4412	10,72	P09439|LOX2_SOYBN
IPSEVLS	33,25	743,4065	744,416	9,29	P04347|GLYG5_SOYBN
KGIFG	26,59	520,3009	521,313	9.69, 9.74	P04776|GLYG1_SOYBN
KGLFG	26,59	520,3009	521,313	9.69, 9.74	Q02920|NO70_SOYBN
KGVITQ	27,49	644,3857	645,4	5.7, 5.74	P22895|P34_SOYBN
KHFLA	30,91	614,354	615,3643	7,27	P04347|GLYG5_SOYBN
KNKPLVVQ	52,22	924,5756	925,5923	7,03	P01070|ITRA_SOYBN
KNPFLFG	52,83	821,4435	822,4519	12,25	P13916|GLCA_SOYBN
KPLVVQ	43,46	682,4377	683,4452	7,52	P01070|ITRA_SOYBN
LPALRQ	26,91	696,4282	697,4458	8,41	P04347|GLYG5_SOYBN
LSKEQIRQ	22,71	1000,5665	501,2937	6,24	P25974|GLCB_SOYBN
NKNPFLFG	39,81	935,4865	936,4974	12,2	P13916|GLCA_SOYBN
NPFLFG	37,78	693,3486	694,3577	13,04	P13916|GLCA_SOYBN
NQLDQ	28,51	616,2816	617,2924	3,58	P04405|GLYG2_SOYBN
NSGPLVNP	75,36	796,4079	797,4288	8,8	P04347|GLYG5_SOYBN
NSLLN	28,4	559,2966	560,3035	8,31	P04405|GLYG2_SOYBN
QELRK	20,92	672,3918	673,3674	2.29, 2.34	Q1W376|PMM_SOYBN
RDPIY	25,02	662,3387	663,3578	7,91	P13916|GLCA_SOYBN
RDPIYS	43,93	749,3708	750,3839	7.37, 7.42, 7.47	P13916|GLCA_SOYBN
RPSYT	45,5	622,3074	623,319	3.97, 4.02, 4.12	P04405|GLYG2_SOYBN
SGDAIR	44,78	617,3132	618,3238	2.98, 3.03	P07135|RR7_SOYBN
SGDALR	44,78	617,3132	618,3238	2.98, 3.03	P13916|GLCA_SOYBN
SNLNFFA	53,27	811,3864	812,3928	12,3	P13916|GLCA_SOYBN
SNRFETLFK	37,09	1140,5928	571,3098	10,77	P13916|GLCA_SOYBN
TPVVA	21,85	485,2849	486,2963	7,07	P04405|GLYG2_SOYBN
VEICT	23,19	563,2625	564,2834	10,13	P09439|LOX2_SOYBN
VNMQIVR	36,09	858,4745	859,4857	9,05	P04405|GLYG2_SOYBN
VPPQE	21,38	568,2856	569,2798	3,67	P04405|GLYG2_SOYBN
VPTGV	20,6	471,2693	472,291	4.56, 4.61	P04405|GLYG2_SOYBN
VQVHT	21,86	582,3126	583,3224	5.2, 5.25	P29531|OLEO2_SOYBN
VVPPAR	43,68	637,3911	638,4017	5,89	P29531|OLEO2_SOYBN
WNPNNKPFQ	64,18	1143,5461	1144,5513	9.39, 9.34	P04405|GLYG2_SOYBN
WWMYNNEDTPVVA	27,25	1623,7028	812,8536	13,19	P04405|GLYG2_SOYBN
Peptides in fractions 3-5 kDa GI					
1IF42 peptides					
AGVTVS	24,66	532,2856	533,2927	6,25	P04347|GLYG5_SOYBN
AGVVPPA	37,62	609,3486	610,3592	8,17	P29531|OLEO2_SOYBN
ANSII	24,08	516,2908	517,2986	9,6	P04776|GLYG1_SOYBN
AVVAGLP	19,41	625,3799	626,3883	9,65	P29531|OLEO2_SOYBN
DFYNPK	60,42	782,3599	783,3716	8,32	P04347|GLYG5_SOYBN
EEGGSV	25,55	576,2391	577,2758	6,99	P04347|GLYG5_SOYBN
FEAPR	41,37	618,3125	619,323	7,14	P29531|OLEO2_SOYBN
GGLIE	21,97	487,2642	488,2733	8,56	P04347|GLYG5_SOYBN
GKGIFG	23,68	577,3224	578,3324	9,5	P04776|GLYG1_SOYBN
GNDTFPYPR	35,64	1065,4879	1066,4934	9,55	P09186|LOX3_SOYBN
HENIARPS	41,87	922,462	923,4683	5,71	P04347|GLYG5_SOYBN
IPSEV	34,34	543,2904	544,2982	8,27	P04347|GLYG5_SOYBN
IPSEVL	32,9	656,3745	657,3767	10,24	P04347|GLYG5_SOYBN
IPSEVLS	36,53	743,4065	744,4142	9,35	P04347|GLYG5_SOYBN
IPSQV	26,61	542,3064	543,318	7,88	P13916|GLCA_SOYBN
ISSEDKPF	43,16	921,4443	922,4562	8,71	P13916|GLCA_SOYBN
KGAIG	27,51	444,2696	445,2782	2,55	P04347|GLYG5_SOYBN
KGALG	27,51	444,2696	445,2782	2,55	P02858|GLYG4_SOYBN
KGIFG	19,64	520,3009	521,3122	9,75	P04776|GLYG1_SOYBN
KGLFG	19,64	520,3009	521,3122	9,75	Q02920|NO70_SOYBN
KGVIT	19,96	516,3271	517,3308	4,57	P22895|P34_SOYBN
KGVITQ	33,55	644,3857	645,3919	5,66	P22895|P34_SOYBN
NIARPS	28,37	656,3605	657,3711	5,56	P04347|GLYG5_SOYBN
NQLDQ	32,51	616,2816	617,2924	3,44	P04347|GLYG5_SOYBN
NQLDQMPR	45,83	1000,476	1001,4887	8,42	P04776|GLYG1_SOYBN
NSGPLVNP	92,61	796,4079	797,4164	8,81	P04347|GLYG5_SOYBN
NSYNLG	23,17	666,2973	667,306	8,12	P04347|GLYG5_SOYBN

NTGNLLG	27,41	687,3552	688,3624	9,11	P22895|P34_SOYBN
QELVQ	23,76	615,3228	616,3422	5,21	P10538|AMYB_SOYBN
RDPIYS	33,05	749,3708	750,3795	7,38	P13916|GLCA_SOYBN
RPSYT	47,13	622,3074	623,3176	3,88	P04776|GLYG1_SOYBN
SGDAI	26,54	461,2122	462,2233	5,9	P07135|RR7_SOYBN
SGDAL	26,54	461,2122	462,2233	5,9	P13916|GLCA_SOYBN
SSEDKPF	40,67	808,3602	809,3687	9,01	P13916|GLCA_SOYBN
SSSIA	20,76	463,2278	464,2499	9,8	Q2PMQ9|PSBB_SOYBN
SSSLA	20,76	463,2278	464,2499	9,8	P09755|CB22_SOYBN
TGSGMGTLL	31,41	835,4109	836,4244	11,03	P28551|TBB3_SOYBN
TISSEDKP	36,04	875,4236	876,4266	6,3	P13916|GLCA_SOYBN
TISSEDKPF	54,35	1022,492	1023,5031	8,96	P13916|GLCA_SOYBN
VEGGLS	28,1	560,2806	561,2917	6,05	P04347|GLYG5_SOYBN
VQVHT	23,51	582,3126	583,3184	5,02	P29531|OLEO2_SOYBN
YNLRQ	31,71	692,3605	693,3647	7,04	P02858|GLYG4_SOYBN
2IF 36 peptides					
AGVTVS	23,04	532,2856	533,2955	6,29	P04347|GLYG5_SOYBN
AGVVPPA	52,88	609,3486	610,3576	8,11	P29531|OLEO2_SOYBN
AGVVPPGA	53,58	666,3701	667,3796	8,41	P29530|OLEO1_SOYBN
AIVTV	25,28	501,3162	502,3236	9,59	P04776|GLYG1_SOYBN
DFYNPK	58,17	782,3599	783,3716	8,31	P04347|GLYG5_SOYBN
DFYNPKA	53,47	853,397	854,4159	8,75	P04347|GLYG5_SOYBN
EEGGSV	20,91	576,2391	577,274	6,98	P04347|GLYG5_SOYBN
EFPPR	22,25	644,3282	645,34	7,57	P09186|LOX3_SOYBN
FEAPR	26,76	618,3125	619,3209	7,18	P29531|OLEO2_SOYBN
FLVPPQE	36,25	828,4381	829,4492	10,18	P04776|GLYG1_SOYBN
GGLIE	28,2	487,2642	488,2757	8,6	P04347|GLYG5_SOYBN
GKGIFG	24,19	577,3224	578,3302	9,49	P04776|GLYG1_SOYBN
IKNNNPF	36,33	845,4395	846,4503	8,65	P04776|GLYG1_SOYBN
IPSEVLS	31,58	743,4065	744,4177	9,34	P04347|GLYG5_SOYBN
IYIQQGK	45,34	848,4756	849,4827	7,42	P04776|GLYG1_SOYBN
KGIFG	21,41	520,3009	521,3115	9,74	P04776|GLYG1_SOYBN
KGLFG	21,41	520,3009	521,3115	9,74	Q02920|NO70_SOYBN
KGVITQ	21,51	644,3857	645,3984	5,7	P22895|P34_SOYBN
KNKNPF	28,4	746,4075	747,4099	6,68	P13916|GLCA_SOYBN
NIARPS	28,83	656,3605	657,3752	5,55	P04347|GLYG5_SOYBN
NQLDQ	28,55	616,2816	617,291	3,58	P04347|GLYG5_SOYBN
NSGPLVNP	70,43	796,4079	797,4184	8,85	P04347|GLYG5_SOYBN
NSYNLG	28,13	666,2973	667,3091	8,11	P04347|GLYG5_SOYBN
RDPIYS	32,99	749,3708	750,3798	7,47	P13916|GLCA_SOYBN
RPSYT	44,09	622,3074	623,3176	4,12	P04776|GLYG1_SOYBN
SGDAI	22,35	461,2122	462,2237	5,94	P07135|RR7_SOYBN
SGDAL	22,35	461,2122	462,2237	5,94	P13916|GLCA_SOYBN
TISSEDKPF	51,15	1022,492	1023,4997	9	P13916|GLCA_SOYBN
TPVVA	22,54	485,2849	486,2946	7,13	P04776|GLYG1_SOYBN
VISPK	24,98	542,3428	543,3516	3,97	P04347|GLYG5_SOYBN
VKGGLS	22,57	559,3329	560,3414	3,04	P04776|GLYG1_SOYBN
VPPQE	24,18	568,2856	569,2925	3,73	P04776|GLYG1_SOYBN
VQVHT	28,3	582,3126	583,3195	5,2	P29531|OLEO2_SOYBN
YEAGVVPPA	32,04	901,4545	902,4636	9,15	P29531|OLEO2_SOYBN
YFVDAQPK	51,44	966,481	967,4904	8,8	P13916|GLCA_SOYBN
YQGNSGPL	76,62	834,3871	835,3953	8,56	P04347|GLYG5_SOYBN
3IF 55 peptides					
AGVVPPA	30,7	609,3486	610,3563	8,17	P29531|OLEO2_SOYBN
AGVVPPGA	45,34	666,3701	667,3817	8,4	P29530|OLEO1_SOYBN
ANSLLN	33,98	630,3337	631,3441	8,5	P04776|GLYG1_SOYBN
DFYNPK	53,15	782,3599	783,3769	8,32	P04347|GLYG5_SOYBN
DQMPR	32,47	645,2904	646,3019	4,47	P04776|GLYG1_SOYBN
EEINK	28,75	631,3177	632,3287	1,86	P13916|GLCA_SOYBN
EFPPR	30,76	644,3282	645,3376	7,53	P09186|LOX3_SOYBN
EGEDK	24,19	576,2391	577,2783	7,03	P04776|GLYG1_SOYBN
FEAPR	32,28	618,3125	619,3256	7,13	P29531|OLEO2_SOYBN
FPGSAQ	35,66	605,2809	606,2933	7,04	P13916|GLCA_SOYBN
FSHNILE	28,26	858,4235	859,4295	9,69	P25974|GLCB_SOYBN
FVDAQPK	40,39	803,4177	804,426	6,89	P13916|GLCA_SOYBN
GINAENNQ	46,18	858,3832	859,392	4,13	P13916|GLCA_SOYBN
GKGIFG	25,45	577,3224	578,3347	9,49	P04776|GLYG1_SOYBN
GNDTFPYPR	43,39	1065,4879	1066,4904	9,55	P09186|LOX3_SOYBN
IKNNNPF	36,73	845,4395	846,449	8,61	P04776|GLYG1_SOYBN
INAENNQ	40,71	801,3617	802,3724	2,5	P13916|GLCA_SOYBN
IPSEVLS	28,61	743,4065	744,4156	9,34	P04347|GLYG5_SOYBN

IPSQV	24,68	542,3064	543,3222	7,87	P13916|GLCA_SOYBN
IPVNKPG	47,09	723,4279	724,4376	6,79	P13916|GLCA_SOYBN
ISSEDKPFN	56,43	1035,4873	1036,4995	8,07	P13916|GLCA_SOYBN
IYIQQGK	45,65	848,4756	849,4837	7,38	P04776|GLYG1_SOYBN
KGIFG	26,43	520,3009	521,3102	9,78	P04776|GLYG1_SOYBN
KGLFG	26,43	520,3009	521,3102	9,78	Q02920|NO70_SOYBN
KNILE	26,77	615,3591	616,3669	7,96	P11827|GLCAP_SOYBN
KNKNPF	31,01	746,4075	747,4158	6,68	P11827|GLCAP_SOYBN
KYEGNWGPL	38,46	1062,5134	1063,5253	11,27	P02858|GLYG4_SOYBN
LAFPGSA	32,39	661,3435	662,3517	10,29	P13916|GLCA_SOYBN
LAFPGSAQ	44,31	789,4021	790,4161	9,99	P13916|GLCA_SOYBN
LVPPQE	26,34	681,3697	682,3839	7,77	P04776|GLYG1_SOYBN
LVPPQES	31,17	768,4017	769,4106	7,48	P04776|GLYG1_SOYBN
NALKPDN	39,58	770,3922	771,4019	4,08	P04776|GLYG1_SOYBN
NALPEE	28,95	671,3126	672,3165	7,73	P04776|GLYG1_SOYBN
NEGNPLEN	31,92	885,3828	886,4249	8,46	P01070|ITRA_SOYBN
NIARPS	26,76	656,3605	657,3763	5,41	P04347|GLYG5_SOYBN
NNQLDQNPR	47,91	1097,5214	1098,5239	6,25	P04347|GLYG5_SOYBN
NQYGHV	48,98	716,3242	717,335	6,29	P11827|GLCAP_SOYBN
NSGPL	38,63	486,2438	487,2521	7,28	P04347|GLYG5_SOYBN
NSGPLVNP	55,72	796,4079	797,4196	8,86	P04347|GLYG5_SOYBN
NSLENQ	35,79	703,3137	704,3228	3,83	P04776|GLYG1_SOYBN
RDPIYS	32,36	749,3708	750,3779	7,43	P13916|GLCA_SOYBN
RPSYT	39,5	622,3074	623,3188	3,93	P04776|GLYG1_SOYBN
SGDAI	25,88	461,2122	462,2223	5,94	P07135|RR7_SOYBN
SGDAL	25,88	461,2122	462,2223	5,94	P11827|GLCAP_SOYBN
SNFNNQ	29,4	722,2983	723,3099	3,54	P04347|GLYG5_SOYBN
SPYPR	35,93	618,3125	619,3239	5,79	P02858|GLYG4_SOYBN
SYFVDAQPK	41,94	1053,5131	1054,5291	9,2	P13916|GLCA_SOYBN
TISSEDKP	46,2	875,4236	876,4354	6,2	P13916|GLCA_SOYBN
TISSEDKPF	45,27	1022,492	1023,4995	9,01	P13916|GLCA_SOYBN
VIQHT	23,98	596,3282	597,338	1,96	P04776|GLYG1_SOYBN
VKNNNPF	40,28	831,4239	832,4362	8,22	P04405|GLYG2_SOYBN
VPPQE	23,74	568,2856	569,2881	3,82	P04776|GLYG1_SOYBN
YEGNWGPL	44,78	934,4185	935,4291	11,91	P02858|GLYG4_SOYBN
YFVDAQPK	46,65	966,481	967,498	8,81	P13916|GLCA_SOYBN
YPVVV	24,26	575,3319	576,3379	10,33	P13916|GLCA_SOYBN
4IF 49 peptides					
VNPESQQGSPR	29,93	1197,5737	599,7953	5,9	P02858|GLYG4_SOYBN
YVVNPDNNEN	53,17	1176,5048	1177,5144	7,83	P13916|GLCA_SOYBN
FPFPRPPHQ	47,47	1121,577	561,798	10,39	P13916|GLCA_SOYBN
GNDTFPYPR	31,96	1065,4879	1066,4988	9,55	P09186|LOX3_SOYBN
LVPPQESQK	22,28	1024,5553	1025,5586	7,09	P04776|GLYG1_SOYBN
TISSEDKPF	42,73	1022,492	1023,5074	9,01	P13916|GLCA_SOYBN
YFVDAQPK	47,9	966,481	967,4903	8,81	P13916|GLCA_SOYBN
NEGNPLEN	30,09	885,3828	886,4201	8,47	P01070|ITRA_SOYBN
TISSEDKP	45,14	875,4236	876,4338	6,35	P13916|GLCA_SOYBN
IYIQQGK	48,48	848,4756	849,4839	7,38	P04776|GLYG1_SOYBN
IKNNNPF	40,29	845,4395	846,4507	8,61	P04776|GLYG1_SOYBN
IYIQQGN	52,24	834,4236	835,4357	7,92	P04405|GLYG2_SOYBN
GSKDNVIS	29,96	818,4134	819,4221	5,95	P11827|GLCAP_SOYBN
FVDAQPK	43,82	803,4177	804,431	6,94	P13916|GLCA_SOYBN
NSGPLVNP	47,29	796,4079	797,4172	8,91	P04347|GLYG5_SOYBN
LAFPGSAQ	41,29	789,4021	790,4109	9,99	P13916|GLCA_SOYBN
DFYNPK	55,92	782,3599	783,3776	8,32	P04347|GLYG5_SOYBN
LVPPQES	32,27	768,4017	769,4158	7,68	P04776|GLYG1_SOYBN
IPVNKPG	50,87	723,4279	724,4395	6,84	P13916|GLCA_SOYBN
SNFNNQ	23,42	722,2983	723,3073	3,64	P04347|GLYG5_SOYBN
NQYGHV	41,88	716,3242	717,3325	6,3	P11827|GLCAP_SOYBN
AGVVPPGA	37,73	666,3701	667,3853	8,42	P29530|OLEO1_SOYBN
NIARPS	26,03	656,3605	657,3737	5,61	P04347|GLYG5_SOYBN
VDAQPK	28,54	656,3493	657,359	1,81	P13916|GLCA_SOYBN
VSIIDT	25,35	646,3538	647,3629	9,6	P04776|GLYG1_SOYBN
VSLIDT	25,35	646,3538	647,3629	9,6	P11828|GLYG3_SOYBN
DQMPR	35,89	645,2904	646,3024	4,77	P04776|GLYG1_SOYBN
DAKVEA	25,81	631,3177	632,3287	3,34	P29531|OLEO2_SOYBN
AVVAGLP	29,47	625,3799	626,3861	9,8	P29531|OLEO2_SOYBN
RPSYT	44,22	622,3074	623,3185	4,18	P04776|GLYG1_SOYBN
NKNPF	24,45	618,3126	619,3234	7,63	P13916|GLCA_SOYBN
AGVVPPA	38,24	609,3486	610,3585	8,12	P29531|OLEO2_SOYBN
NNNPF	23,22	604,2605	605,2722	8,66	P04776|GLYG1_SOYBN

VIQHT	22,93	596,3282	597,3395	2,06	P04776|GLYG1_SOYBN
SAEFGS	22,46	596,2442	597,2564	6,74	P04776|GLYG1_SOYBN
FSVDK	32,51	594,3013	595,3083	7,14	P04776|GLYG1_SOYBN
IAVPTG	21,85	556,322	557,3339	8,52	P04776|GLYG1_SOYBN
IPSEV	25,73	543,2904	544,3007	8,27	P04347|GLYG5_SOYBN
IPSQV	24,22	542,3064	543,3145	7,87	P13916|GLCA_SOYBN
KGIFG	22,02	520,3009	521,3108	9,7	P04776|GLYG1_SOYBN
KGLFG	22,02	520,3009	521,3108	9,7	Q02920|NO70_SOYBN
LPVGG	21,53	441,2587	442,2686	6,1	P29531|OLEO2_SOYBN
YEGNWGPL	40,54	934,4185	935,4261	11,9	P02858|GLYG4_SOYBN
FLVPPQES	41,84	915,4702	916,4792	9,98	P04776|GLYG1_SOYBN
YEAGVVPPA	34,02	901,4545	902,4626	9,14	P29531|OLEO2_SOYBN
INAENNQ	40,01	801,3617	802,3705	2,59	P13916|GLCA_SOYBN
KNKPLV	27,01	697,4486	698,4574	5,35	P01070|ITRA_SOYBN
FEAPR	32,73	618,3125	619,3198	7,17	P29531|OLEO2_SOYBN
FPGSAQ	36,69	605,2809	606,2891	7,07	P13916|GLCA_SOYBN
5IF 81 peptides					
AGVVPPA	34,56	609,3486	610,3604	8,11	P29531|OLEO2_SOYBN
AGVVPPGA	35,72	666,3701	667,3858	8,36	P29530|OLEO1_SOYBN
ALPEE	18,82	557,2697	558,2831	7,27	P04776|GLYG1_SOYBN
DFYNPK	53,26	782,3599	783,3779	8,31	P02858|GLYG4_SOYBN
DFYNPKA	47,14	853,397	854,4146	8,7	P02858|GLYG4_SOYBN
DQMPR	32,44	645,2904	646,2988	4,37	P04776|GLYG1_SOYBN
DQTPR	25,33	615,2976	616,3099	1,41	P02858|GLYG4_SOYBN
EFPPR	20,45	644,3282	645,3361	7,52	P09186|LOX3_SOYBN
FAFGIN	27,12	667,3329	668,3448	11,81	P11827|GLCAP_SOYBN
FAIGIN	22,78	633,3486	634,3582	10,92	P13916|GLCA_SOYBN
FEAPR	34,67	618,3125	619,3263	7,12	P29531|OLEO2_SOYBN
FLVPPQE	30,72	828,4381	829,4523	10,13	P04776|GLYG1_SOYBN
FPFPRPPHQ	36,75	1121,577	561,8013	10,38	P13916|GLCA_SOYBN
FPGSAQ	36,02	605,2809	606,2958	7,03	P13916|GLCA_SOYBN
FSREEGQQQGEQ	25,55	1421,6171	711,819	6,04	P13916|GLCA_SOYBN
FSVDK	28,3	594,3013	595,3105	7,08	P04776|GLYG1_SOYBN
FVDAQPK	38,64	803,4177	804,4278	6,93	P13916|GLCA_SOYBN
FYNPK	30,27	667,3329	668,3412	7,17	P02858|GLYG4_SOYBN
GFAPEF	31,48	666,3013	667,3072	11,42	P04405|GLYG2_SOYBN
GKGIFG	19,23	577,3224	578,3347	9,44	P04776|GLYG1_SOYBN
GNDTFPYPR	25,63	1065,4879	1066,5049	9,49	P09186|LOX3_SOYBN
GSKDNVIS	32,03	818,4134	819,4265	5,84	P11827|GLCAP_SOYBN
HENIARPS	47,75	922,462	923,4722	5,65	P02858|GLYG4_SOYBN
IAVPTG	19,83	556,322	557,3309	8,45	P04776|GLYG1_SOYBN
IETWNPNNKPF	34,34	1358,6619	1359,6689	10,87	P04405|GLYG2_SOYBN
IIDTN	19,04	574,2963	575,3049	5,6	P04776|GLYG1_SOYBN
IKNNNPF	37,61	845,4395	846,451	8,55	P04776|GLYG1_SOYBN
INAENNQ	44,85	801,3617	802,3713	2,5	P13916|GLCA_SOYBN
IPSEV	24,18	543,2904	544,304	8,21	P02858|GLYG4_SOYBN
IPSEVLS	28,79	743,4065	744,4149	9,29	P04347|GLYG5_SOYBN
IPSQV	24,92	542,3064	543,3177	7,81	P13916|GLCA_SOYBN
IPVNKPG	48,54	723,4279	724,4414	6,78	P13916|GLCA_SOYBN
ISSEDKPFN	52,63	1035,4873	1036,5126	8,07	P13916|GLCA_SOYBN
IYIQQG	31,33	720,3806	721,3995	8,21	P04405|GLYG2_SOYBN
IYIQQGK	45,18	848,4756	849,491	7,38	P04776|GLYG1_SOYBN
KGIFG	20,19	520,3009	521,3121	9,69	P04776|GLYG1_SOYBN
KGLFG	20,19	520,3009	521,3121	9,69	Q02920|NO70_SOYBN
KGVITQ	29,34	644,3857	645,3964	5,6	P22895|P34_SOYBN
KNILE	20,92	615,3591	616,3695	7,91	P11827|GLCAP_SOYBN
KYEGNWGPL	50,94	1062,5134	1063,522	11,22	P02858|GLYG4_SOYBN
LAFPGSA	29,18	661,3435	662,3541	10,28	P13916|GLCA_SOYBN
LAFPGSAQ	43,31	789,4021	790,4106	9,98	P13916|GLCA_SOYBN
LAIPV	20,03	511,337	512,3463	11,21	P13916|GLCA_SOYBN
LIDTN	19,04	574,2963	575,3049	5,6	P11828|GLYG3_SOYBN
LVPPQE	23,65	681,3697	682,3755	7,96	P04776|GLYG1_SOYBN
LVPPQES	29,69	768,4017	769,4173	7,47	P04776|GLYG1_SOYBN
LVPPQESQ	41,68	896,4603	897,4763	7,42	P04776|GLYG1_SOYBN
NALPEE	32,4	671,3126	672,3273	7,67	P04776|GLYG1_SOYBN
NEDTPV	31,9	673,2919	674,3049	6,49	P04405|GLYG2_SOYBN
NIARPS	27,79	656,3605	657,3704	5,3	P02858|GLYG4_SOYBN
NKNPF	24,37	618,3126	619,3263	7,57	P13916|GLCA_SOYBN
NQYGHV	39,24	716,3242	717,3304	6,19	P11827|GLCAP_SOYBN
NSGPLVNP	50,98	796,4079	797,4281	8,8	P04347|GLYG5_SOYBN
NSLENQ	35,7	703,3137	704,325	3,83	P04405|GLYG2_SOYBN

RPSYT	38,31	622,3074	623,3173	3,77	P04776|GLYG1_SOYBN
RPSYTNGPQE	20,97	1147,5258	1148,5287	6,88	P04776|GLYG1_SOYBN
SAEFGSL	28,34	709,3282	710,3406	10,48	P04776|GLYG1_SOYBN
SDNFE	22,44	610,2234	611,2339	6,34	P04776|GLYG1_SOYBN
SNFNNQ	28,03	722,2983	723,3092	3,43	P02858|GLYG4_SOYBN
SNRFET	21,07	752,3453	753,3544	6,69	P13916|GLCA_SOYBN
SPQLQ	20,29	571,2966	572,3091	5,94	P13916|GLCA_SOYBN
SVISPK	31,58	629,3748	630,3877	6,44	P02858|GLYG4_SOYBN
SYFVDAQPK	38,06	1053,5131	1054,5229	9,24	P13916|GLCA_SOYBN
SYPTKEE	32,66	852,3865	853,3986	5,36	P22895|P34_SOYBN
SYPTKEESET	32,72	1169,5088	1170,5166	6,34	P22895|P34_SOYBN
TISSEDKP	45,56	875,4236	876,4355	6,29	P13916|GLCA_SOYBN
TISSEDKPF	50,42	1022,492	1023,4943	9	P13916|GLCA_SOYBN
VIQHT	23,85	596,3282	597,3345	2	P04405|GLYG2_SOYBN
VISPK	22,23	542,3428	543,35	3,78	P02858|GLYG4_SOYBN
VKNNNPFS	46,74	918,4559	919,4784	8,26	P04405|GLYG2_SOYBN
VNPESQQGSPR	43,14	1197,5737	599,7999	5,85	P02858|GLYG4_SOYBN
VPYWT	26,79	664,322	665,3289	10,53	P02858|GLYG4_SOYBN
VQVHT	20,19	582,3126	583,3215	5,1	P29531|OLEO2_SOYBN
VSIIDT	23,38	646,3538	647,365	9,54	P04776|GLYG1_SOYBN
VSIIDTNS	60,6	847,4287	848,4372	9,15	P04405|GLYG2_SOYBN
VSLIDT	23,38	646,3538	647,365	9,54	P11828|GLYG3_SOYBN
VSLIDTNS	60,6	847,4287	848,4372	9,15	P11828|GLYG3_SOYBN
YEGNWGPL	47,75	934,4185	935,4261	11,9	P02858|GLYG4_SOYBN
YFVDAQPK	47,99	966,481	967,5004	8,75	P13916|GLCA_SOYBN
YPVVV	23,54	575,3319	576,3419	10,33	P13916|GLCA_SOYBN
YVVNPDNDEN	52,2	1177,4888	1178,5088	7,92	P11827|GLCAP_SOYBN
Peptides in fractions <3 kDa GI					
1IF 36 peptides					
AGVTVS	23,86	532,2856	533,2923	6,24	P04347|GLYG5_SOYBN
AGVVPPA	54,49	609,3486	610,3606	8,12	P29531|OLEO2_SOYBN
AGVVPPGA	57,2	666,3701	667,3766	8,42	P29530|OLEO1_SOYBN
AIVTV	24,81	501,3162	502,3229	9,59	P04776|GLYG1_SOYBN
ANSLLN	26,93	630,3337	631,3411	8,46	P04776|GLYG1_SOYBN
ATPTP	24,86	485,2485	486,2166	1,46	Q42783|BCCP_SOYBN
AVVAGLP	27,51	625,3799	626,3879	9,7	P29531|OLEO2_SOYBN
DFYNPK	82,57	782,3599	783,3722	8,32	P04347|GLYG5_SOYBN
EEGGSV	27,2	576,2391	577,2692	6,99	P04347|GLYG5_SOYBN
FEAPR	51,35	618,3125	619,3233	7,19	P29531|OLEO2_SOYBN
IKNNNPF	36,87	845,4395	846,451	8,66	P04776|GLYG1_SOYBN
IPSEVL	39,85	656,3745	657,3781	10,24	P04347|GLYG5_SOYBN
IPSEVLS	29,04	743,4065	744,4169	9,35	P04347|GLYG5_SOYBN
IPSQV	25,42	542,3064	543,3164	7,92	P13916|GLCA_SOYBN
KGAIG	26,08	444,2696	445,2787	2,51	P04347|GLYG5_SOYBN
KGALG	26,08	444,2696	445,2787	2,51	P02858|GLYG4_SOYBN
KGIFG	22,7	520,3009	521,3112	9,79	P04776|GLYG1_SOYBN
KGLFG	22,7	520,3009	521,3112	9,79	Q02920|NO70_SOYBN
KNKNPF	40,9	746,4075	747,4083	6,64	P13916|GLCA_SOYBN
KSTVP	24,07	530,3064	531,278	5,7	Q96558|UGDH_SOYBN
NQYGHV	45,55	716,3242	717,3338	6,25	P11827|GLCAP_SOYBN
NSGPLVNP	100,12	796,4079	797,4205	8,86	P04347|GLYG5_SOYBN
NTGNLLG	26,44	687,3552	688,3624	9,15	P22895|P34_SOYBN
RDPIYS	24,57	749,3708	750,3795	7,38	P11827|GLCAP_SOYBN
RNPIYS	39,67	748,3868	749,3976	7,57	P25974|GLCB_SOYBN
RPSYT	46,88	622,3074	623,3182	3,98	P04776|GLYG1_SOYBN
SPYPR	30,78	618,3125	619,3204	5,85	P02858|GLYG4_SOYBN
TGSGMGTLL	31,93	835,4109	836,4189	11,03	P28551|TBB3_SOYBN
TISSEDKP	41,75	875,4236	876,4305	6,34	P13916|GLCA_SOYBN
TISSEDKPF	54,4	1022,492	1023,5015	9,05	P13916|GLCA_SOYBN
TVTATT	22,91	592,3068	593,3127	3,43	P29531|OLEO2_SOYBN
VEGGLS	22,36	560,2806	561,2917	6,09	P04347|GLYG5_SOYBN
VISPK	39,39	542,3428	543,3463	3,89	P04347|GLYG5_SOYBN
VQVHT	30,44	582,3126	583,3196	5,12	P29531|OLEO2_SOYBN
VTGVP	23,95	471,2693	472,2873	4,57	Q2PMQ5|CYB6_SOYBN
YFVDAQPK	56,45	966,481	967,4906	8,8	P13916|GLCA_SOYBN
2IF 43 peptides					
AGVTVS	22,33	532,2856	533,2939	6,25	P04347|GLYG5_SOYBN
AGVVPPA	35,95	609,3486	610,3577	8,12	P29531|OLEO2_SOYBN
AGVVPPGA	31,31	666,3701	667,3824	8,41	P29530|OLEO1_SOYBN
AIVTV	27,85	501,3162	502,3226	9,6	P04776|GLYG1_SOYBN
ANSLLN	29,45	630,3337	631,3347	8,51	P04776|GLYG1_SOYBN

DFYNPK	60,82	782,3599	783,3711	8,32	P04347|GLYG5_SOYBN
DQMPR	34,79	645,2904	646,3029	4,57	P04776|GLYG1_SOYBN
EEGGSV	19,11	576,2391	577,2758	6,99	P04347|GLYG5_SOYBN
FAPEF	18,61	609,2798	610,2865	10,98	P04405|GLYG2_SOYBN
FEAPR	35,37	618,3125	619,3215	7,14	P29531|OLEO2_SOYBN
FLVPPQE	38,87	828,4381	829,4459	10,14	P04776|GLYG1_SOYBN
GANSLLN	24,43	687,3552	688,371	8,76	P04776|GLYG1_SOYBN
GKGIFG	22,93	577,3224	578,3334	9,5	P04776|GLYG1_SOYBN
GNDTFPYPR	29,52	1065,4879	1066,5	9,55	P09186|LOX3_SOYBN
IAVPTG	22,62	556,322	557,3289	8,56	P04776|GLYG1_SOYBN
IKNNNPF	45,39	845,4395	846,4513	8,62	P04776|GLYG1_SOYBN
IPSEVLA	23,28	727,4116	728,4222	9,9	P02858|GLYG4_SOYBN
IPSEVLS	35,51	743,4065	744,4176	9,35	P04347|GLYG5_SOYBN
KGAIG	19,02	444,2696	445,2773	2,5	P04347|GLYG5_SOYBN
KGALG	19,02	444,2696	445,2773	2,5	P02858|GLYG4_SOYBN
KGIFG	23,31	520,3009	521,3099	9,75	P04776|GLYG1_SOYBN
KGLFG	23,31	520,3009	521,3099	9,75	Q02920|NO70_SOYBN
KGVITQ	31,86	644,3857	645,3962	5,71	P22895|P34_SOYBN
KHFLA	29,39	614,354	615,3593	7,33	P04347|GLYG5_SOYBN
KNILE	23,14	615,3591	616,3739	7,97	P11827|GLCAP_SOYBN
NIARPS	31,01	656,3605	657,3708	5,56	P04347|GLYG5_SOYBN
NPFLFG	28,96	693,3486	694,3539	13	P13916|GLCA_SOYBN
NQYGHV	31,42	716,3242	717,3309	6,29	P11827|GLCAP_SOYBN
NSGPLVNP	77,18	796,4079	797,4183	8,81	P04347|GLYG5_SOYBN
NSLENQ	22,65	703,3137	704,3232	3,98	P04776|GLYG1_SOYBN
RDPIYS	43,35	749,3708	750,3771	7,43	P13916|GLCA_SOYBN
RDPIYSN	36,52	863,4137	864,424	7,24	P13916|GLCA_SOYBN
RNPIYS	23,56	748,3868	749,3945	7,58	P25974|GLCB_SOYBN
RPSYT	45,9	622,3074	623,3191	4,13	P04776|GLYG1_SOYBN
SGDAI	30,41	461,2122	462,2261	5,91	P07135|RR7_SOYBN
SGDAL	30,41	461,2122	462,2261	5,91	P13916|GLCA_SOYBN
TGNLLG	24,65	573,3122	574,323	8,96	P22895|P34_SOYBN
TISSEDKPF	43,1	1022,492	1023,5007	9,06	P13916|GLCA_SOYBN
TPVVA	23,58	485,2849	486,2935	7,09	P04776|GLYG1_SOYBN
VISPK	26,37	542,3428	543,3471	3,98	P04347|GLYG5_SOYBN
VKGGLS	21,06	559,3329	560,3439	3,05	P04776|GLYG1_SOYBN
WNPNNKPFQ	52,71	1143,5461	1144,556	9,4	P04776|GLYG1_SOYBN
YNLRQ	21,71	692,3605	693,3718	7,04	P02858|GLYG4_SOYBN
3IF 52 peptides					
AGVVPPA	31,35	609,3486	610,3653	8,06	P29531|OLEO2_SOYBN
AGVVPPGA	39,15	666,3701	667,3815	8,41	P29530|OLEO1_SOYBN
AIVTV	27	501,3162	502,3256	9,54	P04776|GLYG1_SOYBN
ANSLLN	35,34	630,3337	631,3446	8,46	P04776|GLYG1_SOYBN
AVVAGLP	20,78	625,3799	626,3907	9,64	P29531|OLEO2_SOYBN
DFYNPK	51,97	782,3599	783,3791	8,26	P02858|GLYG4_SOYBN
DQMPR	34,27	645,2904	646,302	4,31	P04776|GLYG1_SOYBN
EFPPR	27,63	644,3282	645,3406	7,47	P09186|LOX3_SOYBN
FLVPPQE	34,77	828,4381	829,4511	10,08	P04776|GLYG1_SOYBN
FPFPRPPHQ	52,81	1121,577	561,799	10,28	P13916|GLCA_SOYBN
FPGSAQ	39,39	605,2809	606,289	6,98	P13916|GLCA_SOYBN
FVDAQPK	41,97	803,4177	804,4238	6,83	P13916|GLCA_SOYBN
GKGIFG	20,93	577,3224	578,3294	9,5	P04776|GLYG1_SOYBN
GNDTFPYPR	38,67	1065,4879	1066,4996	9,49	P09186|LOX3_SOYBN
IAVPTG	22,12	556,322	557,3354	8,51	P04776|GLYG1_SOYBN
IIIAQGK	39,56	741,4749	742,4865	7,81	P02858|GLYG4_SOYBN
IKNNNPF	41,19	845,4395	846,4506	8,55	P04776|GLYG1_SOYBN
INAENNQ	46,24	801,3617	802,3721	2,39	P13916|GLCA_SOYBN
IPNSI	24,98	542,3064	543,3148	8,85	P08864|NO27_SOYBN
IPSEV	29,78	543,2904	544,2987	8,21	P02858|GLYG4_SOYBN
IPSEVLS	27,91	743,4065	744,4161	9,3	P04347|GLYG5_SOYBN
IPVNKPG	55,15	723,4279	724,4379	6,78	P13916|GLCA_SOYBN
ISSEDKPFN	47,19	1035,4873	1036,5017	8,11	P13916|GLCA_SOYBN
IYIQQGK	45,74	848,4756	849,4885	7,37	P04776|GLYG1_SOYBN
KGVITQ	24,72	644,3857	645,394	5,65	P22895|P34_SOYBN
KNKNPF	22,4	746,4075	747,4132	6,64	P11827|GLCAP_SOYBN
KVARSP	24,2	656,3969	657,3725	5,55	Q10370|HMGYB_SOYBN
LAFPGSA	39,17	661,3435	662,3532	10,23	P13916|GLCA_SOYBN
LAFPGSAQ	49,49	789,4021	790,4092	9,93	P13916|GLCA_SOYBN
LVPPQES	30,48	768,4017	769,4116	7,42	P04776|GLYG1_SOYBN
LVPPQESQ	32,22	896,4603	897,4639	7,52	P04776|GLYG1_SOYBN
NIARPS	29,75	656,3605	657,3717	5,3	P02858|GLYG4_SOYBN

NQLDQ	27,47	616,2816	617,2923	3,58	P04347|GLYG5_SOYBN
NQYGHV	43,34	716,3242	717,3337	6,14	P11827|GLCAP_SOYBN
NSGPLVNP	60,4	796,4079	797,4183	8,85	P04347|GLYG5_SOYBN
QELRK	24,57	672,3918	673,3636	2,3	Q1W376|PMM_SOYBN
QGNGIFG	22,07	691,3289	692,3369	9,99	P04405|GLYG2_SOYBN
RDPIYS	37,71	749,3708	750,3816	7,32	P13916|GLCA_SOYBN
RNPIYS	25,39	748,3868	749,3958	7,52	P25974|GLCB_SOYBN
RPSYT	40,77	622,3074	623,3173	3,77	P04776|GLYG1_SOYBN
SNFNNQ	36,65	722,2983	723,3096	3,43	P02858|GLYG4_SOYBN
SPYPR	32,93	618,3125	619,3242	5,75	P02858|GLYG4_SOYBN
SSEDKPFN	44,13	922,4032	923,4099	7,02	P13916|GLCA_SOYBN
SYFVDAQPK	44,6	1053,5131	1054,5293	9,19	P13916|GLCA_SOYBN
SYPTKEE	35,23	852,3865	853,394	5,2	P22895|P34_SOYBN
TISSEDKP	45,41	875,4236	876,4305	6,19	P13916|GLCA_SOYBN
TISSEDKPF	48,35	1022,492	1023,5038	9	P13916|GLCA_SOYBN
VEGGLS	23,11	560,2806	561,2916	6,05	P04347|GLYG5_SOYBN
VISPK	25,99	542,3428	543,3506	3,93	P04347|GLYG5_SOYBN
VKGGLS	22,33	559,3329	560,3422	3,04	P04776|GLYG1_SOYBN
YEGNWGPL	44,95	934,4185	935,426	11,85	P02858|GLYG4_SOYBN
YFVDAQPK	45,69	966,481	967,4907	8,75	P13916|GLCA_SOYBN
4IF 49 peptides					
AGVVPPA	35,6	609,3486	610,3586	8,12	P29531|OLEO2_SOYBN
AGVVPPGA	35,27	666,3701	667,3798	8,42	P29530|OLEO1_SOYBN
AIPVNKPG	52,17	794,465	795,4733	7,33	P13916|GLCA_SOYBN
DFYNPK	52,26	782,3599	783,3744	8,32	P04347|GLYG5_SOYBN
DQMPR	32,66	645,2904	646,2997	4,72	P04776|GLYG1_SOYBN
EFPPR	22,48	644,3282	645,3389	7,53	P09186|LOX3_SOYBN
FEAPR	33,79	618,3125	619,323	7,19	P29531|OLEO2_SOYBN
FLVPPQE	34,42	828,4381	829,4487	10,14	P04776|GLYG1_SOYBN
FLVPPQES	40,37	915,4702	916,4774	9,99	P04776|GLYG1_SOYBN
FPFPRPPHQ	43,53	1121,577	561,7978	10,34	P13916|GLCA_SOYBN
FPGSAQ	34,43	605,2809	606,2909	7,09	P13916|GLCA_SOYBN
FSVDK	34,26	594,3013	595,3127	7,13	P04776|GLYG1_SOYBN
FVDAQPK	40,68	803,4177	804,4258	6,99	P13916|GLCA_SOYBN
GNDTFPYPR	40,74	1065,4879	1066,4974	9,55	P09186|LOX3_SOYBN
HENIARPS	50,83	922,462	923,4698	5,75	P02858|GLYG4_SOYBN
IKNNNPF	38,38	845,4395	846,4487	8,66	P04776|GLYG1_SOYBN
IPSEV	28,94	543,2904	544,3005	8,22	P02858|GLYG4_SOYBN
IPSQV	27,24	542,3064	543,3162	7,87	P13916|GLCA_SOYBN
IPVNKPG	53,42	723,4279	724,4344	6,79	P13916|GLCA_SOYBN
IYIQQGK	48,81	848,4756	849,4899	7,43	P04776|GLYG1_SOYBN
IYIQQGN	53,22	834,4236	835,4388	7,92	P04405|GLYG2_SOYBN
KYEGNWGPL	47,91	1062,5134	1063,5242	11,27	P02858|GLYG4_SOYBN
LVPPK	22,5	552,3635	553,3713	6,44	P11828|GLYG3_SOYBN
LVPPQE	28,56	681,3697	682,3797	7,77	P04776|GLYG1_SOYBN
LVPPQES	35,83	768,4017	769,4088	8,02	P04776|GLYG1_SOYBN
NALPEE	27,16	671,3126	672,3252	7,72	P04776|GLYG1_SOYBN
NIARPS	30,95	656,3605	657,3699	5,61	P02858|GLYG4_SOYBN
NKNPF	23,86	618,3126	619,3255	7,68	P13916|GLCA_SOYBN
NLGQSQV	46,4	744,3766	745,39	7,63	P04347|GLYG5_SOYBN
NQLDQMPR	47,76	1000,476	1001,486	8,46	P04776|GLYG1_SOYBN
NQYGHV	41,75	716,3242	717,3303	6,3	P11827|GLCAP_SOYBN
NSGPL	31,24	486,2438	487,2543	7,33	P04347|GLYG5_SOYBN
NSGPLVNP	58,58	796,4079	797,4187	8,86	P04347|GLYG5_SOYBN
NSLENQ	32,24	703,3137	704,322	3,98	P04776|GLYG1_SOYBN
RDPIYS	34,91	749,3708	750,3836	7,43	P13916|GLCA_SOYBN
RDPIYSN	32,64	863,4137	864,4163	7,23	P13916|GLCA_SOYBN
RPSYT	37,02	622,3074	623,3197	4,08	P04776|GLYG1_SOYBN
SNFNNQ	24,57	722,2983	723,3079	3,59	P02858|GLYG4_SOYBN
SYFVDAQPK	37,69	1053,5131	1054,5254	9,26	P13916|GLCA_SOYBN
TISSEDKP	44,22	875,4236	876,4344	6,35	P13916|GLCA_SOYBN
TISSEDKPF	46,7	1022,492	1023,5007	9,01	P13916|GLCA_SOYBN
TISSEDKPFN	37,38	1136,5349	1137,5469	8,47	P13916|GLCA_SOYBN
VIQHT	22,65	596,3282	597,3378	2,06	P04776|GLYG1_SOYBN
VISPK	25,4	542,3428	543,3511	3,98	P02858|GLYG4_SOYBN
VKNNNPF	37,9	831,4239	832,4316	8,27	P04405|GLYG2_SOYBN
VPPQES	38,61	655,3177	656,3187	3,83	P04776|GLYG1_SOYBN
VSIIDT	27,02	646,3538	647,3633	9,6	P04776|GLYG1_SOYBN
VSLIDT	27,02	646,3538	647,3633	9,6	P11828|GLYG3_SOYBN
YFVDAQPK	44,11	966,481	967,4971	8,81	P13916|GLCA_SOYBN
5IF 72 peptides					

AGNPDIE	34,14	714,3184	715,3355	6,73	P02858|GLYG4_SOYBN
AGVVPPA	35,24	609,3486	610,3558	8,12	P29531|OLEO2_SOYBN
AIPVNKPG	52,59	794,465	795,4747	7,29	P13916|GLCA_SOYBN
ANSLLN	30,21	630,3337	631,3513	8,47	P04776|GLYG1_SOYBN
DFYNPK	53,36	782,3599	783,3809	8,32	P04347|GLYG5_SOYBN
DQMPR	32,04	645,2904	646,3023	4,48	P04776|GLYG1_SOYBN
DQTPR	24,82	615,2976	616,3073	1,46	P02858|GLYG4_SOYBN
FAFGIN	29,08	667,3329	668,337	11,8	P11827|GLCAP_SOYBN
FAPEF	27,86	609,2798	610,2905	10,98	P04405|GLYG2_SOYBN
FEAPR	36,2	618,3125	619,3217	7,14	P29531|OLEO2_SOYBN
FGPMIQ	24,05	691,3363	692,3466	10,54	P19594|2SS_SOYBN
FKTNDTPMIG	30,23	1122,538	1123,5538	9,64	P04776|GLYG1_SOYBN
FLVPPQE	31,87	828,4381	829,4533	10,08	P04776|GLYG1_SOYBN
FLVPPQES	40,13	915,4702	916,4763	9,93	P04776|GLYG1_SOYBN
FPFPRPPHQ	45,82	1121,577	561,7995	10,39	P13916|GLCA_SOYBN
FPGSAQ	36,52	605,2809	606,3268	6,05	P13916|GLCA_SOYBN
FVDAQPK	39,89	803,4177	804,4269	6,94	P13916|GLCA_SOYBN
FYNPK	27,48	667,3329	668,3448	7,22	P02858|GLYG4_SOYBN
GFAPEF	27,01	666,3013	667,3143	11,46	P04405|GLYG2_SOYBN
HKNKNPF	36,68	883,4664	884,4692	5,32	P13916|GLCA_SOYBN
HQQEEENEGGSI	23,5	1355,559	1356,5715	6,78	P04776|GLYG1_SOYBN
IIDTN	27,17	574,2963	575,3059	5,69	P04776|GLYG1_SOYBN
IKNNNPF	38,21	845,4395	846,4517	8,62	P04776|GLYG1_SOYBN
INAENNQ	43,52	801,3617	802,3711	2,51	P13916|GLCA_SOYBN
IPSEV	24,14	543,2904	544,3044	8,22	P04347|GLYG5_SOYBN
IPSEVLS	33,26	743,4065	744,4178	9,31	P04347|GLYG5_SOYBN
IPSQV	23,39	542,3064	543,3207	7,86	P13916|GLCA_SOYBN
IPVNKPG	47,58	723,4279	724,439	6,83	P13916|GLCA_SOYBN
KHFLA	21,87	614,354	615,3644	7,27	P02858|GLYG4_SOYBN
KNKPLV	27,32	697,4486	698,459	5,22	P01071|ITRB_SOYBN
KTNDTPMIG	46,96	975,4695	976,4839	8,45	P04776|GLYG1_SOYBN
KYEGNWGPL	45,48	1062,5134	1063,5167	11,21	P02858|GLYG4_SOYBN
LAFPGSA	33,97	661,3435	662,353	10,29	P13916|GLCA_SOYBN
LAFPGSAQ	36,7	789,4021	790,4149	10	P13916|GLCA_SOYBN
LHENIARPS	35,72	1035,5461	1036,5492	7,09	P04347|GLYG5_SOYBN
LIDTN	27,17	574,2963	575,3059	5,69	P11828|GLYG3_SOYBN
LKYEGNWGPL	29,1	1175,5974	1176,6165	11,85	P02858|GLYG4_SOYBN
LVPPKE	22,3	681,4061	682,4186	7,07	P11828|GLYG3_SOYBN
LVPPQE	25,9	681,3697	682,3807	7,78	P04776|GLYG1_SOYBN
LVPPQES	29,4	768,4017	769,4194	7,48	P04776|GLYG1_SOYBN
LVPPQESQ	36,19	896,4603	897,4782	7,47	P04776|GLYG1_SOYBN
NIARPS	27,12	656,3605	657,3741	5,41	P04347|GLYG5_SOYBN
NNNPFSF	35,51	838,361	839,3672	11,9	P04405|GLYG2_SOYBN
NPIYS	22,31	592,2856	593,2929	7,83	P25974|GLCB_SOYBN
NQLDQ	26,83	616,2816	617,2919	3,54	P04347|GLYG5_SOYBN
NSGPLVNP	61,72	796,4079	797,4204	8,81	P04347|GLYG5_SOYBN
NSLENQ	34,15	703,3137	704,326	3,87	P04776|GLYG1_SOYBN
NSLENQL	36,87	816,3977	817,4109	9,29	P04776|GLYG1_SOYBN
NTGDEPVVA	43,35	900,4188	901,4369	7,91	P02858|GLYG4_SOYBN
NTGNLLG	35,32	687,3552	688,3698	9,11	P22895|P34_SOYBN
RDPIYS	37,79	749,3708	750,3836	7,39	P13916|GLCA_SOYBN
RPSYT	37,73	622,3074	623,3187	4,08	P04776|GLYG1_SOYBN
SNFNNQ	28,47	722,2983	723,3082	3,53	P02858|GLYG4_SOYBN
SPYPR	25,34	618,3125	619,3217	5,86	P02858|GLYG4_SOYBN
SVISPK	31,76	629,3748	630,3861	6,45	P04347|GLYG5_SOYBN
SYFVDAQPK	36,84	1053,5131	1054,527	9,24	P13916|GLCA_SOYBN
SYPTKEE	38,19	852,3865	853,3915	5,36	P22895|P34_SOYBN
TISSEDKP	45,6	875,4236	876,4353	6,25	P13916|GLCA_SOYBN
TISSEDKPF	50,11	1022,492	1023,5031	9,01	P13916|GLCA_SOYBN
TISSEDKPFN	51,58	1136,5349	1137,5586	8,4	P13916|GLCA_SOYBN
VIQHT	21,14	596,3282	597,3375	2,05	P04776|GLYG1_SOYBN
VIQHTFNL	44,63	970,5236	971,5314	10,67	P04776|GLYG1_SOYBN
VISPK	22,62	542,3428	543,3536	3,89	P04347|GLYG5_SOYBN
VKNNNPF	38,87	831,4239	832,4361	8,21	P04405|GLYG2_SOYBN
VKNNNPFS	44,12	918,4559	919,4776	8,26	P04405|GLYG2_SOYBN
VKNNNPFSF	31,56	1065,5243	1066,5385	11,41	P04405|GLYG2_SOYBN
VNPESQQGSPR	46,51	1197,5737	599,7978	5,84	P02858|GLYG4_SOYBN
VSIIDT	23,16	646,3538	647,3649	9,55	P04776|GLYG1_SOYBN
VSLIDT	23,16	646,3538	647,3649	9,55	P11828|GLYG3_SOYBN
YFVDAQPK	48,28	966,481	967,5009	8,76	P13916|GLCA_SOYBN
YVVNPDNDEN	23,07	1177,4888	1178,5088	7,98	P11827|GLCAP_SOYBN

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References for Article 3:

[1] M. Castro-Puyana, C. García-Ruiz, A.L. Crego, M. L. Marina, Development of a CE-MS2 method for the enantiomeric separation of L/D-carnitine: Application to the analysis of infant formulas, Electrophoresis 30 (2009) 337-348.

[2] M.C. García, M. Torre, M.L. Marina, F. Laborda, Composition and characterization of soyabean and related products, Crit. Rev. Food Sci. Nutr. 37 (1997) 361-391.

[3] R. Merritt, B. Jenks, Safety of soy-based infant formulas containing isoflavones: The clinical evidence, J. Nutr. 134 (2004) 1220S-1224S.

[4] M. Mendez, M. Anthony, L. Arab, Soy-based formulae and infant growth and development: A review, J. Nutr. 132 (2002) 2127-2130.

[5] B. Hernández-Ledesma, M. M. Contreras, I. Recio, Antihypertensive peptides: Production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23-35.

[6] A.R. Madureira, T. Tavares, A.M.P. Gomes, M.E. Pintado, F.X. Malcata, Invited review: Physiological properties of bioactive peptides obtained from whey proteins, J. Dairy Sci. 93 (2010) 437-455.

[7] M. Zimecki M, M.L. Kruzel ML, Milk-derived proteins and peptides of potential therapeutic and nutritive value, J. Exp. Ther. Oncol. 6 (2007) 89-106.

[8] B. Hernández-Ledesma, A. Quiros, L. Amigo, I. Recio, Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin, Int. Dairy J. 17 (2007) 42-49.

[9] S. Catala-Clariana, F. Benavente, E. Gimenez, J. Barbosa, V. Sanz-Nebot, Identification of bioactive peptides in hypoallergenic infant milk formulas by capillary electrophoresis-mass spectrometry, Anal. Chim. Acta 683 (2010) 119-125.

[10] M. Martin, A. Wellner, I. Ossowski, T. Henle, Identification and quantification of inhibitors for angiotensin-converting enzyme in hypoallergenic infant milk formulas, J. Agric. Food Chem. 56 (2008) 6333-6338.

[11] B. Hernández-Ledesma, L. Amigo, M. Ramos, I. Recio, Release of angiotensin converting enzyme-inhibitory peptides by simulated gastrointestinal digestion of infant formulas, Int. Dairy J. 14 (2004) 889-898.

[12] Z. Chen, C. Peng, R. Jiao, Y.M. Wong, N. Yang, Y. Huang, Anti-hypertensive nutraceuticals and functional foods, J. Agric. Food Chem. 57 (2009) 4485-4499.

[13] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[14] P. Puchalska, M.L Marina, M.C. García, Isolation and characterization of peptides with antihypertensive activitiy in foodstuffs, Crit. Rev. Food Sci. Nutr. (DOI 10.1080/10408398.2012.664829).

[15] S.Y. Park, J. Lee, H. Baek, H.G. Lee, Purification and characterization of antioxidant peptides from soy protein hydrolysate, J. Food Biochem. 34 (2010) 120-132.

[16] E. Domínguez-Vega, O. Kotkowska, M.C. García, A.L. Crego, M.L. Marina, Fast determination of the functional peptide soymetide in different soybean derived foods by capillary-high performance liquid chromatography, J. Chromatogr. A 1218 (2011) 4928-4933.

[17] P. Puchalska, M.L. Marina, M.C. García, Isolation and identification of antioxidant peptides from commercial soybean based infant formulas, Food Chem. 1 (2014) 147-154.

[18] D.D. Kitts, K. Weiler, Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery, Curr. Pharm. Des. 9 (2003) 1309-1323.

[19] D. Wang, L. Wang, F. Zhu, J. Zhu, X.D. Chen, L. Zou, M. Saito, L. Li, In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food), Food Chem. 107 (2008) 1421-1428.

[20] D. Cushman, H. Cheung, Spectrophotometric assay and properties of angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637.

[21] D. Garrett, M. Failla, R. Sarama, Development of an in vitro digestion method to assess carotenoid bioavailability from meals, J. Agric. Food Chem. 47 (1999) 4301-4309.

[22] L. You, M. Zhao, J.M. Regenstein, J. Ren, In vitro antioxidant activity and in vivo anti-fatigue effect of loach (Misgurnus anguillicaudatus) peptides prepared by papain digestion, Food Chem. 124 (2011) 188-194.

[23] C. Wiriyaphan, B. Chitsomboon, J. Yongsawadigul, Antioxidant activity of protein hydrolysates derived from threadfin bream surimi byproducts, Food Chem. 132 (2012) 104-111.

[24] Y. Nakamura, N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, T. TAKANO, Purification and characterization of angiotensin I-converting enzyme-inhibitors from sour milk, J. Dairy Sci. 78 (1995) 777-783.

[25] H. Chen, K. Muramoto, F. Yamauchi, Structural-analysis of antioxidative peptides from soybean beta-conglycinin, J. Agric. Food Chem. 43 (1995) 574-578.

[26] E. Escudero, M. Angel Sentandreu, F. Toldrá, Characterization of peptides released by in vitro digestion of pork meat, J. Agric. Food Chem. 58 (2010) 5160-5165.

[27] K. Erdmann, W.Y. Cheung Belinda, H. Schroder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease, J. Nutr. Biochem. 19 (2008) 643-654.

[28] B. Sarmadi, A. Ismail, M. Hamid, Antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of cocoa (Theobroma cacao L.) autolysates, Food Res. Int. 44 (2011) 290-296.

III.1.2.

Quantification of highly potent antihypertensive peptides in soybean and maize crops

III.1.2. Quantification of highly potent antihypertensive peptides in soybean and maize crops.

Preface

Most studies related with bioactive peptides are focused on their separation, purification, identification, and characterization from different sources in order to implement them for the preparation of functional foods. In addition, there are already few reports devoted to the generation of transgenic lines (especially for soybean) that expressed modified forms of proteins containing tandem repeats of selected bioactive peptides [250-253]. Quick development of transgenic crop varieties with a higher content in certain bioactive peptides or the presence of natural protein content differences within crops varieties open a new line of investigation of bioactive peptides. Therefore, one of the very emerging research topics within the study of bioactive peptides is their absolute quantification. These methods are very interesting for regulatory agencies in order to develop appropriate policies to regulate contents of bioactive peptides in foodstuffs for human consumption. Antihypertensive peptides present an especial interest since they are highly dosage dependence. Consequently, the development of reliable standardized methods for the determination of antihypertensive peptides in foods is crucial.

At the time, there are just very few methods to determine antihypertensive peptide content in foods for human consumption. As already presented, most of these methods are devoted to animal origin antihypertensive peptides. However, antihypertensive peptides from vegetable origin may exhibit much more potent activities, especially those present in soybean and maize crops. Indeed, soybean contains the peptide (VLIVP), identified in protease P hydrolysates of the 11S glycinin protein fraction. High antihypertensive activity of this peptide has been demonstrated (IC_{50} value 1.69 μM) [85]. In the case of maize, three peptides (LRP, LSP, and LQP) derived from the α-zein protein fraction with demonstrated high antihypertensive activity (IC_{50} values 0.29, 2.0, 1.7 μM, respectively) have been identified [84]. Interestingly, all these peptides have demonstrated higher antihypertensive activity than famous VPP and IPP tripeptides (IC_{50} values 9.13 and 5.15 μM, respectively) from fermented milk [55, 64, 194]. Nevertheless, there is no work devoted to the absolute quantification of these potent antihypertensive peptides in soybean and maize.

Objectives

The specific objectives of this work were:

- To propose a rapid and efficient analytical method using high intensity ultrasonic waves for the extraction of proteins from soybean and α-zeins from maize crops.
- To optimize a fractionation method for targeted soybean and maize protein groups.
- To design and to optimize adequate methodologies for the enzymatic digestion of extracted proteins in order to obtain the highest recovery of targeted peptides from the digested extracts.
- To obtain quick and efficient chromatographic separations of targeted peptides in highly complex digested protein extracts using novel stationary phases.
- To unambiguously identify targeted antihypertensive peptides (VLIVP in soybean and LRP, LSP, and LQP in maize) in soybean and maize hydrolysates using chromatographic separation techniques and mass spectrometry systems.
- To optimize mass spectrometric parameters in order to obtain highly sensitive and selective detection of targeted peptides enabling their unambiguous determination in complex food hydrolysates at a low concentration level.
- To implement different approaches to avoid ionization interferences affecting the determination of targeted peptides in complex samples.
- To evaluate the analytical characteristics of the developed methodologies (linearity, LOD, LOQ, precision, matrix effect, and recovery).
- To apply these new analytical methodologies to the determination and quantification of highly antihypertensive peptides in different genotypes of soybean and maize.

Results

The results obtained in this research work are included in the following scientific articles:

- **Article 4:** *Development of a RP-LC analytical methodology for the determination of antihypertensive peptides in maize crops.*
  P. Puchalska, M. L. Marina, M. C. García.
  J. Chromatogr. A, 2012, 1234, 64-71

- **Article 5:** *Development of a HPLC-ESI-Q-ToF-MS methodology for the determination of three highly antihypertensive peptides in maize crops.*
  P. Puchalska, M. L. Marina, M. C. García.
  J. Chromatogr. A, 2013, 1285, 69-77.

- **Article 6:** *Development of a capillary HPLC-IT-MS method for the determination of VLIVP antihypertensive peptide in soybean crops.*
  P. Puchalska, M. C. García, M. L. Marina.
  J. Chromatogr. A, in press.

Article 4

Development of a RP-LC analytical methodology for the determination of antihypertensive peptides in maize crops

P. Puchalska, M. L. Marina, M. C. García

J. Chromatogr. A, 2012, 1234, 64–71

Abstract

The aim of this work was to estimate the content of three highly antihypertensive peptides (LQP, LSP, and LRP) in different maize crops. For that purpose, a method consisting of the extraction of the protein containing these peptides (α-zeins), releasing of peptides by thermolysin digestion, and separation and detection of peptides was designed. The rapid and efficient ultrasound assisted extraction of α-zeins proteins from whole maize kernels was achieved using 70% of ethanol followed by precipitation with acetone. A 10 mM Tris–HCl (pH 8.0) buffer containing 8 M urea enabled to dissolve the precipitated α-zeins. This buffer was diluted to reach a 6 M urea concentration before digestion to keep active the enzyme. Other digestion parameters that were optimized were: enzyme to substrate ratio (5:100 was selected), digestion temperature (50 °C) and digestion time (6 h). The RP-LC separation in a fused-core column was also optimized allowing the separation of the three peptides extracted from maize kernels in 6 min. The presence of the three antihypertensive peptides in the digested extract was confirmed using HPLC–Q-ToF-MS analysis and by comparison with peptide standards. Clear differences were observed in the content of the three antihypertensive peptides and, thus, in the antihypertensive activity of the analyzed crops. The content of LRP peptide was very low regardless of the maize variety while the content of LQP and LSP significantly varied among studied maize lines.

Keywords:

Antihypertensive peptides; HPLC; Maize; Thermolysin; Fused-core column

1. Introduction

Hypertension appears as a leading cause of cardiovascular diseases (CVDs) and is known as 'silent killer' since over 50% of hypertensive population is unaware of their condition [1]. According to the World Health Organization, hypertension is a highly prevalent cardiovascular risk factor worldwide and its treatment has been shown to prevent CVDs [2]. Different therapies can be applied to prevent hypertension being the use of angiotensin converting enzyme inhibitors (ACE inhibitors (*e.g.* captopril)) the first choice [3]. ACE inhibitor compounds act on the renin-angiotensin system associated in the control of blood pressure in living organisms. Angiotensin I is hydrolyzed in the presence of angiotensin I converting enzyme (ACE) to angiotensin II, leading to an increase in blood pressure. The ACE also removes a dipeptide from the C-terminus of bradykinin resulting in the inactivation of this vasodilator. As a consequence, ACE inhibitors cause effective reduction of blood pressure by decreasing the angiotensin II level and rising up bradykinin level [4-6]. Most antihypertensive drugs employ mechanism of ACE inhibition. Synthetic drugs are very potent but they also provoke several adverse effects [5, 7]. An alternative can rise from those foods naturally containing antihypertensive peptides which do not yield adverse effects [8, 9].

Most ACE inhibitory peptides contain 2-12 amino acids residues with a noticeably amount of hydrophobic amino acids such as proline, especially at C-terminal position [10]. ACE inhibitors were found in marine foods [11], fishes [8], meat [12], vegetable foods [13], mushrooms [14], and processed products [15]. Most studied antihypertensive peptides are from foods of animal origin, specially dairy products [16-18], although the most active peptides were found in maize. Indeed, maize contains three peptides (Leucine-Glutamine-Proline (LQP), Leucine-Serine-Proline (LSP), and Leucine-Arginine-Proline (LRP)) derived from the α-zein protein fraction with extremely high antihypertensive activity (IC_{50} value (the half maximal inhibitory concentration) 2.0, 1.7, 0.29 μM, respectively) [19] which is much higher than the popular Valine-Proline-Proline (VPP) (IC_{50} = 9.13 μM) and Isoleucine-Proline-Proline (IPP) (IC_{50} = 5.15 μM) found in milk. Taking into account that the protein content of maize crops can vary [20] and that antihypertensive activity of peptides is highly dosage dependent [21, 22], the development of analytical methodologies for estimating peptide contents in different crops is required.

Worldwide corn or maize (*Zea mays* L.) is a major crop for both livestock feeding and human nutrition [23]. Maize protein content is in the range 6-12% (as dry basis) [20]. Zeins, according

to Osborne nomenclature, are the prolamin fraction of maize, representing 60% of total maize proteins [24]. Zeins can be classified as: α-zeins (21-25 kDa), β-zeins (17 kDa), γ-zeins (18 and 27 kDa), and δ-zeins (10 kDa). The most abundant zein is the α fraction accounting for 75–85% of total zeins [25]. Two major groups of α-zeins can be separated using SDS-PAGE: Z19 zein migrating at 19 kDa and Z22 zein 22 kDa. Nevertheless, the studies of zein sequences obtained from cloned cDNAs and genes, have shown that those two groups of zeins had a Mw around 23-24 and 26-27 kDa, respectively [26].

Several different attempts were made in order to obtain total-zeins from maize kernels, where extraction using aqueous solutions of ethanol or isopropanol with or without a reducing agent are the most frequent [27-29]. Moreover, despite there is one methodology enabling α-zeins extraction, it was applied to maize product with high protein content (corn gluten meal, CGM) [19]. Nevertheless, to our best knowledge, none of these procedures have been applied to exclusively extract the α-zeins from maize kernels.

Next step in the isolation of antihypertensive peptides would be the digestion of α-zeins. Different enzymes have been employed for the digestion of proteins containing antihypertensive peptides being thermolysin the most preferred due to its broad specificity to hydrophobic amino acids [30]. Reports about digestion of CGM by trypsin [31], alcalase [32], thermolysin [19], and six different commercial proteases [33] or zeins by trypsin or thermolysin [34- 35] can be found in the literature. Nevertheless, in all cases CGM or zeins were purchased, and no extraction procedure was previously applied. The lack of methodologies where zein proteins were digested after extraction from whole maize kernels need to be highlighted since the selection of a suitable buffer enabling to dissolve the alcohol soluble α-zeins and to keep active the enzyme had to be overcome. Moreover, comparison of digestion protocols using thermolysin revealed that digestion conditions differed significantly from one work to the other.

Regarding peptide separation, a new trend in HPLC is focused to the development of stationary phases enabling high sample throughput analysis of peptides. Several strategies have been developed being the use of fused-core or superficially porous silica particles very interesting for the reduction of analysis times while keeping column efficiency and low back pressure. Columns with 2.7 μm fused-core particles produce approximately half of the back pressure of the 1.8 μm conventional columns allowing the use of traditional HPLC systems [36]. This fused-core particles start to play important role in chromatography and their use in bio-analytical methods have already been reviewed [37]. Nevertheless, the use of this

innovative approach is still not common, and has scarcely been employed for peptide separation.

The aim of this work was to develop an analytical methodology for the rapid extraction of α-zeins from maize kernels allowing their further digestion by thermolysin and their separation by RP-LC in order to evaluate the content of three highly active antihypertensive peptides (LQP, LRP, and LSP) in maize crops.

2. Materials and methods

2.1. Chemicals and samples

Water, was freshly taken every day from a Milli-Q system (Millipore, Bedfore, MA, USA). All used reagents were of analytical grade purity. Acetic acid (AA), AA with purity for LC-MS, acetone, acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), isopropanol (IPA), and urea were supplied from Scharlau Chemie (Barcelona, Spain). Formic acid (FA), hydrochloric acid, sodium dodecyl sulfate (SDS), sodium hydroxide, tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), and β-mercaptoethanol (B-ME) were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide, dithiothreitol (DTT), iodoacetamide (IAM), thermolysin, and trifluoroacetic acid (TFA) were from Sigma (St. Luis, MO, USA). Heptafluorobutyric acid (HFBA), and sodium acetate were acquired from Fluka (Burchs, Switzerland) and trichloroacetic acid (TCA) was from Panreac (Barcelona, Spain). All chemicals and gels for SDS-PAGE analysis were acquired in Bio-Rad (Hercules, CA, USA): Laemmli buffer (62.5 mM Tris–HCl, 25% (v:v) glycerol, 2% (m:v) SDS, 0.001% (m:v) bromophenol blue), Mini-Protean Precast Gels, running buffer (25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS, pH 8.3), Precision Plus Protein Standards, and Bio-Safe Coomassie stain. Standards and samples employed were: corn gluten meal (CGM) (Sigma, St. Luis, MO, USA), peptides LQP, LSP, and LRP (GeneScript Corp., Piscataway, NJ, USA), standard of zeins (Sigma, St. Luis, MO, USA), and maize lines that were kindly donated by a Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain): EZ6, B73, EZ11A, EZ9, A632.

Prepared solutions were stored in the fridge at 4 °C with the exception of urea, IAM, and DTT solutions that were always freshly prepared. Additionally, thermolysin powder or thermolysin stock solution (2.5 mg/mL in water) and peptides were stored always at −20 °C. Standards of peptides (1 mg/mL or 0.1 mg/mL) were dissolved in water (LRP, LQP) or in ACN

(LSP) according to the recommendation guide supplied by Genscript. Standard of zeins (100 mg/mL) was dissolved in 70% of ethanol prior to analysis. Maize kernels (around thirty whole kernels for each line) were grounded with a domestic miller during 1 min at ambient temperature. All milled maize powders were stored at 4 °C.

2.2. Extraction and fractionation of zeins from CGM

Main maize proteins (zeins) were fractionated following a method developed by Parris and Dickey [38] and improved by Rodríguez-Nogales *et al.* [39] with some modifications. The method was applied to fractionate zeins from CGM. The method consisted of extracting 2 g of CGM with 20 mL of 60% IPA containing 1% B-ME at room temperature and centrifuging for 1 min at 4000 × *g*. This extraction was repeated three additional times. Next, three volumes of 100% IPA were added and the resulting solution was left overnight at 4 °C. Afterwards, the solution was centrifuged for 10 min at 4000 × *g* and 4 °C. The resulting pellet containing β- and γ-zeins was separated from the supernatant. The supernatant was mixed with two volumes of water and 0.01 volumes of sodium acetate (pH 6.0). The solution was left for 2 h followed by centrifugation for 10 min at 4000 × *g* and 4 °C. The pellet containing α-zeins was separated.

2.3. Extraction and purification of α-zeins from maize lines

α-zeins from the EZ6 maize line were extracted using a method developed by Yano *et al.* [19] to isolate α-zeins from CGM. The method was carried out by extracting 1 g of pulverized maize line with 10 mL of 70% EtOH. For a more efficient and fast extraction, an ultrasonic probe (VCX.130, Sonic Vibra-Cell, Hartord, CT, USA) was employed for 10 min (amplitude 90%) followed by centrifugation (4000 × *g*, 10 min, 20 °C). α-Zeins in the previous extract were purified by precipitation with 80 mL of acetone containing 0.07% B-ME followed by centrifugation (4000 × *g*, 15 min, 4 °C). The resulting pellet was dissolved in 20 mL of a buffer (10 mM Tris-HCl (pH 8.0) containing 8 M urea) and left overnight at 4 °C.

2.4. Ultrafiltration

The protein extract obtained with 70% EtOH was ultrafiltrated through semi permeable membranes with Mwco of 3 kDa (Amicon® Ultra, Millipore) and 10 kDa (Centricon®, Millipore), for 20 min by centrifugation at room temperature (4000 × *g*).

2.5. α-Zein digestion

Purified α-zeins dissolved in a buffer containing 8 M urea were diluted in water to obtain a final urea concentration of 6 M. Optimized digestion protocol consisted of mixing 1.5 mL of protein extract with thermolysin stock solution (2.5 mg/mL), so that the enzyme to substrate ratio was 5:100 (w:w). Solution was incubated in a hot air oven (Memmert, model 300, Schwabach, Germany) for 6 h at 50 °C, then boiled for 10 min, and centrifuged for 5 s (mini centrifuge Nahita, model 2507, 7200 rpm).

2.6. HPLC analysis

Separation of zein proteins was performed on a modular Agilent Technologies liquid chromatograph (Pittsburg, PA, USA). The chromatographic assembly consisted of a degassing system, a quaternary pump, a thermostatized compartment for the column, an injection system, and an UV detector (series 1100). HP Chemstation software was used to control HPLC instrument. All experiments were made by duplicate and injected twice into the HPLC system.

Extracted and purified zeins were separated in a POROS R2/10 perfusion column (100 mm × 2.1 mm I.D.) (Perspective Biosystem, Framingham, MA, USA) using a flow-rate of 1 mL/min, a temperature of 25 °C, a binary gradient from 5–50.2% B in 7.2 min, 50.2–65.4% B in 2.94 min, 65.4–95% B in 1 min, and 95–5% B in 1 min. Mobile phases consisted of Milli-Q water/0.1% (v:v) TFA (mobile phase A) and ACN/0.1% (v:v) TFA (mobile phase B). The injected volume was 5 µL and the detection was performed at a wavelength of 280 nm.

Separation of digested α-zein and target peptides was performed by HPLC with UV detection using an Ascentis Express Peptide ES-C18 column (100 mm × 2.1 mm I.D., with 2.7 µm particle size) with an Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 µm particle size) both from Supelco (Bellefonte, PA, USA). The optimized chromatographic conditions for the separation of peptides were: 3% B for 5 min, 3–5% B in 5 min, 5–97% B in 2 min, and 97–3% B in 2 min; mobile phase A, Milli-Q water/20 mM AA; mobile phase B, ACN/20 mM AA; temperature, 40 °C; flow-rate, 0.4 mL/min; injected volume, 5 µL; UV detection at 210 nm. Digested extracts were filtrated through 0.45 µm pore size regenerated cellulose filter membranes (Titan 2, Eatontown, NJ, USA) before injections.

2.7. MS analysis

MS analysis was performed using a Quadrupole-Time-of-Flight (Q-ToF) MS (instrument series 6530) from Agilent Technologies coupled to a liquid chromatograph 1100 series also from Agilent Technologies. HPLC separation was made on the Ascentis Express column previously employed, using mobile phases: A, Milli-Q water/0.3% (v:v) AA and B, ACN/0.3% (v:v) AA. The elution gradient was 5–20% B in 15 min, 20–95% B in 2 min, and 95–5% B in 2 min with a flow-rate of 0.5 mL/min and a column temperature of 25 °C. The injected volume was 20 μL for the digested extract and 1 μL for the standards of peptides. Simultaneous UV (210 nm) and MS detection were registered. The mass spectrometer was operated with the ESI source Jet Stream in the positive ion mode using only ToF analyzer (only MS mode) and a mass range of 100–3200 *m*/*z*. The dry gas flow-rate was 10 L/min and its temperature was 300 °C. The nebulizer gas pressure was 30 psig and the sheath gas flow and temperature were set up at 12 L/min and 400 °C, respectively. MS conditions were: capillary voltage, 3500 V; fragmentator, 200 V; skimmer voltage, 60 V; octopole voltage, 750 V, and nozzle voltage, 0 V. Mass spectrometer control, data acquisition, and data analysis were carried out with the MassHunter Software.

2.8. SDS-PAGE analysis

A Bio-Rad Mini-Protean system was used for the electrophoretic separation of proteins. Samples were prepared as follows: 15 μL of each sample was added to 15 μL of Laemmli buffer containing 0.5% of B-ME, vortexed, boiled at 95 °C for 5 min, and centrifuged for 10 s. Separation was carried out on commercial Mini-Protean Precast Gels using a solution consisting of 25 mM Tris–HCl, 192 mM glycine, and 0.1% SDS (pH 8.3) as running buffer. Electrophoresis was performed by applying 200 V for 30 min. Protein standards (Precision Plus Protein Standards) consisting of recombinant proteins expressed by *Escherichia coli* (with Mw 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa) were injected in the first lane and used as a ladder to estimate molecular weights of proteins in the following lanes. After electrophoresis, proteins were fixed by gentle agitation in 100 mL solution consisting of 10% (v:v) glacial acetic acid and 40% (v:v) MeOH for 30 min, stained for 1 h (gently agitating) with 50 mL of Bio-Safe Coomassie stain, and washed with Milli-Q water for at least 2 h.

3. Results and discussion

3.1. Selection fo the extraction procedure and identification of α-zeins

There is no quick and efficient procedure to extract α-zeins from maize kernels. Existing extraction procedures are mainly focused to the extraction of whole maize proteins or total-zeins from maize kernels or to the extraction of α-zeins from maize products with high protein content, *e.g.* CGM.

The procedure developed by Parris and Dickey [38] and improved by Rodríguez-Nogales *et al.* [39] enabled the fractionation of zeins from CGM based on their different solubility. We have modified the original fractionation procedure reducing the second overnight precipitation to 2 h. Moreover, we have also tried the use of this method to fractionate zeins from maize kernels. Nevertheless, zein fractionation from maize kernels was not possible due to their low protein content (6–12%) in comparison with maize products (CGM contained 60% protein) employed in the original procedure. Then, the method was applied to the extraction of zeins from CGM such as it was previously performed by Parris and Dickey [38]. The precipitated α, and β/γ-zeins, and the supernatant obtained from CGM, were injected into the HPLC system. Although, in each fraction different peaks were distinguished, small signals supposedly from α-zeins, could also be observed in the β/γ-zein fraction, and in the supernatant. These results could indicate that part of the analytes remain in the solution after precipitation. These results suggested that this fractionation method was not quantitative and could not fully separate α-zeins from β and γ-zeins. In order to obtain a clear identification of peaks corresponding to α-zeins, an extraction procedure used for extracting α-zeins from CGM [19] was applied to maize kernels from the EZ6 line. The extraction consisted of mixing 1 g of maize kernel with 10 mL of 70% EtOH. The extraction procedure was accelerated by the employ of an ultrasonic probe (20 min, 100% amplitude). Finally, the extract was centrifuged for 10 min (4000 × *g*, 20 °C). Furthermore, the 70% EtOH extract, supposedly containing α-zeins, was subjected to ultrafiltration through membranes with Mwco 3 and 10 kDa, where in both cases, results were identical. On the other hand, a commercially available zeins standard mainly made up from α-zeins (due to the fact that other types of zeins (β, γ, δ) are thought to contribute to gelling [40]) was also used in order to clarify the identification of α-zeins. The commercially available zeins standard, the precipitated β/γ-zeins, the extract of α-zeins obtained from maize kernels extracted with 70% of EtOH, and the same extract passing through the membrane with Mwco 3 kDa were injected into the HPLC system (Fig. 1). α-Zeins eluted in three peaks at 3, 3.7, and 8 min (peaks

C, D, and H/I). As previously stated, β and γ-zeins fraction also yielded small peaks C, D, and H/I in addition to a big signal at 6 min (peak F) and two small signals (peaks E/G). Extract obtained from maize kernels with 70% of EtOH using the method of Yano *et al.* [19] accelerated with an ultrasonic probe, yielded mainly peaks C, D and H/I in addition to small signals A, B, and E.

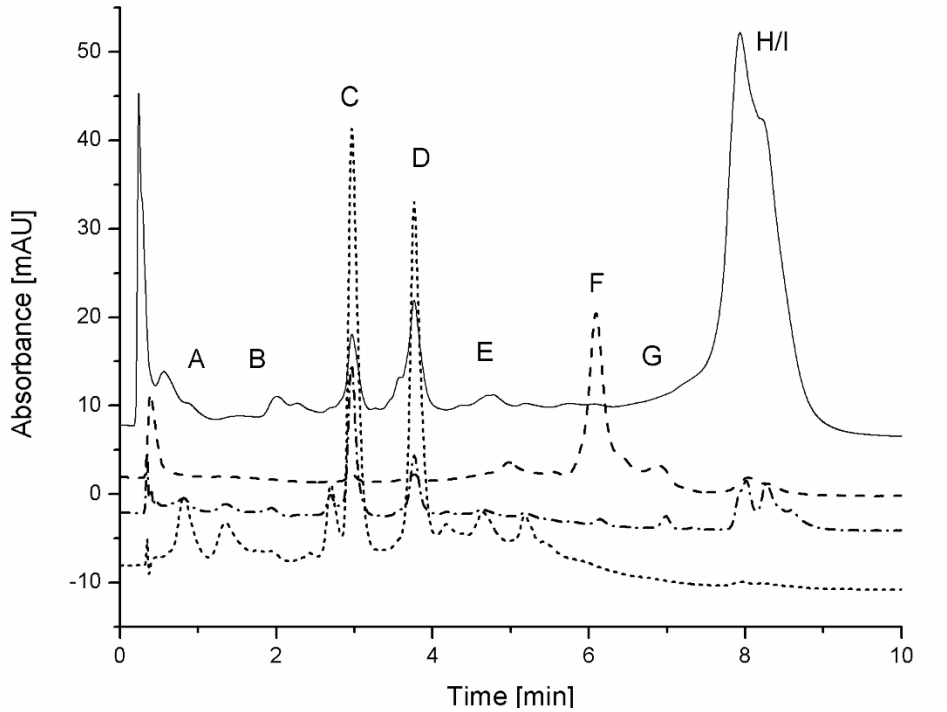

Fig. 1. Chromatograms obtained for: the commercial α-zein standard (solid line); fraction β/γ-zeins obtained by the fractionation of CGM (dash line); the extract of α-zeins obtained from the maize kernel with 70% of EtOH (dash dot line); extract when passing through a membrane with Mwco of 3 kDa (dot line). Chromatographic conditions: column: POROS R2/10 perfusion (100 mm × 2.1 mm I.D.), mobile phases: A, Milli-Q water/0.1% (v:v) TFA; B, ACN/0.1% (v:v) TFA; gradient: 5–50.2% B in 7.2 min, 50.2–65.4% B in 2.94 min, 65.4–95% B in 1 min, 95–5% B in 1 min; T = 25 °C; inj. vol. = 5 μL; F = 1 mL/min; λ = 280 nm.

Interestingly, when this extract was subjected to ultrafiltration through a 3 kDa Mwco membrane, peaks H/I disappeared and only signals A–E were observed. From these results, α-zeins seemed to elute mainly in peaks H and I, while peaks F and G could correspond to β- and γ-zeins, and peaks A–E (passing through the 3 kDa filter) could be small molecules. These small molecules could correspond to impurities like polyamines putrescine compounds which have very high UV absorbance at 320 nm [27]. As reported Moreau *et al.* [41] when maize kernels are extracted with common polar organic extractants (*e.g.* ethanol or methylene chloride), high levels of polyamine conjugates such as diferuloylputrescine (DFP) and *p*-coumaroylferuloyputrescine (CFP) were also extracted. Moreover, LC–MS analysis of these

compounds reported molecular weights of 440 and 410 mass units, for DFP and CFP, respectively [42] demonstrating these compounds passed through the 3 kDa cut-off filter. Furthermore, the chromatogram obtained when registering the signal at 320 nm (specific wavelength for polyamine putrescine compounds) showed just peaks A, B, C, D, and E and not the peaks assigned to zeins (F, G, and H/I). Moreover, the extract obtained from maize kernel using 70% of ethanol and accelerated with ultrasonic probe was also analyzed by SDS-PAGE. The electrophoretic pattern of this extract confirmed the presence of only α-zeins bands appearing at molecular weights of 19 and 22 kDa.

3.2. Optimization of α-zeins extraction

Once demonstrated that the use of an organic solvent as ethanol could extract α-zeins from maize kernels, following parameters were next optimized: organic solvent, percentage of organic solvent in extractant, time of extraction, amplitude of the ultrasonic probe, and maize to extractant ratio. According to the literature, an alternative solution to EtOH for the extraction of α-zeins could be IPA and ACN [28]. ACN was discharged due to the low amount of α-zeins that were extracted and due to the high amount of interferences that were obtained. Regarding IPA and EtOH, different solutions ranging from 40% to 90% were tried for the extraction of α-zeins. Maximum α-zein extraction with EtOH was reached at 70% while for IPA, the highest extraction was obtained at 60%. Taking into account that IPA extraction provided higher amount of interferences and the higher price of this solvent, 70% EtOH was chosen as the optimum extractant for α-zeins. Next, the influence of the extracting time using the ultrasonic probe was evaluated in the range from 0 (sample mixed with solvent and centrifuged) to 60 min in 10 min intervals. Results indicated that α-zein solubility increased up to 10 min and afterwards decreased, concluding the optimum time for extraction was 10 min. Next, the ultrasonic energy was modified to evaluate its effect on the amount of extracted proteins. Following amplitudes were tested: 20%, 30%, 50%, 70%, 90%, and 100%. Results showed that higher ultrasonic energies accelerated the extraction of α-zeins up to 90% of amplitude, that was selected as the optimum value. Moreover, different sample to solvent ratios were evaluated: 1:3, 1:5, 1:10, 1:15, and 1:20 (w:v). Extractions under sample to solvent ratios 1:5 and 1:3 were not possible and gave unreliable results. A 1:10 (w:v) sample to solvent ratio was further used as optimum since it enabled to obtain the maximum peak area. After optimization of the extraction procedure, a study on the stability of samples was performed. The same sample was injected over a long period of time (26 h). No change in the peak area corresponding to α-zeins was observed. Estimated repeatability for the ten first injections of the same sample yielded a

RSD of 0.4%. The precision obtained when injecting eight different samples in the same day was 2.2%. The inter-day precision when injecting eight maize samples in two different days (16 analysis) was 3.4%.

3.3. Purification of α-zeins

In order to clean the α-zeins extract from interferences and also to transfer α-zeins to other suitable solution for enzymatic digestion, the precipitation of proteins was proposed. Two different precipitation methods were designed. Precipitation methods consisted of mixing α-zeins extract with 50 mL of 10% TCA/acetone containing 0.07% B-ME or with 50 mL of acetone containing 0.07% B-ME, both at −20 °C for 1 h. Both pellets were dissolved again in 70% EtOH and were injected into the chromatographic system. Pellets obtained by the TCA/acetone method were very difficult to redissolve. Unlike this procedure, pellets from the acetone methodology were quickly redissolved in 70% EtOH. Moreover, a recovery close to 100% was observed by comparing the signal corresponding to the protein before its precipitation with that of the protein after its precipitation and redissolution. In order to use the best conditions for the acetone precipitation of α-zeins, the volume of acetone and the precipitation time were optimized. The recoveries of α-zeins and interferences were estimated taking into account signals obtained before and after precipitation with different volumes (50–100 mL) of acetone. Results data showed that all samples, regardless to the volume of acetone employed, were cleaned from interferences at approximately the same level. Finally, an acetone volume of 80 mL was considered as the optimum one since it yielded the highest protein recovery. Moreover, different precipitation times at −20 °C were tested (0–90 min in 30 min intervals). Results at each precipitation time were very similar and no waiting time for precipitation was considered necessary.

3.4. Solubilization of precipitated α-zeins in an aqueous buffer

The selection of an appropriate solvent for the enzymatic digestion of a protein is usually not a big problem since most proteins are soluble in aqueous buffers where enzymes are working. Nevertheless, since zeins are alcohol soluble proteins, choosing a medium in which zeins were soluble and, simultaneously, enzyme was active, is quite challenging. Information found in the literature indicated that zeins were soluble in aqueous solutions containing high concentrations of urea, salts, and ammonia [20]. Moreover, thermolysin has unusual properties and remains active in unfolding conditions such as high concentrations of urea (6–8 M urea)

[43] or in the presence of some salts [44]. In addition, anionic surfactants like SDS at concentrations up to 1% had been proven to be useful for solubilization of proteins [41]. Taking into account this bibliographic information, the following media were employed for the solubilization of the α-zeins obtained from the maize kernels: 4 M urea/0.1% NH_3; 10 mM Tris–HCl/6 M urea (pH 8.5); 10 mM Tris–HCl/8 M urea (pH 8.5); 50 mM Tris–HCl/5 mM $CaCl_2$ (pH 8.0); 8 M urea; 1% NH_3; 10 mM Tris–HCl/0.1% SDS (pH 8.5); 10 mM Tris–HCl/8 M urea + 0.6% SDS (pH 8.5); 10 mM Tris–HCl/0.6% SDS (pH 8.5). Nevertheless, only the buffer consisting of 10 mM Tris–HCl/8 M urea (pH 8.5) could dissolve α-zeins and enabled the digestion with thermolysin by the previous dilution of urea to 6 M. This solubilization process was accelerated when temperature decreased. Hence, the precipitated α-zeins were best dissolved when keeping in the fridge overnight. Obtained digested extract was injected into the HPLC system using UV detection at 210 nm which is a suitable wavelength for detecting peptides. Comparison of chromatograms demonstrated (data not shown) that α-zeins peaks appearing in the initial extract disappeared after thermolysin digestion and, at the same time, new signals corresponding to peptides appeared. Nevertheless, the perfusion column did not permit a good separation of peptides and it was replaced by a fused-core column in next experiments.

3.5. Identification of target peptides in the α-zeins digested extract

In order to confirm that target peptides LQP, LRP, and LSP were in the digested extract, HPLC–MS was used. For that purpose, the chromatographic conditions were chosen in order to obtain the best peptide separation with the fused-core column and, at the same time, a nice environment for the MS detection. At this point, it is important to highlight that the ion-pairing reagent used in this work up to now in RP-LC separations was not suitable for the MS detection. In fact, TFA creates strong complexes with peptides which are enhancing the separation in the RP-LC column, but at the same time, these complexes are strong enough to inhibit peptide ionization in MS resulting in signal suppression. Different alternatives such as reduction of the TFA concentration or the use of other ion-pairing reagent can be proposed. Typical mobile phases used for HPLC with MS detection are 0.3% AA, 0.2% HFBA, 0.025% TFA or 0.2% FA [45]. These ion-pairing reagents were tested for the separation of the α-zein digested extract. The chromatogram obtained using AA as ion-pairing reagent seemed to enable the best separation of peptides.

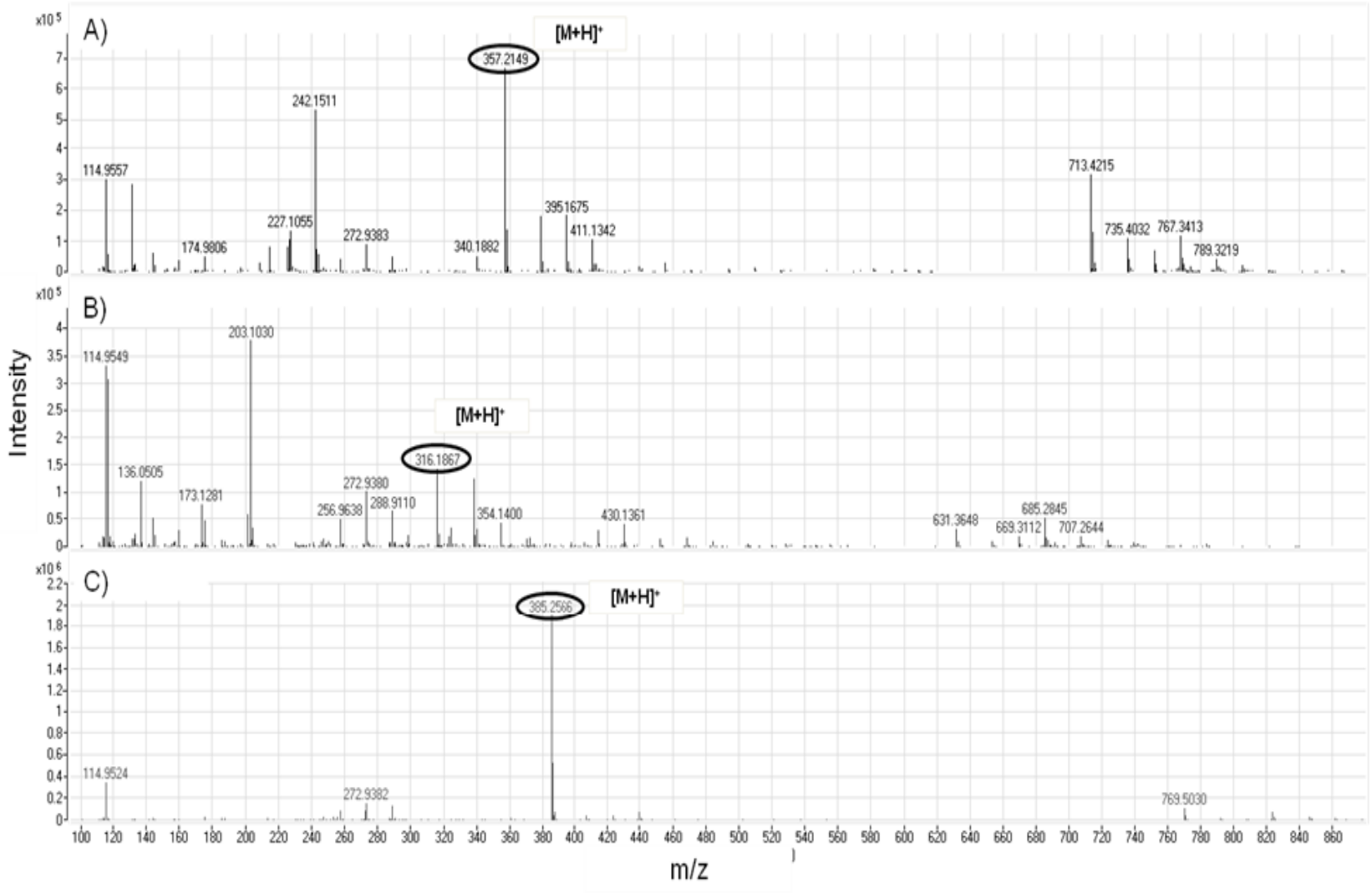

Fig. 2. MS spectra of standard peptides: (A) LQP, (B) LSP, and (C) LRP. Identified ions from each peptide are marked by circle.

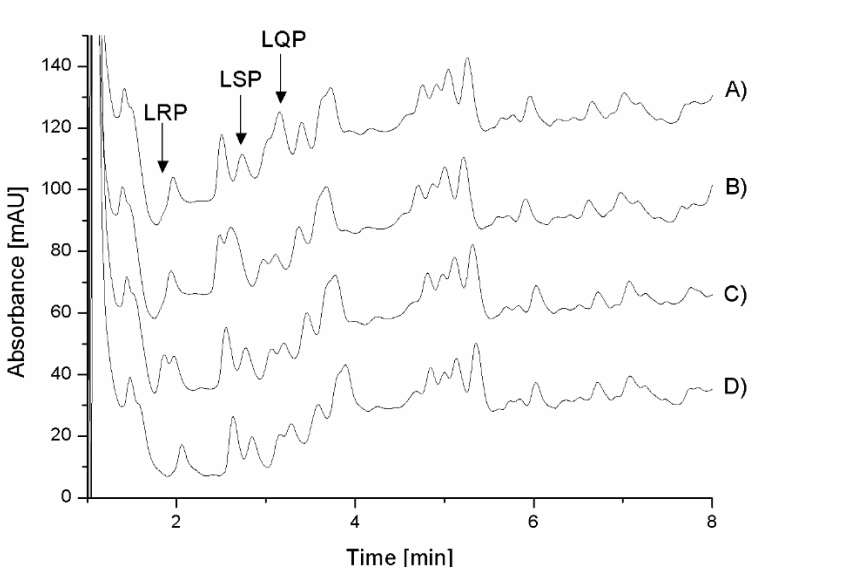

Fig. 3. Chromatograms corresponding to the digested extract spiked with (A) LQP, (B) LSP, (C) LRP and to (D) the digested extract. Chromatographic conditions: column: Ascentis Express Peptide ES-C18 (100 mm × 2.1 mm I.D., with 2.7 μm particle size) with Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 μm particle size), mobile phases: A, Milli-Q water/0.3% AA (v:v), B, ACN/0.3% AA (v:v). Gradient: 5–20% B in 15 min, 20–95% B in 2 min, 95–5% B in 2 min, T = 25 °C, inj. vol. = 10 μL, F = 0.5 mL/min, λ = 210 nm.

As a consequence, 0.3% of AA in the mobile phase was chosen for further experiments using the following gradient: 5–20% B in 15 min, 20–95% B in 2 min, and 95–5% B in 2 min. The comparison of the elution times of these peaks with the elution times of the standard peptides enabled a tentative identification of the peaks corresponding to LQP, LSP and LRP. Moreover, the MS spectra of these peptides in the maize sample and in the standards were compared for a better identification. Fig. 2 shows the spectra corresponding to the three standard peptides. All three peptides in the standards resulted singly protonated. In the case of LQP and LRP, this protonated ion was the base peak. Moreover, additional signals were also observed in every peptide standard, especially in the case of LQP and LSP. These additional signals could correspond to fragments of peptide and other reagents that have resulted during their synthesis. The total ion chromatogram (TIC) of the digested extract, and the extracted ion chromatograms (EICs) of each peptide ion obtained from the separation of digested extract, and peptide standards were compared in order to identify target peptides in the digestion extract. Moreover, the digested extract of α-zeins was spiked with every peptide standard to confirm the identity of peaks corresponding to these peptides in the digested extract. The digested extract (250 μL) was enriched with 2 μL of LQP (1 mg/mL), LSP (0.1 mg/mL) or LRP (1 mg/mL) (Fig. 3).

Comparison of spiked and non-spiked extracts enabled the clear identification of LQP, LSP, and LRP peptides.

3.6. Optimization of the chromatographic separation of the α-zeins digested extract by RP-LC

Once target peptides have been identified in the digested extract, the chromatographic conditions were optimized. Since target peptides still eluted closely in the first minutes of the chromatogram, the slope of the gradient in the first part of the separation was reduced. Finally, a gradient consisting of a first isocratic step at 3% B for 5 min and followed by 3–5% B in 5 min, 5% B in 5 min and 5–97% B in 2 min was chosen for further experiments. Different concentrations of AA in the mobile phase (20, 40, 50 (0.3%), 60, and 80 mM) were also examined. Separation was not possible when removing the AA reagent, while high concentrations resulted in very low retention. Therefore, a concentration of 20 mM of AA was found to be the best. Moreover, four different temperatures were tried: 25 °C, 30 °C, 35 °C, and 40 °C. Best separation was obtained at 40 °C. Nevertheless, despite the appropriate separation of standard peptides (LRP peptide eluted at 2.1 min, LSP at 2.7 min, and LQP at 3.9 min), the digested extract from the maize kernel showed a limited sensitivity and very small peaks were observed. Thus, next step was focused to increase the sensitivity.

3.7. Optimization of thermolysin digestion of α-zeins

The following parameters were optimized: concentration of Tris–HCl buffer, pH, substrate to enzyme ratio, temperature, and digestion time. Also, an attempt of enhancing the digestion using an ultrasonic probe and the influence of reduction and alkylation of proteins previous to digestion, have been evaluated. Digestion can be enhanced and accelerated if using ultrasonic energy. The digested extract prepared under the initial conditions (α-zeins dissolved in a 10 mM Tris–HCl/8 M urea buffer (pH 8.5) and diluted to 6 M urea was mixed with thermolysin at an enzyme to substrate ratio of 0.1:100 (w:w) and digested at 37 °C for 3 h) was subjected to the action of the ultrasonic probe. Both the amplitude (20%, 40%, 60%, 80%) and the time (3, 5, 7, 10 min) were studied. Surprisingly, in all cases proteins remained undigested. These results suggested that thermolysin was not able to stand the ultrasonic energy resulting inactive under these conditions. Therefore, precipitated zeins were dissolved in 20 mL of a buffer containing 8 M urea and different concentrations (10, 20, 50 or 100 mM) of Tris–HCl (pH 8.5). Best results were obtained when using the 10 mM Tris–HCl buffer. On the other hand, according to the literature, the stability pH range of thermolysin ranges from 5.0 to 9.5 [46]. In order to confirm

the pH which was optimum for the digestion of α-zeins, the following buffer pHs were evaluated: 7.5, 8.0, 8.5, and 9.5. Results showed that thermolysin worked best at pH 8.0, while at pH 9.0 the digestion was inhibited due to instability of the enzyme. Following, the ratio enzyme to substrate was optimized. Several enzyme to substrate ratios were tested: 0.02:100, 0.1:100, 0.5:100, 1:100, 2.5:100, 5.0:100, and 10:100 (w/w). Moreover, blank digestions without α-zeins were also performed for every ratio in order to evaluate the existence of autodigestion [47]. Rising up the level of enzyme resulted in an increase of detected peptides till ratio 5:100. No autodigestion was observed at enzyme to substrate values below 5:100 while at a ratio 10:100, peaks probably corresponding to the hydrolyzed enzyme were observed. As a consequence, a 5:100 ratio was chosen. Furthermore, the influence of temperature in the digestion was next studied. Since themolysin can stand temperatures up to 70 °C, examined temperatures were: room temperature, 37 °C, 50 °C, 60 °C, and 70 °C. Maximum signals were observed at 50 °C that was chosen as optimum temperature value. Different digestion times were also tested (1, 3, 6, 12, 18, 24 h). Despite there were not significant changes in the profiles obtained, results showed a slight improvement in the hydrolysis of α-zeins till 6 h. Therefore, 6 h of digestion was chosen as optimum time. Moreover, we evaluated the effect of reduction and alkylation of α-zeins previously to their digestion. Interestingly, the hydrolysis of α-zeins did not occur when reduction and alkylation took place. Finally, the injected volume was optimized. Volumes ranging from 2 to 20 μL were employed. Reduction of the injected volume decreased the height of the detected signals. However, signals were much better resolved at reduced injected volumes and an injection volume of 5 μL was finally adopted. Fig. 4 shows the separation of the digested extract under the final chromatographic and digesting conditions. The arrows show the peaks in which target peptides are eluting.

3.8. Application of developed methodology to the analysis of maize varieties

The developed methodology for the extraction and purification of α-zeins was applied to different maize varieties to evaluate the performance of the optimized methodology for isolating α-zeins. The worst purification was obtained for the crop B73 and the best for the EZ9 crop. However, the recovery of proteins after their precipitation with acetone was always higher than 93%. Peptidic profiles were qualitatively very similar and only differences on the size of the peaks corresponding to the target peptides were observed. The comparison of the average areas of every antihypertensive peptide in the studied maize crops is shown in Fig. 5.

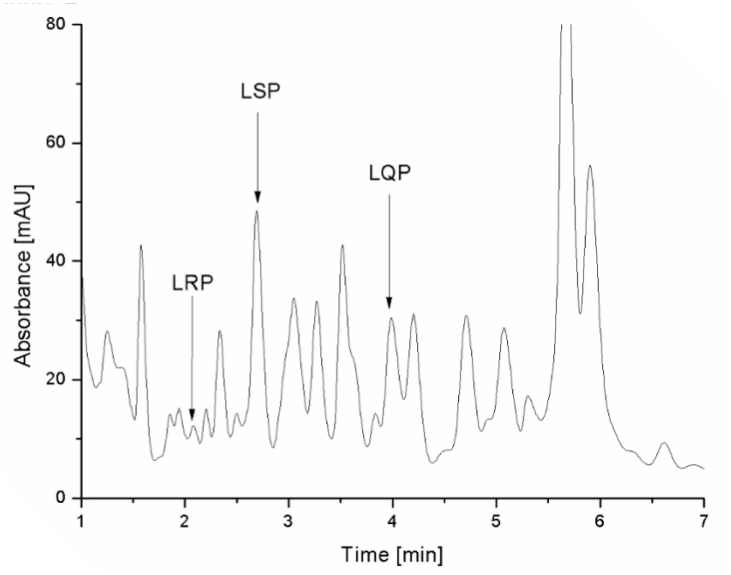

Fig. 4. Separation of LRP, LSP, and LQP peptides from the digested extracts corresponding to maize line B73 under the final chromatographic conditions: column: Ascentis Express Peptide ES-C18 (100 mm × 2.1 mm I.D., with 2.7 µm particle size) with Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 µm particle size), mobile phases: A, Milli-Q water/20 mM AA; B, ACN/20 mM AA; gradient: 3% B for 5 min, 3–5% B in 5 min, 5% B for 5 min, 5–97% B in 2 min, and 97–3% B in 2 min, T = 40 °C, inj. vol. = 5 µL, F = 0.4 mL/min, λ = 210 nm.

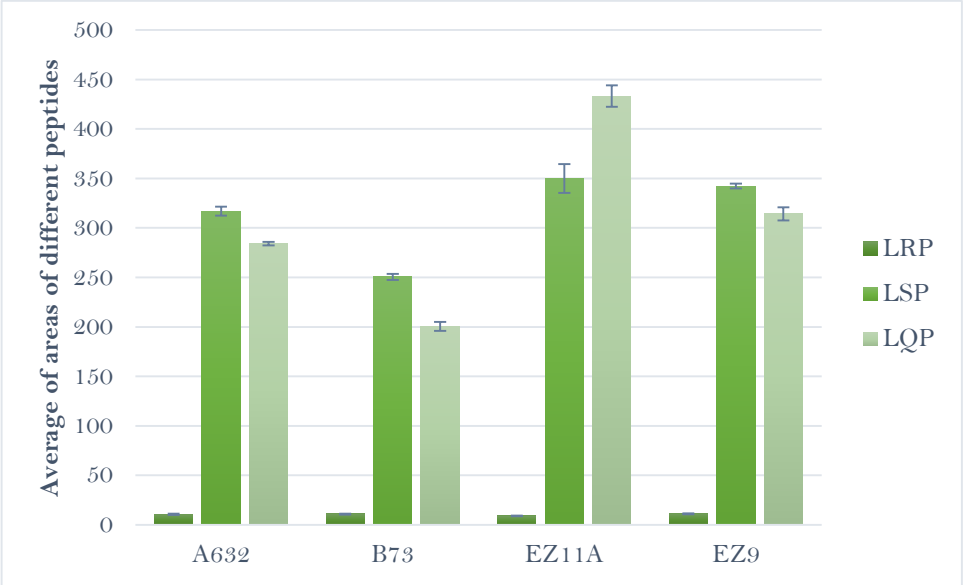

Fig. 5. Comparison of average areas of peptides LQP, LSP, and LRP in different maize varieties (four maize varieties, prepared twice and injected two times in the HPLC; 16 analysis).

There were statistically significant differences among results obtained for every antihypertensive peptide in the studied maize crops (ANOVA, $P < 0.05$). The content in LRP peptide was very low in all the analyzed maize lines. Nevertheless, LRP is the most potential

antihypertensive peptide (IC_{50} = 0.29 μM) causing a decrease in blood pressure of about 15 mmHg by intravenously injection of a small amount of peptide (30 mg/kg) in SHR (spontaneously hypertensive rats) after 2 min intake [35]. It should be highlighted that a reduction of the diastolic blood pressure of 5 mmHg decreases the risk of heart disease by approximately 16% [5]. In the case of peptides LQP (IC_{50} = 2.0 μM) and LSP (IC_{50} = 1.7 μM), their content significantly varied among the studied maize lines. The highest yield of LSP peptide was found in the EZ9 and EZ11A crops, while the smallest in the B73. The highest level of LQP was observed in the EZ11A line, and the lowest in the B73. Concluding the crop with the highest yield of antihypertensive peptides seemed to be the EZ11A line while the B73 crop showed the lowest content in antihypertensive peptides. The reproducibility of the method for each peptide and for every crop was calculated based on the area of two independently prepared samples injected twice into the HPLC. The reproducibility, expressed as RSD values, for each peptide was 7.28% for LRP, 1.43% for LSP, and 1.27% for LQP.

4. Conclusions

According to the results presented in this work, it can be concluded that, a new methodology has been developed for extracting and purifying α-zeins from whole maize kernels. The method resulted precise, effective, and quick. A solvent has been selected enabling the suitable solubilization of precipitated α-zeins and their suitable digestion (after urea dilution to 6 M) with thermolysin. A method has been optimized for the digestion of α-zeins with thermolysin. Three antihypertensive peptides (LQP, LSP, and LRP) have been identified by HPLC-Q-ToF in the digests of extracted α-zeins from maize kernels. A RP-LC analytical methodology using a fused-core column was optimized enabling the separation of the three antihypertensive peptides in maize crops in less than 6 min after the optimized extraction and digestion of α-zeins from maize crops. The contents of LQP, LRP, and LSP peptides have been estimated in different maize varieties. The content of LRP peptide (IC_{50} = 0.29 μM) was very low regardless of the maize variety. LQP (IC_{50} = 2.0 μM) and LSP (IC_{50} = 1.7 μM) peptides, presenting an activity more than twice that of the most known and studied VPP and IPP peptides (IC_{50} = 9.13 and 5.15 μM, respectively), were detected in all maize varieties. Significant differences in the content of LQP and LSP were observed among studied maize lines which clearly demonstrated the different antihypertensive activity of maize lines.

Acknowledgments

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References for Article 4:

[1] A. Chockalingam, World hypertension day and global awareness, Can. J. Cardiol. 24 (2008) 441-444.

[2] www.who.int.

[3] L.M. Souza, R. Riera, H. Saconato, A. Demathé, A.N. Atallah, Oral drugs for hypertensive urgencies: Systematic review and meta-analysis, Sao Paulo Med. J. 127 (2009) 366-372.

[4] Z.Y. Chen, C. Peng, R. Jiao, Y.M. Wong, N. Yang, Y. Huang, Antihypertensive nutraceuticals and functional foods, J. Agric. Food Chem. 57 (2009) 4485-4499.

[5] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[6] R.E. Schmieder, K.F. Hilgers, M.P. Schlaich, B.M.W. Schmidt, Renin-angiotensin system and cardiovascular risk, Lancet 369 (2007) 1208-1219.

[7] A.B. Atkinson, J.I. Robertson, Captopril in the treatment of clinical hypertension and cardiac failure, Lancet 2 (1979) 836-839.

[8] S.H. Lee, Z.J. Qian, S.K. Kim, Using combinations of principal component scores from different spectral ranges in near-infrared region to improve discrimination for samples of complex composition, Food Chem. 118 (2010) 96-101.

[9] T. Nakahara, A. Sano, H. Yamaguchi, K.R.I. Sugimoto, H. Chikata, E. Kinoshita, R. Uchida, Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances, J. Agric. Food Chem. 58 (2010) 821-827.

[10] H. Meisel, Biochemical properties of regulatory peptides derived from milk proteins, Biopolymers 43 (1997) 119-128.

[11] Y.K. Wang, H.L. He, X.L. Chen, C.Y. Sun, Y.Z. Zhang, B.C. Zhou, Production of novel angiotensin I-converting enzyme inhibitory peptides by fermentation of marine shrimp Acetes chinensis with Lactobacillus fermentum SM 605, Appl. Microbiol. Biotechnol. 79 (2008) 785-791.

[12] E. Escudero, M.A. Sentandreu, K. Arihara, F. Toldrá, Angiotensin I-Converting enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat, J. Agric. Food Chem. 58 (2010) 2895-2901.

[13] S.J. Rho, J.S. Lee, Y. Il Chung, Y.W. Kim, H.G. Lee, Purification and identification of an angiotensin I-converting enzyme inhibitory peptide from fermented soybean extract, Process Biochem. 44 (2009) 490-493.

[14] K.C. Koo, D.H. Lee, J.H. Kim, H.E. Yu, J.S. Park, J.S. Lee, Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from Pholiota adiposa, J. Microbiol. Biotechnol. 16 (2006) 757-763.

[15] J.H. Zhang, E. Tatsumi, C.H. Ding, L.T. Li, Neural network based PID control on NC milling process with high-speed, Food Chem. 98 (2006) 551-555.

[16] E. Smacchi, M. Gobbetti, Bioactive peptides in dairy products: Synthesis and interaction proteolytic enzymes, Food Microbiol. 17 (2000) 129-141.

[17] S.M. Kamau, S.C. Cheisonn, W. Chen, X.M. Liu, R.R. Lu, Alpha-lactalbumin: its production technologies and bioactive peptides, Compr. Rev. Food Sci. F. 9 (2010) 197-212.

[18] R. Nagpal Behare, R. Rana, A. Kumar, M. Kumar, S. Arora, F. Morota, S. Jain, H. Yadav, Bioactive peptides derived from milk proteins and their health beneficial potentials: An update, Food Funct. 2 (2011) 18-27.

[19] S. Yano, K. Suzukim, G. Funatsum, Isolation from α-Zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity, Biosci. Biotech. Biochem. 60 (1996) 661-663.

[20] R. Shukla, M. Cheryan, Zein: The industrial protein from corn, Ind. Crop Prod. 13 (2001) 171-192.

[21] M.M. Contreras, I. López-Exposito, B. Hernández-Ledesma, M. Ramos, I. Recio, Application of mass spectrometry to the characterization and quantification of foodderived bioactive peptides, J. AOAC Int. 91 (2008) 981-994.

[22] G.S. Gilani, C. Xiao, N. Lee, Need for accurate and standardized determination of amino acids and bioactive peptides for evaluating protein quality and potential health effects of foods and dietary supplements, J. AOAC Int. 91 (2008) 894-900.

[23] B.M. Prasanna, S.K. Vasal, B. Kassahun, N.N. Singh, Quality protein maize, Curr. Sci. India. 81 (2001) 1308-1319.

[24] T.J. Anderson, B.P. Lamsal, Zein extraction from corn, corn products, and coproducts and modifications for various applications: A review, Cereal Chem. 88 (2011) 159-173.

[25] A. Esen, An immunodominant site of γ-zein is in the region of tandem hexapeptide repeats, J. Protein Chem. 9 (1990) 453-460.

[26] A.S. Tatham, J.M. Field, V.J. Morris, K.J. I'Anson, L. Cardle, M.J. Dufton, P.R. Shewry, Solution conformational analysis of the α-zein proteins of maize, J. Biol. Chem. 268 (1993) 26253 -26259.

[27] A. Kale, F. Zhu, M. Cheryan, Separation of high-value products from ethanol extracts of corn by chromatography, Ind. Crop Prod. 26 (2007) 44-53.

[28] S.R. Bean, G.L. Lookhart, J.A. Bietz, Acetonitrile as a buffer additive for free zone capillary electrophoresis separation and characterization of maize (Zea mays L.) and Sorghum (Sorghum bicolor L. Moench) storage proteins, J. Argic. Food Chem. 48 (2000) 318-327.

[29] J. Landry, S. Delhaye, C. Demerval, Comparative efficiencies of isopropyl and tert-Butyl alcohols for extracting zeins from maize endosperm, J. Agric. Food Chem. 50 (2002) 4131-4134.

[30] M.T.H. Khan, W. Yimingjiang, I. Sylte, Molecular recognition of thermolysin and homologous zinc-metalloproteinases, Minerva Biotecnol. 19 (2007) 139-150.

[31] X.X. Lu, X.H. Chen, J.Z. Tang, Studies on the functional property of enzymatic modified corn protein, Food Sci. 21 (2000) 13-15.

[32] X.Q. Zheng, L.T. Li, X.L. Liu, X.J. Wang, J. Lin, D. Li, Production of hydrolysate with antioxidative activity by enzymatic hydrolysis of extruded corn gluten, Appl. Microbiol. Biotechnol. 73 (2006) 763-770.

[33] H.J. Suh, J.H. Whang, D.B. Suh, S.H. Bae, D.O. Noh, Preparation of angiotensin I converting enzyme inhibitor from corn gluten, Process Biochem. 38 (2003) 1239-124.

[34] M. Gobbetti, E. Smacchi, A. Corsetti, M. Bellucci, Inhibition of proteolytic enzymes from Pseudomonas fluorescens ATCC 948 and angiotensin I-converting enzyme by peptides from zein, hordein, and gluten hydrolysates, J. Food Protect. 60 (1997) 499-504.

[35] S. Miyoshi, H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka, S. Maruyama, Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate, Agric. Biol. Chem. 55 (1991) 1313-1318.

[36] A. Abrahim, M. Al-Sayah Skrdla, P.Y. Bereznitski, Y. Chen, N. Wu, Practical comparison of 2.7 μm fused-core silica particles and porous sub-2 μm particles for fast separations in pharmaceutical process development, J. Pharm. Biomed. Anal. 51 (2010) 131-137.

[37] L. Novakova, H. Vlckova, A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation, Anal. Chim. Acta 656 (2009) 8-35.

[38] N. Parris, L.C. Dickey, Extraction and solubility characteristics of zein proteins from dry-milled corn, J. Agric. Food Chem. 49 (2001) 3757-3760.

[39] J.M. Rodríguez-Nogales, M.C. García, M.L. Marina, Development of a perfusion reversed-phase high performance liquid chromatography method for the characterisation of maize products using multivariate analysis, J. Chromatogr. A 1104 (2006) 91-99.

[40] J.W. Lawton, Zein: A history of processing and use, Cereal Chem. 79 (2002) 1-18.

[41] R.A. Moreau, A. Nuñez, V. Singh, Diferuloylputrescine and p-coumaroyl-feruloylputrescine, abundant polyamine conjugates in lipid extracts of maize kernels, Lipids 36 (2001) 839-844.

[42] S. LeClere, E.A. Schmelz, P.S. Chourey, Phenolic compounds accumulate specifically in maternally-derived tissues of developing maize kernels, Cereal Chem. 84 (2007) 350-356.

[43] R.J. Simpson, Protein and Proteomics, A laboratory manual cold spring harbor Laboratory Press, New York (2003).

[44] J.R. Whitaker, A.G.J. Vorageb, D.W.S. Wong, Handbook of food enzymology, Marcel Dekker Inc., New York (2003).

[45] M.C. García, A.C. Hogenboom, H. Zappey, H. Irth, Effect of the mobile phase composition on the separation and detection of intact proteins by reversed-phase liquid chromatography-electrospray mass spectrometry, J. Chromatogr. A 957 (2002) 187-199.

[46] W.D. Chiang, M.J. Lee, W.S. Guo, T.C. Tsai, Protein hydrolysate batch production with angiotensin I-converting enzyme inhibitory activity form egg whites, J. Food Drug Anal. 14 (2006) 385-390.

[47] J. Duan, Z. Liang, C. Yang, J. Zhang, L. Zhang, W. Zhang, Y. Zhang, Rapid protein identification using monolithic enzymatic microreactor and LC-ESI-MS/MS, Proteomics 6 (2006) 412-419.

Article 5

Development of a HPLC-ESI-Q-ToF methodology for the determination of three highly antihypertensive peptides in maize crops

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Abstract

The simultaneous quantification of three highly antihypertensive peptides (LRP, LSP, and LQP) in six maize crops using novel HPLC–ESI-Q-ToF methodology is presented. The method included the extraction of α-zein proteins from maize, their purification by acetone precipitation, digestion with thermolysin and HPLC separation in a fused-core column. Several MS parameters were optimized to increase sensitivity and reduce spontaneous fragmentation of peptide ions into the ESI source. The ions with *m*/*z* 193.1315, 385.2558 (for LRP), 316.1867 (for LSP), and 357.2132 (for LQP) were monitored in the optimization and characterization of the method. In order to improve the repeatability, sensitivity, and the stability of peptides in the sample, the removal of urea was required. The use of two solid-phase extraction methods to remove urea from digested extract was evaluated. For the first time filter aided sample preparation approach for the study of bioactive peptides in foodstuffs has been proposed. The optimized HPLC–ESI-Q-ToF method was characterized by the evaluation of linearity, LOD, LOQ, precision, and recovery. A study on the existence of matrix interferences was also performed. The developed method was applied to the quantification of LRP, LQP, and LSP peptides in maize lines using the standard addition method. The results showed the highest yield of LSP peptide in EZ11A line and LRP and LQP peptides in A632 line.

Keywords:

Antihypertensive peptides; HPLC; ESI-Q-ToF; Fused-core column; Maize; Quantification

1. Introduction

High blood pressure or hypertension is one of the most spread health problems worldwide. Treatment of hypertension has been shown to prevent cardiovascular diseases (CVDs), which were estimated in 2010 by World Health Organization (WHO) as the main cause of death in developing countries. Last clinical studies have shown that, in addition to synthetic drug therapy, the ingestion of fermented milk with antihypertensive peptides enables to reduce blood pressure in patients with first stage of hypertension [1]. Antihypertensive compounds act on renin-angiotensin system which is at the core of blood pressure regulation in living organisms. Briefly, angiotensin I converting enzyme (ACE) converts angiotensin I to a potent vasoconstrictor angiotensin II, and at the same time inactivates a potential vasodilator bradykinin. In this regards, those compounds inhibiting ACE activity would cause the drop of blood pressure [2]. ACE inhibitors can be classified like synthetic drugs (*e.g.* captopril and enapril) and naturally existing antihypertensive peptides, with the difference that the first one causes several side effects [3]. Common features of antihypertensive peptides are short sequences (2–12 amino acid residues) containing significant amount of hydrophobic residues [4] and the presence of proline at the C-terminal [5].

Corn or maize (*Zea mays* L.) is a major crop which accounts 15–56% of the total daily calories in human diets, particularly in Africa and Latin America [6]. Maize contains 6–12% of proteins depending on the line, where zeins (the prolamin fraction) account to 60% of total proteins [7]. According to Esen classification, zeins can be distinguished based on their molecular masses as α-zeins (21–25 kDa), β-zeins (17 kDa), γ-zeins (18 and 27 kDa), and δ-zeins (10 kDa). Almost 75–85% of total zeins are α-zeins [8]. Maize contains three highly antihypertensive peptides derived from α-zeins [9]. These peptides exhibit one of the highest half maximal inhibitory concentration (IC_{50}) values, namely leucine–arginine–proline (LRP = 0.29 μM), leucine–serine–proline (LSP = 1.7 μM), and leucine–glutamine–proline (LQP = 2.0 μM). Nevertheless, the presence of these peptides can vary in relation with the maize genotype being very interesting the development of analytical methodologies for their reliable quantification. In our previous work, we have developed a quick and effective analytical methodology for the extraction and purification of α-zeins from whole maize kernels, their further solubilization, and digestion by thermolysin [10]. Nevertheless, in order to perform the absolute peptide quantification in such a high complex sample, better method selectivity and sensitivity are required.

The food peptidome is an ambitious challenge to be analyzed in the food matrix. Two main parameters need to be considered, namely peptide length and dynamic range. In nutritional peptidomic, a great peptide length distribution with high dynamic range is attributed to the diverse selectivity and specificity at the processing level (enzymatic digestion or fermentation) [11]. In this regards, antihypertensive peptides, usually composed by di- or tri-peptides, could be considered as a difficult task in nutritional peptidomic. In order to obtain antihypertensive peptides, enzymes with low specificity are used, which leads to very complex matrices. For the analysis of bioactive peptides in a food matrix, high performance liquid chromatography (HPLC) has been the technique most commonly applied [11]. Significant advantage in the analysis of complex matrices is the use of chromatographic columns with small particle size, which enable the separation with high efficiency and short analysis times. Nevertheless, at the same time these columns generate high back pressure which requires a special instrumentation (ultra high performance liquid chromatography, UPLC). Fused-core particles constitute a new type of stationary phases offering high efficiency and short analysis times while maintaining back pressure at a reasonable level [12]. In addition, the use of mass spectrometry (MS) coupled to HPLC has shown to provide several advantages in the analysis of complex matrices [13].

Functional foods containing antihypertensive peptides are having a growing interest since diet is the factor with the most profound life-long influence on health. There are 24 patented and commercially available antihypertensive functional foods described in literature [2, 14, 15]. Despite the great interest on the discovery of new antihypertensive peptides in foodstuffs [16-19], their quantification is quite scarce [20]. However, the establishment of standardized methodologies in order to quantify these targeted peptides in foodstuffs is essential, since their content can significantly vary with genotype [20- 21]. The relevance of analytical methods dealing with the quantification of peptides in food matrices is often emphasized. Nevertheless, the quantification of antihypertensive peptides is not common, probably due to the complexity of food matrices from which targeted peptides need to be distinguished in an unambiguous way. HPLC has been the only technique used for this purpose using UV detection [22-24] or more frequently MS detection (HPLC–MS [25-28], HPLC–MS/MS [29-32], or HPLC–MS^3 [33-34]). Nevertheless, in no case quantification of LRP, LSP, and LQP in maize crops was performed.

The aim of this work was the development of a sensitive analytical methodology enabling the quantification of highly antihypertensive peptides LRP, LSP, and LQP in maize crops by high-performance liquid chromatography coupled with electrospray ionization-quadrupole-

time-of-flight-mass spectrometry (HPLC–ESI-Q-ToF-MS) using a fused-core column. The method was characterized and the evaluation of the content of these three antihypertensive peptides in different maize crops lines was performed.

2. Materials and methods

2.1. Chemicals and samples

Acetonitrile (ACN) (MS and HPLC grade), trifluoroacetic acid (TFA), and thermolysin were purchased from Sigma (St. Louis, MO, USA). Tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), β-mercaptoethanol, formic acid (FA), and sodium hydroxide were from Merck (Darmstadt, Germany). Acetic acid (AA), acetone, ethanol, and urea were supplied by Scharlau Chemie (Barcelona, Spain). HPLC grade solvents (unless otherwise stated) were use. Peptide standards (LRP, LSP, and LQP) were synthesized by Genescript (Genescript Corp., Piscataway, NJ, USA), all with more than 98% of purity. Maize lines (EZ6, EZ9, B73, EZ11A, A632, and Millo Corvo (MC)) were kindly donated by a Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain) and stored in the fridge at 4 °C. The moisture of samples was determined using official reference method [35]. Thermolysin powder and stock solution (2.5 mg/mL) and peptide standards were always stored at -20 °C.

2.2. α-Zeins extraction

Whole maize kernels were ground with a domestic miller and extracted using 70% of ethanol with assistance of an ultrasonic probe (VCX.130, Sonic Vibra-Cell, Hartford, CT, USA) as in a previous work [10]. Afterwards, sample was centrifuged (4000 × *g*, 10 min, 20 °C) and separated from the pellet.

2.3. Purification and in-solution digestion of α-zeins

α-Zein extract was purified by precipitation with acetone containing 0.07% of β-mercaptoethanol as in a previous work [10]. Air dried pellet was dissolved in 20 mL of buffer (10 mM Tris–HCl (pH 8.0) containing 8 M urea) and left overnight at 4 °C. Dissolved α-zeins were diluted in water in order to reach an urea concentration of 6 M. Therefore, protein extract was mixed with thermolysin stock solution (enzyme to substrate ratio 5:100 (w:w)), and incubated for 6 h at 50 °C in a hot air oven (Memmert, model 300, Schwabach, Germany). The reaction was stopped and sample was desalted using solid phase extraction (SPE).

2.4. Filter aided sample preparation (FASP)

A FASP protocol [36] that had never been applied to food matrices was tested for the extraction and digestion of antihypertensive peptides with modifications. Filters with Mwco of 10 kDa (Amicon® Ultra, Millipore) were washed with 0.25 mL of freshly taken water from a Milli-Q system (Millipore, Bedford, MA, USA) and centrifuged for 20 min (12,100 × *g*). Therefore, 93 µL of α-zein extract was diluted till 0.25 mL with 8 M urea in 0.1 M Tris–HCl buffer (pH 8.5). Next, 0.23 mL of this mixture was applied onto the filters and centrifuged (20 min, 12,100 × *g*). Filters retaining proteins were washed once with 0.20 mL of 8 M urea in 0.1 M Tris–HCl buffer (pH 8.5) and twice with 0.10 mL of 8 M urea in 0.1 M Tris–HCl buffer (pH 8.0) and centrifuged after each step for 20 min (12,100 × *g*). The thermolysin solution (0.22 mL) dissolved in 10 mM Tris–HCl buffer (pH 8.0) containing 6 M urea was applied onto the filters (enzyme to substrate ratio 5:100 (w:w)), and incubated for 6 h at 50 °C in the hot air oven. After incubation, filters were centrifuged (20 min, 12,100 × *g*) and the eluates were collected. Filters were washed with a solution containing 50 µL of 0.5 M NaCl and centrifuged (20 min, 12,100 × *g*). Both eluates were combined. The reaction was stopped and the eluates were analyzed.

2.5. Solid phase extraction (SPE)

Two different kinds of C18 cartridges were tested for the desalting of extracts using SPE: C18 Sep-Pak (*Waters* Associates Inc., Milford, MA, USA) and C18 Supelco (Sigma, St. Louis, MO, USA). Sample (0.25 mL) acidified with 10% of AA was loaded onto SPE columns, previously conditioned with 2 mL of ACN and 2 mL of solvent A. Solvent A for Sep-Pak cartridge was 0.1 M AA and for Supelco cartridge was 0.1% TFA. SPE columns were washed twice with 1 mL of solvent A. Peptides from cartridges were eluted with 0.5 mL (Sep-Pak cartridge) or 3 mL (Supelco cartridges) of solvent B. Solvent B consisted of 80% ACN in solvent A. Collected eluates from Sep-Pak or Supelco cartridges were evaporated and resuspended into 0.25 mL of 10% AA.

2.6. HPLC analysis

The separations were performed on a modular Agilent Technologies liquid chromatograph (Pittsburgh, PA, USA) consisting of a degassing system, a quaternary pump, a thermostabized compartment for the column, an injection system, and an UV detector (series 1100). Separation was performed using previously optimized conditions [10]. The Ascentis Express Peptide ES-

C18 column (100 mm × 2.1 mm I.D., with 2.7 µm particle size) with an Ascentis Express Guard column (5 mm × 2.1 mm I.D., with 2.7 µm particle size), both from Supelco (Bellefonte, PA, USA), were employed. The optimized conditions for the separation of peptides were: mobile phase A, Milli-Q water/20 mM AA; mobile phase B, ACN/20 mM AA; flow-rate, 0.4 mL/min; temperature, 40 °C. The optimized gradient was: 3% B for 5 min, 3–5% B in 5 min, 5–97% B in 2 min, and 97–3% B in 2 min. The injection volume was 5 µL. Peptides were detected using quadrupole-time-of-flight (Q-ToF) MS (instrument series 6530) with electrospray ionization (ESI) jet stream ionization from Agilent Technologies.

2.7. MS analysis

The mass spectrometer operated in the positive ion mode using only ToF analyzer and a mass range 100-3200 *m*/*z*. Optimization of ESI conditions was performed by injecting in triplicate a standard solution containing the three peptides at a concentration of 10 ppm. Samples were analyzed immediately after preparation. The extracted ion chromatogram (EIC) peak areas obtained for each peptide ion and their fragments were compared using one-way ANOVA test. Optimized ESI conditions were: fragmentator voltage, 100 V for 3.5 min and 150 V till the end of analysis; nozzle voltage, 0 V; nebulizer pressure, 50 psig; capillary voltage, 3500 V; gas temperature, 300 °C; gas flow, 10 L/min. The jet stream sheath gas flow and temperature were 5.5 L/min and 250 °C, respectively.

2.8. Characterization of the method and calibration

All solutions were injected, at least by triplicate. Calibration was performed by the external standard, the standard addition, and the single point calibration methods. The external calibration method was performed in ranges 1–59 ppb (9 points) for LRP, 25–2297 ppb (10 points) for LSP, and 3–304 ppb (8 points) for LQP. Each peptide calibration solution was prepared by individual dilution of peptide standard stock solution to desired peptide concentration. For each injection, the EIC of peptides was extracted with different extraction windows (EW) (10–200 ppm) and EIC peak areas were averaged. The EZ6 maize line was employed for the characterization of the method and the EZ6 and EZ9 lines for the study of the existence of the matrix interferences. The standard addition method was performed by the addition of known amounts of peptide standard solution to two individual digested extract samples so that the concentration of peptides in the sample was 0%, 10%, 20% and 30%. Samples were injected in triplicate and EIC peak areas of peptides were extracted, averaged,

and plotted against the amount of added peptide standard. The comparison of standard addition method with external standard method enabled to establish the existence of matrix interferences. The quantification of the three antihypertensive peptides in different maize lines was performed by means of the single point calibration method [37]. Single point calibration was performed by the injection of two different sample solutions for every maize sample: one maize solution and an identical maize solution spiked with known amounts of peptide standards. This kind of calibration was carried out after checking that the intercept did not significantly differ from zero. EIC peak areas for LRP, LSP, and LQP were extracted, respectively, using 200 ppm, 100 ppm, and 100 ppm extraction windows. Limits of detection and quantification (LOD and LOQ) were calculated using two approaches. Repeatability was evaluated by five repeated injections (n = 5) of standard solutions at two concentration levels. First solution contained 8 ppb, 53 ppb, and 62 ppb of LRP, LSP, and LQP, respectively, and the second one consisted of 41 ppb, 1159 ppb, and 155 ppb of these peptides. The EIC signals were extracted using different EW (10–200 ppm). Inter-day precision was determined by the triplicate injection of an extract in two consecutive days (n = 6). Inter-sample precision was established using five individually desalted maize samples. The relative standard deviation (RSD) was calculated using different EW. Selectivity was evaluated by the comparison of total ion chromatogram (TIC) obtained for the digested extract with its corresponding EIC of peptides using different EW. Recovery was estimated by the analysis of a digested extract spiked with peptide standards. Added standard concentrations corresponded to 0%, 20%, or 40% of each peptide concentration in the maize line.

2.9. Data analysis

MassHunter® Workstation Software (B.04.00) was used to carry out mass spectrometer control, data acquisition, and data analysis. After optimization, peptide ions for LRP (*m*/*z* 193.1315, and 385.2558) were extracted with 200 ppm EW while peptide ions for LSP (*m*/*z* 316.1867) and LQP (*m*/*z* 357.2132) were extracted using 100 ppm extraction window. Statistical analysis was performed using Software Statgraphics Plus 5.1 (*Statpoint Technologies, Inc*., Warrenton, VA, USA).

3. Results and disscusion

MS spectra of LRP, LSP, and LQP standard peptides were obtained using the following MS starting conditions: positive ion mode; mass range 100–3200 *m*/*z*: (just ToF mode)

fragmentator: 200 V; skimmer: 60 V; OCT 1 RF: 750 V; Gas temp.: 300 °C; drying gas: 10 L/min; Nebulizer: 30 psig; Capillary voltage: 3500 V; Sheath gas temp. and flow: 400 °C, 12 L/min; nozzle: 0 V. In all cases, in-source fragmentation of peptides was observed. Fig. 1 depicts the MS spectrum of LRP (Fig. 1A), LSP (Fig. 1B), and LQP (Fig. 1C). The MS spectrum of LRP contained singly $[M+H]^+$ and doubly charged $[M+2H]^{2+}$ peptide ions with *m*/*z* values 385.2558 and 193.1315, respectively. Moreover, the LRP ion fragment y_2^+ with *m*/*z* 272.1717 was also observed as a consequence of in-source ion fragment. This effect is probably due to the strong effect of basic arginine (R) which maintains the positive charge of the peptide. Spectra corresponding to LSP and LQP were much more complex than the LRP spectrum. In both cases just singly charged peptide ions were observed, with *m*/*z* 316.1867 and 357.2132 for LSP and LQP, respectively. Moreover, sodium adducts $[M+Na]^+$ and dimmer ions $[2M+H]^+$ (part of the spectrum not shown) were also present in both peptide spectra. Due to similar elution times, LSP spectrum showed signals corresponding to LQP and vice versa (signals marked with an X). Furthermore, highly abundant y_2^+ and b_2^+ ions were observed after in-source fragmentation of singly charged peptides. For LQP, the b_2^+ ion was more intense than y_2^+ ion which would suggest the preferable cleavage between Q and P. Strong ion with *m*/*z* 116 would suggest the presence of y_1^+ ion in both spectra. The extraction of EIC for the proline residue ion (*m*/*z* 116.0706) confirmed that this ion was present just during the elution time of LSP/LQP peptides. However, due to the high co-elution of these two peptides the y_1^+ ion could not be assigned to one peptide. The same approach was applied for ion with *m*/*z* 114 (possible b_1^+ ion). However, in this case, the ion was present during all analysis and was labeled as background (BS). Moreover, both peptides also showed the neutral loss of CO (−28 Da) from b_2^+ ions (a_2^+ ions). The specific neutral loss of ammonia (−17 Da) from y_2^+ ion was also observed in the spectra.

Optimization of ESI parameters. In order to reduce the spontaneous fragmentation of peptides and to increase the intensity of peptide signals these starting ESI conditions were next optimized. The instrumental parameters to optimize were divided into two groups. First group was constituted by parameters depending on the analyte nature (fragmentator voltage, capillary voltage, and nozzle voltage) and the second group included the parameters depending on the flow and composition of the mobile phase (nebulizer pressure, temperature and flow of the drying gas, and temperature and flow of sheath gas).

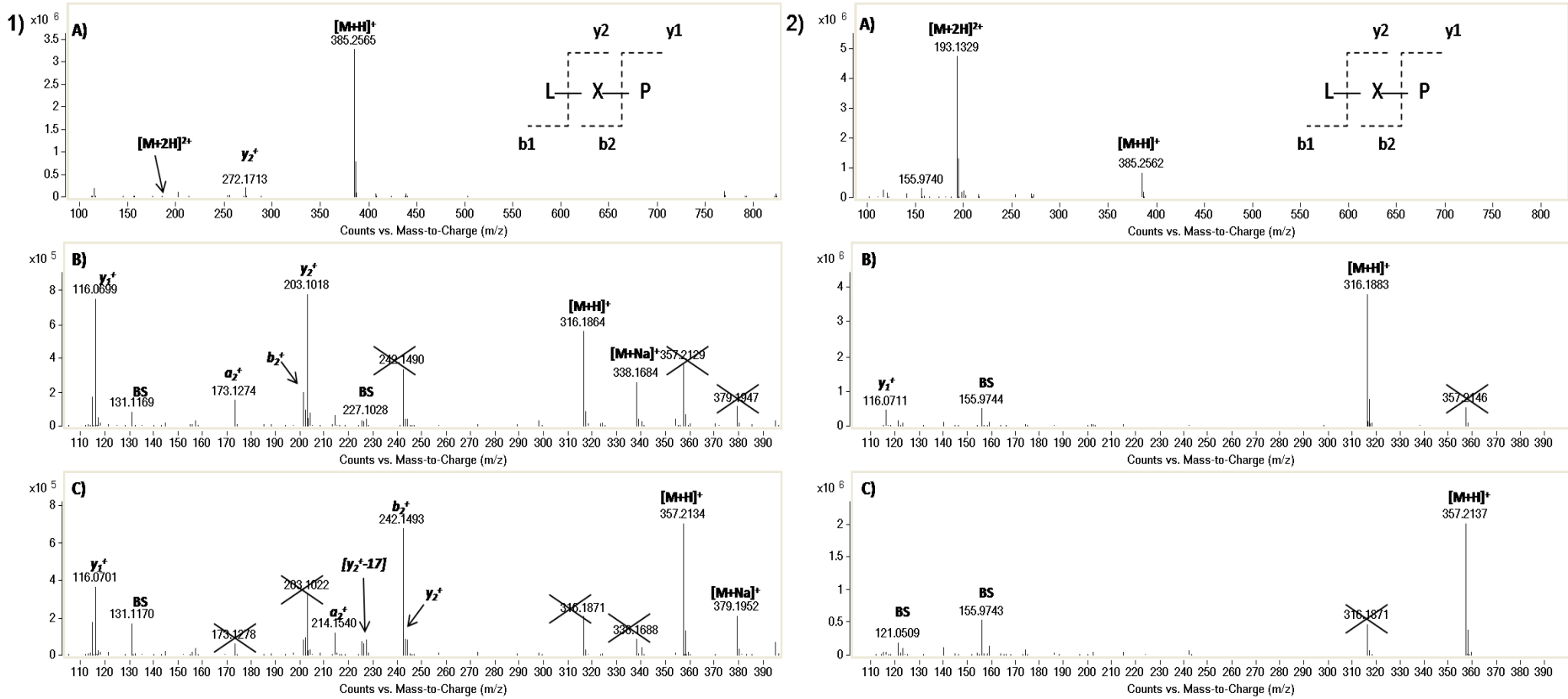

Fig. 1. MS spectra of peptide standards (10 ppm): (A) LRP, (B) LSP, and (C) LQP obtained: (1) before optimization of the ESI parameters and (2) after its optimization. MS conditions: positive ion mode; mass range 100–3200 *m/z*; just ToF mode. ESI conditions before optimization (1): fragmentator: 200 V; skimmer: 60 V; OCT 1 RF: 750 V; gas temp.: 300 °C; drying gas: 10 L/min; nebulizer: 30 psig; capillary voltage: 3500 V; sheath gas temp. and flow: 400 °C, 12 L/min; nozzle: 0 V. ESI conditions after optimization (2): fragmentator: 100 V and then 150 V; skimmer: 60 V; OCT 1 RF: 750 V; gas temp.: 300 °C; drying gas: 10 L/min; nebulizer: 50 psig; capillary voltage: 3500 V; sheath gas temp. and flow: 250 °C, 5.5 L/min; nozzle: 0 V. Chromatographic conditions: mobile phases: A, Milli-Q water/20 mM AA; B, ACN/20 mM AA; gradient: 3% B for 5 min, 3–5% B in 5 min, 5% B for 5 min, 5–95% B in 2 min, and 95–3% B in 2 min, T = 40 °C, inj. vol. = 20 µL, F = 0.4 mL/min (X is R in A, S in B, and Q in C). BS, background.

Fig. 2 depicts the influence of optimization of ESI parameters on the relative abundance of peptide signals. Since at the beginning of the optimization, peptide ions underwent the spontaneous fragmentation in the ESI source, all its fragmented ions has been merged in order to calculate a reliable intensity of presented peptide ion in the analysis. Relative abundance corresponding to the first measurement (*) was considered 100% and abundance signals obtained with the different parameters were related to this first abundance signal. The voltage of fragmentator is responsible for the introduction of the analyte into the MS system. Application of high fragmentator voltages can improve the sensitivity of detected ions by their faster transmission into the MS. At the same time, too high voltages can break the molecule and produce spontaneous fragmentation. Four fragmentator voltages were studied: 50 V, 100 V, 150 V, and 200 V. Decreasing the voltage of fragmentator to 100 V, slightly decreased the intensities of singly charged LRP ion, and noticeably of the y_2^+ ion. In addition, it rose up the intensity of doubly charged LRP ion by 400%. For LSP and LQP peptides, the use of 150 V eliminated almost all spontaneous fragmentation. It also enabled the improvement of intensities corresponding to peptide ions by 500% for LSP and by 270% for LQP. Taking into account these results, the analysis was divided into two MS time segments. The fragmentator voltage was maintained at 100 V during the first 3.5 min of analysis, at which LRP is eluting. Afterwards, the voltage rose up till 150 V for the elution of LSP (4.2 min) and LQP (4.5 min). Following capillary voltages were tested: 2500 V, 3000 V, 3500 V, 4000 V, and 4500 V. No change in the LRP signal was observed when varying the capillary voltage. For LSP and LQP peptides, just a slight improvement was observed for 3500 V, which was selected as the optimum for further analysis. Three nozzle voltages were tried: 0 V, 500 V, and 1000 V. The nozzle voltage can improve the ionization of compounds with heteroatoms. However, for all targeted peptide ions, there were no changes (500 V), or the intensities of ions were slightly decreased (1000 V) in comparison with the absence of nozzle voltage. In this regards, the nozzle voltage was maintained at 0 V. Nebulizer pressure was optimized in the range 30–60 psig. Highest pressure values (50 psig and 60 psig) resulted in a better ionization of peptides. Finally, a 50 psig nebulizer pressure was selected for next investigations. In order to improve the evaporation of the mobile phase and to reduce the diameter of micro-drops, the influence of gas temperature was studied in the range 200–350 °C. While LSP and LQP did not show any significant improvement when increasing gas temperature, LRP yielded the best ionization at 300 °C. This gas temperature was selected as the optimum.

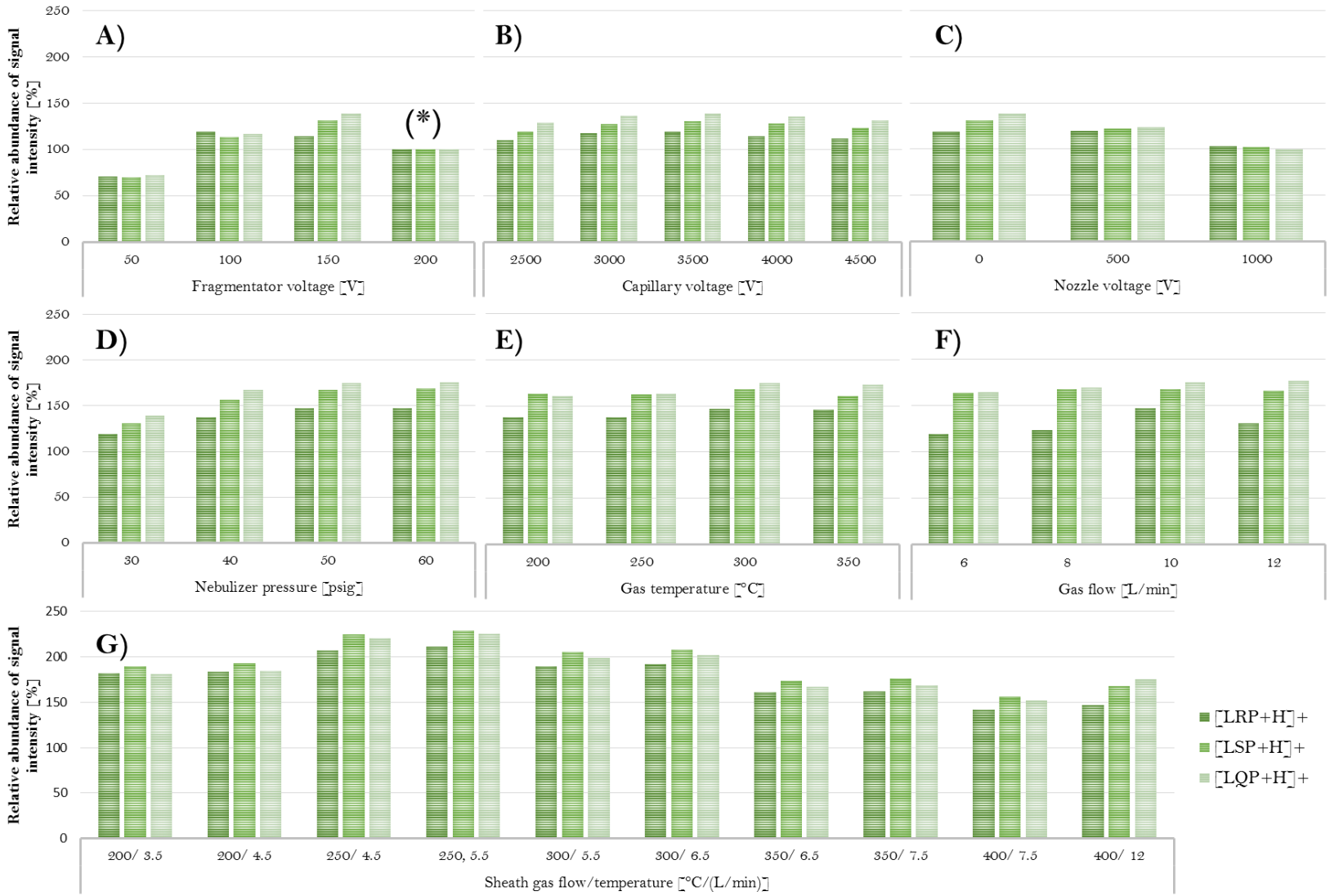

Fig. 2. The influence of ESI parameters: (A) fragmentator voltage [V], (B) capillary voltage [V], (C) nozzle voltage [V], (D) nebulizer pressure [psig], (E) gas temperature [°C], (F) gas flow [L/min], and (G) sheath gas temperature/flow [°C/(L/min)] on the relative signal abundances [%] of LRP, LSP, and LQP ions $[M+H]^+$. Chromatographic conditions as in Fig. 1.

Together with the gas temperature, higher nitrogen gas flow enhances the ionization of ions. Examined gas flows were: 6 L/min, 8 L/min, 10 L/min, and 12 L/min. The highest signal intensities were observed with 10 L/min (for LRP), with 8–12 L/min (for LSP), and with 10–12 L/min (for LQP). As a consequence, 10 L/min gas flow was chosen. Next optimized parameters were sheath gas temperature and flow. Both parameters are connected and cannot be studied separately since the use of higher sheath gas flows involves the use of higher sheath gas temperatures. The sheath gas is applied around the nebulizer needle, focusing the nebulizer cone, and improving the signal to noise ratio. Studied temperatures were in the range 200–400 °C and flow rates were between 3.5 and 12 L/min. In all cases, improved peptide intensities were observed for 250 °C with 5.5 L/min sheath gas flow. After optimization of all ESI parameters, the in-source fragmentation decreased significantly (see Fig. 1 and Fig. 2). The peptide LRP was detected as singly (*m*/*z* 385.2558) and doubly charge (*m*/*z* 193.1315) peptide ions, which represented, respectively, 14% and 83% of all detected LRP ions. On the other hand, LSP and LQP were detected just as singly protonated peptides, representing 95% of all detected ions in their MS spectra. Finally, the ions with *m*/*z* 193.1315, and 385.2558 for LRP, *m*/*z* 316.1867 for LSP, and *m*/*z* 357.2132 for LQP were selected for their determination. In comparison with the first measurement considered as 100% (*), the abundance obtained using the optimized parameters is more than twice higher.

3.1. Stability of peptide standard solutions

Stability of standard solutions of LRP, LSP, and LQP was studied by the comparison of chromatograms obtained when they were injected into the chromatographic system at different times. Peptide standards at a concentration of 20 ppm were dissolved in water (LRP and LQP) or in ACN (LSP) (according to the recommendation guide of Genescript). Samples were injected twice immediately after preparation, and after 4 h or 24 h kept to room temperature. Changes in the EIC peak areas of peptides were observed in all cases. After one day the most dramatic differences were observed for LRP (a decrease in EIC peak area of 55% relative to the initial EIC peak area was observed) and LSP (an increase in EIC peak area of 220% relative to the initial EIC peak area was observed) while just slight modifications were detected in the LQP area (106%). In order to increase this stability, a 10% of FA or AA was added to each solution. A standard solution containing the three peptides (10 ppm) was injected in triplicate after 0 h, 7 h, 24 h, and 96 h from the preparation. Experiments were performed with samples stored at room temperature, 4 °C or −20 °C. Comparison of EIC peak areas showed that peptides were more stable when prepared with 10% AA than with 10% FA or with water or ACN. The

EIC peak areas for peptide standards stored at 4 °C or −20 °C did not keep. The application of one-way ANOVA test and Multiple Range Test showed that LSP and LQP were stable during 7 h while LRP was stable during 96 h (*P*-value 0.16) at room temperature.

3.2. Sample preparation

An analytical methodology for the extraction of α-zeins from whole maize kernels, their further purification, and digestion by thermolysin has been recently developed [10]. For the first time zein proteins were digested after their extraction from whole maize kernels. The challenge to select a suitable buffer to dissolve alcohol soluble proteins and to keep active the enzyme had been overcome. This analytical methodology showed to be precise, effective, and robust being its main drawback the time required for the acetone precipitation of proteins, their further air drying, and overnight dissolution in an appropriate buffer. In order to reduce sample preparation time, filter aided sample preparation (FASP) approach was tested in this work. The base of this recently introduced approach is the enzymatic digestion of retained proteins on a weight cut-off membrane [36]. In our case, direct introduction of α-zeins, on a membrane with molecular weight cut-off of 10 kDa, would avoid the previous purification of proteins by acetone precipitation and overnight dissolution. Small molecular weight interferences should pass through the membrane while α-zeins (21–25 kDa) should be retained. After thermolysin digestion on a membrane, peptides could be recovered by simple centrifugation since their molecular masses would be smaller than 10 kDa. This was the first time that the FASP approach was tested for the analysis of bioactive peptides in foods. Two extracts of α-zeins were also introduced on a membrane and digested using the FASP protocol. The same extracts were purified, dried, dissolved, and in-solution digested by thermolysin, using previously optimized conditions [10]. Afterwards, extracts were diluted to an appropriate volume in order to reach the same peptide concentration of the extracts obtained by FASP. Samples were injected in triplicate. Comparison of peptide profiles obtained with the FASP protocol and with in-solution digestion showed, at a glade, a high similarity. Nevertheless, the attempt to obtain the EIC signals for LRP, LSP, and LQP was not successful, probably because these peptides were fully or partly retained on the membrane. As a consequence, the in-solution digestion protocol was selected in this work.

3.3. Desalting of digested extract

Several compounds such as urea are well known to suppress ionization in ESI. In order to remove high concentration of urea (6 M) in the digested extract of α-zeins, solid-phase extraction (SPE) was proposed. Two different C18 cartridges containing different amounts of beads were compared. Two digested extracts of α-zeins (0.25 mL) were subjected to Sep-Pak (50 mg of beads) or Supelco (500 mg of beads) cartridges. Peptides were retained and desalted from both C18 cartridges by employing a 0.1 M AA solution. Peptides were eluted with a 80% ACN/0.1 M AA solution. After evaporation and dilution of pellet in 0.25 mL with a 10% AA solution, samples were injected by triplicate into the HPLC–MS system. Elimination of urea from sample resulted in higher intensities, better defined EIC peaks, improved ionization of ions (particularly ion with *m*/*z* 193.1315), and improved repeatability. Indeed, RSDs of two samples injected by triplicate improved from 10.25% (in urea) to 5.09% (Sep-Pak) and 4.12% (Supelco) for LRP, from 7.87% (in urea) to 2.07% (Sep-Pak) and 0.46% (Supelco) for LSP, and from 10.14% (in urea) to 3.75% (Sep-Pak) and 1.05% (Supelco) for LQP. On the other hand, the comparison of Sep-Pak and Supelco cartridges was not so straightforward. In fact, injection repeatability and TICs and base peak chromatograms (BPCs) were very similar. Nevertheless, the EIC peak area for LRP was around three times lower in the sample extracted with the Sep-Pak cartridge than in the sample obtained with the Supelco cartridge. Unlike LRP, the signals for LSP and LQP were around three times higher when the Sep-Pak cartridge was applied. In order to explain this behavior, the elution order of these peptides when using similar conditions in a C18 HPLC column was taken into account. Smaller signal obtained for LRP on the Sep-Pak cartridge and higher for LSP and LQP could be due to LRP lost during the cleaning step, while LSP and LQP were totally eluted. Regarding the Supelco cartridge, with ten times more C18 beads than the Sep-Pak one, LRP seemed to be properly eluted which would explain the higher signal observed for this peptide using this cartridge. Nevertheless, the LSP and LQP peptides were probably much retained in this cartridge which would explain their lower recovery. In order to recover all peptides with the highest score, experimental conditions using the Supelco cartridge were optimized.

3.4. Optimization of new SPE method on Supelco cartridge

Optimization of new SPE method was performed using a standard solution containing the three peptides. Firstly, different ion pairing agents (20 mM AA and 0.1% TFA) and ACN concentrations were tested. The stepwise elution was performed using 5%, 30%, and two times

80% ACN (each time 0.25 mL) in 0.1% TFA. In addition, the loading and washing eluates were collected and analyzed. All eluates were evaporated and resuspended in 0.25 mL of 10% AA. Results showed higher effectiveness of 0.1% TFA to retain peptides on the bead, especially for LRP. All peptides eluted with 80% of ACN. Moreover, less than 1.4% of each peptide was lost during the loading and washing steps when using 0.1% TFA. In order to obtain a higher recovery, the elution volume was optimized. The use of 3 mL of 80% ACN with 0.1% TFA enabled to obtain recoveries of the standard peptides very close to 100%. Selectivity of the two SPE methods was demonstrated by the comparison of TIC with EIC of peptides in a maize line digest (see Fig. 3). Both methods were highly selective for LSP and LQP peptides, since just one intense signal was observed (E and F). In addition, the EIC signals for both peptides were detected at exactly the same retention time as their peptide standards. The results for LRP peptide showed that just the use of Sep-Pak protocol provided selectivity for this peptide (Fig. 3, 1C) while the Supelco optimized method did not enable its detection in the digested sample. Although, some additional signals in the EIC of LRP peptide were detected, its unambiguous identification was undisputed. The comparison of TIC and BPC for both SPE methodologies demonstrated that the sample obtained with the Supelco cartridge and 0.1% TFA yielded more intense signals, especially in the zone where first eluting compound, like LRP appeared while sample obtained with the Sep-Pak cartridge resulted in a more clean profile. The greater amount of compounds eluting at the same elution time of LRP, when the sample was obtained with the Supelco cartridge, could suppress LRP ionization which would explain the obtained results. From these results, the Sep-Pak protocol, which proved to be a robust approach for all three peptides, was selected for their quantification.

3.5. Method characterization

The optimized method was characterized by the evaluation of linearity in the working concentration range, limits of detection and quantification, repeatability, intermediate precision, recovery, inter-sample precision, and matrix effects. In all cases, sample was injected at least by triplicate and different EW values (from 10 to 200 ppm) were employed. Table 1 groups the results obtained. Calibration by the external standard method yielded correlation coefficients close to unit for the three peptides. Slopes of calibration line were highly dependent on the EW.

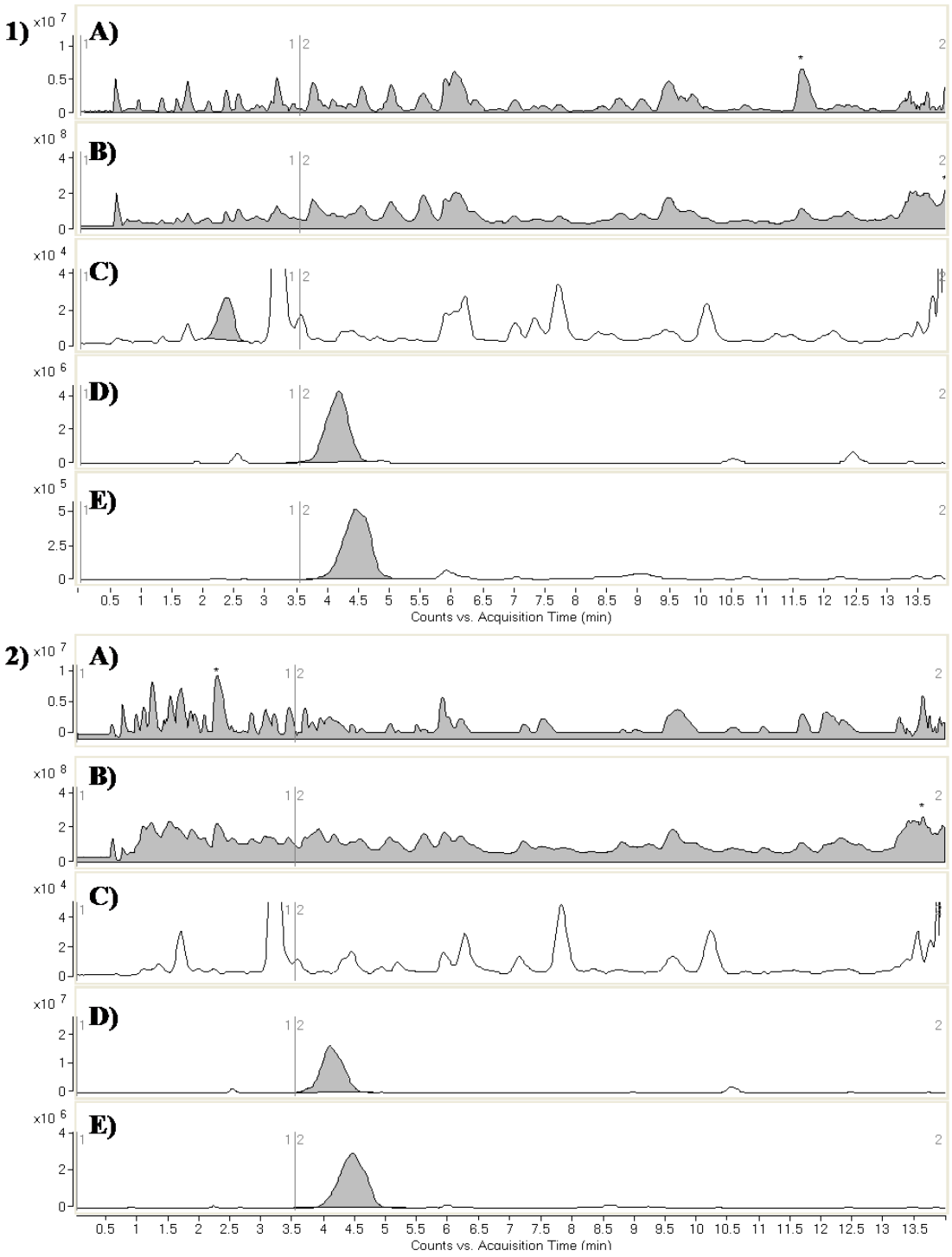

Fig. 3. Comparison of digested extract of α-zeins obtained after SPE methods using Sep-Pak or Supelco cartridges: (1) Sep-Pak cartridge with 0.1 M AA as solvent A, or (2) Supelco cartridge with 0.1% TFA as solvent A. Comparison of BPC (A), TIC (B) and EICs for LRP (C), LSP (D), and LQP (E) peptide ions, using extraction window 100 ppm. MS conditions: positive ion mode; mass range 100–3200 *m/z*; just ToF mode. ESI conditions: fragmentator: (1) 100 V then (2) 150 V; skimmer: 60 V; OCT 1 RF: 750 V; gas temp.: 300 °C; drying gas: 10 L/min; nebulizer: 50 psig; capillary voltage: 3500 V; sheath gas temp. and flow: 250 °C, 5.5 L/min; nozzle: 0 V. Chromatographic conditions as in Fig. 1. The line 1|2 indicated the change in capillary voltage.

The application of wider EW increased the slopes of all calibration curves. LOD and LOQ were calculated as the standard deviation divided by the slope value and multiplied by 3 or 10, respectively, and as the minimum concentration yielding a SNR equal to 3 and 10 times, respectively. LOD and LOQ were estimated at different EW values and when using the first approach they showed a strong dependence with EW while it did not have any influence on the LOD and LOQ calculated from the SNR. Thus, this was the method finally selected to calculate these parameters. Estimated LODs using EW 200 ppm for LRP and 100 ppm for LSP, and LQP were 0.0039 μg/g maize, 0.0003 μg/g maize, and 0.0073 μg/g maize, respectively. Moreover, estimated LOQs using the same EWs were 0.0021 μg/g maize, 0.0130 μg/g maize, and 0.0010 μg/g maize for LRP, LSP, and LQP, respectively. Instrumental repeatability was evaluated using two standard solutions. RSD values for EIC peak areas for the most concentrated solution were always better than the obtained with the less concentrated solution. Furthermore, the RSD for all peptides using different EW did not vary significantly. Determined repeatability with 10 ppm EW was 0.51%, 0.73%, and 0.72%, while with 200 ppm EW was 0.64%, 0.69% and 0.71%, for LRP, LSP and LQP, respectively. Inter-day precision yielded RSD values of 5.66%, 3.54%, and 5.10%, for LRP, LSP, and LQP, respectively. This result demonstrated the stability of the cleaned extract. A similar experiment was performed with an extract stored without the previous removing of urea observing RSD (n = 6) values of 87.75%, 66.13%, and 75.75% for LRP, LSP, and LQP, respectively. These results indicate that peptides are not stable in the sample containing urea. Inter-sample precision was determined using five individual maize samples extracted, purified, digested by thermolysin, and desalted using the Sep-Pak. The RSDs (n = 15) did not depend on the extraction window for LSP, LQP, and LRP. Evaluated inter-sample precision for LSP and LQP peptides using EW 100 ppm showed RSD lower than 9.36%, while for LRP and EW 200 ppm it is equal to 11.43%. On the other hand, although the use of different EW did not have any significant influence on correlation coefficients, LOD/LOD calculated using SNR ratio approach, repeatability, and inter-sample precision it significantly influenced the slopes of the calibration curves and selectivity. In order to choose the suitable EW for every peptide, the area of every EIC peptide signal obtained in a sample was divided by the area of every EIC peptide signal obtained for the standard injected at a similar concentration. Suitable EW will be that extracting exclusively the peptide ions in the sample. In that case, the ratio between areas of peptide ions in the standard and the sample will be equal to 1. In this case, no additional noises from sample will be extracted. The estimated ratios have shown to be the closest to 1 when EW was 200 ppm for LRP and 100 ppm for LSP and LQP peptides. Therefore, selected EWs have been used to refine

and summarized results obtained for the three peptides in the characterization of the method (Table 1).

Table 1. Analytical characterictics of the developed method.

Characterization parameter			LRP	LSP	LQP
Extraction window			200 ppm	100 ppm	100 ppm
External calibration curve		***Range***	1-59 ppb	25-2297 ppb	3-304 ppb
		Slopes	152,708.0	97,394.4	113,243.0
		Linearity (R^2)	0.9996	0.9998	0.9989
LOD [µg/g maize][1]			0.0039	0.0021	0.0003
LOQ [µg/g maize][2]			0.0130	0.0073	0.0010
Repeatability %RSD (n=5)[3]		***1***	1.94	0.74	0.69
		2	0.64	0.62	0.69
Inter-day precision %RSD (n=6)[4]			5.66	3.54	5.10
Inter-sample precision %RSD (n=15)[5]			11.43	9.36	8.81
Standard addition calibration curve	***Slopes***	***EZ6***	148,894.0	46,257.9	50,385.4
		EZ9	$2.75*10^6$	$4.02*10^7$	$7.30*10^6$
	Linearity (R^2)	***EZ6***	0.8387	0.9913	0.9892
		EZ9	0.9763	0.9921	0.9963
Recovery (%)		***20%***	51.7	109.1	92.8
		40%	48.2	110.7	94.4

In order to study the effect of the matrix, digested extracts prepared from two maize lines (EZ6 and EZ9) were spiked with peptide standards so that the concentration of peptides in the sample increased by 10%, 20%, and 30%. The slopes estimated by the standard addition method were compared with slopes obtained for the external calibration curves (Table 1). The results showed no effect of the matrix just in case of LRP peptide in the EZ6 maize line. Rest of assays

[1] Determined as the minimum concentration which yielded an SNR equal to 3. Expressed in the µg/g units which was determined relative to 1 g of maize sample.
[2] Determined as the minimum concentration which yielded an SNR equal to 10. Expressed in the µg/g units which was determined relative to 1 g of maize sample.
[3] The repeatability was measured at two concentration levels: 1) LRP, 8 ppb; LSP, 53 ppb; LQP, 62 ppb; and 2) LRP, 41 ppb; LSP, 1159 ppb; LQP, 155 ppb.
[4] Precision determined in two consecutive days by the injection of desalted digested extract by triplicate.
[5]Determined as the RSD (%) value calculated for five individually prepared digested extracts of maize proteins injected by triplicate.

demonstrated the need of standard addition calibration for the quantification of these peptides. In order to evaluate the recovery of peptides, two individual digested extracts were spiked before the desalting step with different amounts of standard peptides. Recoveries were very close to 100% for LSP and LQP. Only for LRP resulted in a low recovery (averaged recovery 49,99%). In order to be able to calculate the final absolute concentration of this peptide, the use of a correction coefficient for this peptide (estimated as "2") can be applied.

3.6. Analysis of maize crops

The optimized method was applied to the quantification of the three peptides in six maize lines (seeTable 2) using the single point calibration method.

Table 2. Concentration of LRP, LSP, and LQP peptides in different maize lines[6].

Maize line	LRP [µg/g maize]	LSP [µg/g maize]	LQP [µg/g maize]	AEF [µg/g maize][7]
EZ6	0.11±0.02	20.37±0.05	2.74 ±0.09	3.98
EZ9	0.80±0.03	19.46 ±0.06	2.68 ±0.11	4.51
MC	0.84±0.01	38.40 ±0.05	3.03 ±0.27	7.83
A632	1.26±0.21	24.56 ±0.41	6.54 ±0.15	6.40
B73	0.40±0.05	28.65 ±0.26	3.01 ±0.19	5.73
EZ11A	1.10±0.07	41.06 ±0.59	5.78 ±0.24	8.94

The highest concentration of LRP peptide was found in A632 and EZ11A lines while the lowest concentration was observed in the EZ6 variety. Moreover, EZ11A and MC showed the highest content of LSP peptide. The highest yield of LQP peptide was found in the A632 variety. Thus, the EZ11A line could be assumed as the maize variety with the strongest antihypertensive capacity, which is in accordance with our previous results [10]. According to the Uniprot protein database, 19 α-zein proteins have been fully sequenced and confirmed. In addition, there are several α-zein proteins, with no confirmed sequences, that can also be present in maize. Study of these sequences demonstrated that within 19 α-zein proteins, LSP sequence

[6] For every maize line two individually prepared extracts were obtained. Every sample was injected by triplicate.
[7]AEF (antihypertensive equivalent factor)- total antihypertensive activity expressed in terms of equivalent concentration of the most active peptide, LRP.

was present 24 times, LQP 9 times, and LRP was absent. However, the comparison of not confirmed α-zein sequences showed the presence of the LRP peptide. These findings are in accordance with the concentration order obtained in our samples: most concentrated peptide is LSP, followed by LQP, and LRP. In order to do more straightforward comparison of antihypertensive activities of maize lines containing different antihypertensive peptides, a unique parameter is needed. For that purpose, total antihypertensive activity was expressed in terms of equivalent concentration of the most active peptide, LRP. Thus, antihypertensive equivalent factors (AEF) were defined for LSP and LQP using the following expression:

$$AEF = \frac{IC_{50}(LRP)}{IC_{50}}$$

AEF was "1" for LRP, "0.17" (0.29/1.7) for LSP, and "0.14" (0.29/2.0) for LQP. Using these AEF values is possible to express total antihypertensive activity of maize lines as a single number resulting from the product of each peptide concentration and each peptide AEF. As example, for maize line EZ6, the equivalent concentration of antihypertensive peptide in terms of LRP is obtained from:

$$AEF_{(EZ6\ line)} = 0.11\ \mu g/g \times 1 + 20.37\ \mu g/g \times 0.17 + 2.74\ \mu g/g \times 0.14 = 3.98\ \mu g/g$$

This means that the ingestion of 1 g of maize line containing 0.11 μg of LRP, 20.37 μg of LSP, and 2.74 μg of LQP has the same antihypertensive effect to the ingestion of 1 g of maize line containing just 3.98 μg of LRP peptide. Taking into account AEFs, the maize line with the highest antihypertensive activity is line EZ11A while the maize line with the lowest antihypertensive activity is line EZ6. Due to the fact that studied maize varieties were grown in the same conditions, it can be postulated that observed variability between maize lines is due to their different genetic background. This fact is particularly important in terms of production of functional foods.

4. Conclusions

This work proposes novel analytical methodology for the determination of three highly antihypertensive peptides (LRP, LQP, and LSP) in maize lines by HPLC–ESI-Q-ToF-MS. Optimization of ESI parameters enabled a significant reduction of spontaneous fragmentation. Peptide standard solutions prepared in 10% AA demonstrated to be stable for at least 7 h at room temperature. A FASP approach, applied for the first time for the digestion of food proteins, was investigated as an alternative to the conventional sample preparation procedure

for the extraction and digestion of α-zeins from maize lines observing that approach was not useful in this case. Ionization suppression in the MS due to the presence of urea and other compounds employed during the extraction and digestion of α-zeins forced to desalt extracts previously to their analysis. Two different C18 cartridges were used and compared observing that Supelco gave the best results with the peptide standards. Nevertheless, application of such cartridge to desalt maize extracts resulted in a higher extraction of other compounds suppressing the ionization of peptide LRP. The method using the Sep-Pak cartridge was then selected and linearity, LOD, LOQ, repeatability, inter-sample precision, and recovery were evaluated observing adequate results. Comparison of external standard and standard addition calibration curves demonstrated the existence of matrix interferences. Quantification of LRP, LQP, and LSP in six different maize crops showed that the EZ11A variety presented the highest antihypertensive power. Since all maize genotype grow under the same agronomic conditions, peptide content difference could be attributed mainly to their different genetic background.

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References for Article 5:

[1] M.T. Kurosawa, Y. Nakamura, N. Yamamoto, K. Yamada, T. Iketani, Effects of Val-Pro-Pro and Ile-Pro-Pro on nondipper patients: A preliminary study, J. Med. Food. 14 (2011) 538-542.

[2] K. Erdmann, W.Y. Cheung Belinda, H. Schroder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular diseasem, J. Nutr. Biochem. 19 (2008) 643-654.

[3] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[4] H. Meisel, Biochemical properties of regulatory peptides derived from milk proteins, Biopolymers 43 (1997) 119-128.

[5] D. Kitts, K. Weiler, Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery, Curr. Pharm. Des. 9 (2003) 1309-1323.

[6] B.M. Prasanna, S.K. Vasal, B. Kassahun, N.N. Singh, Quality protein maize, Curr. Sci. 81 (2001) 1308-1319.

[7] R. Shukla, M. Cheryan, Zein: The industrial protein from corn, Ind. Crops Prod. 13 (2001) 171-192.

[8] A. Esen, An immunodominant site of γ-Zein is in the region of tandem hexapeptide repeats, J. Protein Chem. 9 (1990) 453-460.

[9] S. Miyoshi, H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka, S. Maruyama, Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate, Agric. Biol. Chem. 55 (1991) 1313-1318.

[10] P. Puchalska, M. L. Marina, M. C. García, Development of a reversed-phase high-performance liquid chromatography analytical methodology for the determination of antihypertensive peptides in maize crops, J. Chromatogr. A 1234 (2012) 64-71.

[11] A.M. Panchaud, F.A.U. Affolter, M.M. Affolter, F.A.U. Kussmann, M.J. Kussmann, Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects, Proteomics 75 (2012) 3546 -3559.

[12] J.M. Cunliffe, T.D. Maloney, Fused-core particle technology as an alternative to sub-2-μm particles to achieve high separation efficiency with low backpressure, J. Sep. Sci. 30 (2007) 3104-3109.

[13] G. Mamone, G. Picariello, S. Caira, F. Addeo Ferranti, Analysis of food proteins and peptides by mass spectrometry-based techniques J. Chromatogr. A 1216 (2009) 7130-7142.

[14] L.F. De, S. Panarese, R. Gallerani, L.R. Ceci, Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food, Curr. Pharm. Des. 15 (2009) 3622-3643.

[15] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[16] J. Chen, Y. Wang, Q. Zhong, Y. Wu, W. Xia, Purification and characterization of a novel angiotensin-I converting enzyme (ACE) inhibitory peptide derived from enzymatic hydrolysate of grass carp protein, Peptides 33 (2012) 52-58.

[17] X. Zhu, K. Watanabe, K. Shiraishi, T. Ueki, Y. Noda, T. Matsui, K. Matsumoto, Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers, Peptides 29 (2008) 338-344.

[18] Z. Yu, B. Liu, W. Zhao, Y. Yin, J. Liu, F. Chen, Primary and secondary structure of novel ACE-inhibitory peptides from egg white protein, Food Chem. 133 (2012) 315-322.

[19] M. Kang, J. Kim, B. Ahn, J. Lee, Characterization of antihypertensive angiotensin I-converting enzyme inhibitory peptides from Korean traditional rice wine, J. Microbiol. Biotechnol. 22 (2012) 339-342.

[20] M.M. Contreras, I. López-Exposito, B. Hernández-Ledesma, M. Ramos, I. Recio, Application of mass spectrometry to the characterization and quantification of foodderived bioactive peptides, J. AOAC Int. 91 (2008) 981-994.

[21] C.S. Gilani, C. Xiao, N. Lee, Need for accurate and standardized determination of amino acids and bioactive peptides for evaluating protein quality and potential health effects of foods and dietary supplements, J. AOAC Int. 91 (2008) 894-900.

[22] K.H. Tokunaga, C. Yoshida, K.M. Suzuki, H. Maruyama, Y. Futamura, Y. Araki, S. Mishima, Antihypertensive effect of peptides from Royal Jelly in spontaneously hypertensive rats, Biol. Pharm. Bull. 27 (2004) 189-192.

[23] N. Yamamoto, M. Maeno, T. Takano, Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4, J. Dairy Sci. 82 (1999) 1388-1393.

[24] I.M.P.L.V.O. Ferreira, R. Eca, O. Pinho Tavares, A. Pereira, A.C. Roque, Development and validation of an HPLC/UV method for quantification of bioactive peptides in fermented milks, J. Liq. Chromatogr. Relat. Technol. 30 (2007) 2139-2147.

[25] K. Inoue, T. Gotou, H. Kitajima, S. Mizuno, T. Nakazawa, N. Yamamoto, Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein, J. Biosci. Bioeng. 108 (2009) 111-115.

[26] H. Enari, Y. Takahashi, M. Kawarasaki, M. Tada, K. Tatsuta, Identification of angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their antihypertensive effect, Fisheries Sci. 74 (2008) 911-920.

[27] J.A. Gómez-Ruiz, M. Ramos, I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1054 (2004) 269-277.

[28] M. Sato, T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, T. Nakano, Angiotensin I-converting enzyme inhibitory peptides derived from wakame (Undaria pinnatifida) and their antihypertensive effect in spontaneously hypertensive rats, J. Agric. Food Chem. 50 (2002) 6245-6252.

[29] T. Nakahara, A. Sano, H. Yamaguchi, K. Sugimoto, H. Chikata, E. Kinoshita, R. Uchida, Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances, J. Agric. Food Chem. 58 (2010) 821-827.

[30] A. Quiros, M. Ramos, B. Muguerza, M.A. Delgado, P.J. Martín-Alvarez, A. Aleixandre, I. Recio, Determination of the antihypertensive peptide LHLPLP in fermented milk by high-performance liquid chromatography-mass spectrometry, J. Dairy Sci. 89 (2006) 4527-4535.

[31] Y. Hu, A. Stromeck, J. Loponen, D. Lopes-Lutz, A. Schieber, M.G. Gaenzle, LC-MS/MS quantification of bioactive angiotensin I-converting enzyme inhibitory peptides in rye malt sourdoughs, J. Agric. Food Chem. 59 (2011) 11983-11989.

[32] J.M. Curtis, D. Dennis, D.S. Waddell, T. MacGillivray, H.S. Ewart, Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS, J. Agric. Food Chem. 50 (2002) 3919-3925.

[33] U. Buetikofer, J. Meyer, R. Sieber, D. Wechsler, Quantification of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses, J. Int. Dairy. 17 (2007) 968-975.

[34] J. Meyer, U. Buetikofer, B. Walther, D. Wechsler, R. Sieber, Hot topic: Changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese varieties, J. Dairy Sci. 92 (2009) 826-836.

[35] B.O.E., Métodos Oficiales de análisis de aceites y grasas, cereales y derivados, productos lácteos y productos derivados de la uva, 19-07-1977, and 20-07-1977.

[36] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359-362.

[37] L. Anta, M. L. Marina, M. C. García, Simultaneous and rapid determination of the anticarcinogenic proteins Bowman-Birk inhibitor and lectin in soybean crops by perfusion RP-HPLC, J. Chromatogr. A 1217 (2010) 7138-7143.

Article 6

Development of a capillary HPLC-IT-MS method for the determination of VLIVP antihypertensive peptides in soybean crops.

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Abstract

Soybean peptide VLIVP presents a very high antihypertensive activity (IC_{50} value 1.69 μM), even higher than extensively studied IPP and VPP peptides from milk. Nevertheless, no much attention has been paid to this peptide and there is no method enabling its determination in soybeans. The aim of this work was the development of an analytical methodology for this purpose. A methodology consisting of the extraction of soybean proteins, their digestion with protease P enzyme, their chromatographic separation using capillary-HPLC, and IT-MS detection was optimized. Protein extraction was performed by the use of high intensity focused ultrasounds to obtain a reduced extraction time. Optimization of chromatographic and mass spectrometry parameters enabled the separation of VLIVP peptide within just 7 min and its sensitive detection. The analytical characteristics of the capillary-HPLC-IT-MS method were evaluated through the study of linearity, LOD, LOQ, study of the presence of matrix interferences, precision, and recovery. The method enabled to detect as low as 3.6 ng of peptide and to determine as low as 12 ng of peptide in 1 g of soybean (as dry basis). Finally, the developed method was applied to the determination of the antihypertensive peptide VLIVP in different soybean varieties. The results showed the highest yield of VLIVP peptide in variety Mazowiecka II from Poland.

Keywords:

Antihypertensive peptide; Soybean; Quantification; Ion Trap; Micro-HPLC

1. Introduction

World Health Organization (WHO) recognizes hypertension as a serious global health problem which is associated with the development of cardiovascular disease [1]. The treatment of hypertension is mainly based on the blockage of an enzyme (angiotensin-I converting enzyme, ACE) involved in the renin-angiotensin system [2-4]. Different drugs have been designed as effective inhibitors of ACE (*e.g.* captopril, enalapril, alecepril and lisinopril *etc.*) but they are usually accompanied by undesirable side effects, such as hypotension, cough, or reduced renal function [5,6]. On the other hand, some peptides present in foods have also been recognized as ACE inhibitors [4]. In fact, milk and dairy products, chicken, pork, eggs, fishes like tuna or bonito and soybean are just some examples of food products containing antihypertensive peptides. These peptides are of great interest since they do not present side effects like synthetic drugs. Nevertheless, the interest for these peptides should not be only focused on their identification but also on their quantitative determination since, obviously, the performance of these peptides is highly dosage dependence. Despite this, the literature concerning this topic is surprisingly scarce.

The quantification of peptides in hydrolyzed food protein matrices is a huge challenge. This difficultly increases when food proteins are hydrolyzed using low specificity enzymes, which are those mostly required to obtain antihypertensive peptides. This kind of enzymatic digestion leads to a great peptide length distribution, a high peptide dynamic range, and high sample complexity [7]. Therefore, highly sensitive, selective, and effective peptide separation, and detection methods are required. HPLC has shown to be the only technique applied for this purpose. Despite the miniaturization of the column dimension in LC provides significant enhancement of method sensitivity [8], there is just one work where capillary- HPLC was used for the quantification of bioactive peptide in food hydrolysate [9]. Typically conventional HPLC is hyphenated with MS detector, where MS [10-15], MS/MS [16-19], or MS^3 modes [20, 21] are applied. Indeed, the use of highly accurate mass spectrometers, and/or monitoring of peptide fragmented ions, can ensure high selectivity of the method.

Soybean contains a highly antihypertensive peptide with sequence VLIVP. This peptide is derived from 11S globulin soybean protein fraction and is obtained by hydrolysis with protease P enzyme. ACE inhibitory activity of VLIVP peptide (IC_{50} value (half maximal inhibitory concentration) is 1.69 μM) [22] is much higher than the activity of famous VPP (IC_{50} = 9.13 μM) or IPP (IC_{50} = 5.15 μM) peptides from milk. Taking into account that soybean protein

content, and thus, peptide content, is greatly affected by genotype [23], it is clear the need for methods enabling the reliable quantitation of VLIVP peptide [24, 25].

The aim of presented work was the development of an analytical methodology for a sensitive quantification of the highly antihypertensive peptide VLIVP in different soybean varieties. For that purpose, the extraction and digestion with protease P enzyme were optimized. Moreover, a capillary HPLC separation method in combination with mass spectrometry detection using an ion trap mass spectrometer in pseudo selected reaction monitoring (SRM) mode was developed. The characterization and critical evaluation of the performance of the method together with its application to the determination of VLIVP peptide in different soybean varieties was also planned.

2. Materials and methods

2.1. Chemicals and samples

HPLC grade solvents were used. Water was freshly taken from a Milli-Q system (Millipore, Bedfore, MA, USA). Tris- (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), sodium hydroxide, and hydrochloric acid were supplied by Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA) and sodium bisulfite (SBS) were purchased from Sigma (St. Luis, MO, USA), acetic acid (AA) from Scharlau Chemie (Barcelona, Spain), andacetonitrile (ACN) from Fischer Scientific (Madrid, Spain). Protease P was kindly donated by Amano (Amano Enzyme Inc., Nagoyo, Japan). VLIVP standard was synthesized by Genescript (Genescript Corp. Piscataway, NJ, USA). Soybean varieties (A, B, C, and D) were kindly donated by CRF-INIA (Centro de Recursos Fitogenéticos del Instituto Nacional deInvestigaciones Agrarias, Madrid, Spain). One commercial soybean was acquired in a market at Alcala de Henares. Moisture content of soybean samples was determined using an official reference method [26].

Protease P powder was stored in the fridge at 4 °C while its stock solution (3 mg/mL in water) was kept at -20°C, both protected from light. VLIVP peptide powder was stored at -20°C while its standard solution prepared in 10% AA was kept at 4°C.

2.2. Soybean protein extraction

Soybean seeds were ground with a domestic miller, sieved through a 60-mesh sieve, and defatted with *n*-hexane. Protein extraction was carried out by mixing 0.5 g of defatted soybean powder with 7.5 mL of 0.03 M Tris-HCl buffer, pH 8.5 as previously described [27]. Extraction

method was accelerated using ultrasonic probe (VCX.130, Sonic Vibra-Cell, Hartord, CT, USA). Optimized extraction time and probe amplitude were 2 min and 40%, respectively. Sample was, then, centrifuged (4000 × *g*, 10 min, 20 °C) and the procedure was repeated. The two supernatants were collected and combined.

2.3. Isolation of 11S globulins from whole soybean protein extract

11S globulins were isolated from the soybean protein extract using the procedure of Liu *et al.* [27] with modifications. For that purpose, SBS was added to the protein extract up to a SBS concentration of 0.01M. Then, the pH was adjusted to 6.4 with 2 N HCl and the extract was left at 4°C overnight. Obtained pellet (11S globulins) was centrifuged (4000 × *g*, 10 min, 4 °C), dissolved in an appropriate volume of water, and, together with supernatant and whole extract, it was injected into the HPLC-UV system.

2.4. Digestion of soybean proteins

In order to optimize protein digestion protocol, experiments were performed in duplicate and injected twice into the capillary HPLC-MS system. Prior to the digestion, the pH of whole soybean protein extract was adjusted to pH 9.0 with 1 N NaOH. To decrease the complexity of sample, extract was diluted five times with 0.03 M Tris-HCl buffer, pH 9.0. Then, 1 mL of protein extract was mixed with protease P stock solution (3.0 mg/mL) in order to reach enzyme to substrate ratio 0.1:100 (w:w). Mixture was incubated in a hot air oven (Memmert, model 300, Schwabach, Germany) for 18 h, at 40°C. To stop the reaction, solution was boiled for 10 min and centrifuged (21130 × *g*, 1 min, and 20 °C). Digested extract was diluted twenty times with 0.03 M Tris-HCl buffer (pH 9.0) just before its injection.

2.5. HPLC analysis

Separation of soybean proteins extracts and their fractions was performed on a modular Agilent Technologies liquid chromatograph (Pittsburg, PA, USA) consisting of a degassing system, a quaternary pump, a thermostatized compartment for the column, an injection system, and an UV detector (series 1100). HP Chemstation software was used to control HPLC instrument. Experiments were performed in duplicate and injected twice. Protein separation was obtained using a method developed by García *et al.* [3]. Whole protein extract and its fractions were separated in a POROS R2/10 perfusion column (50 mm × 4.6 mm I.D.) (Perspective Biosystem, Framingham, MA, USA) using the following chromatographic

conditions: mobile phases, Milli-Q water/0.1% (v:v) TFA (A) and ACN/0.1% (v:v) TFA (B); binary gradient, 5-25% B in 1.7 min, 25-45% B in 1.3 min, 45-95% B in 1 min, and 95-5% B in 1 min; flow rate, 3 mL/min; temperature, 60 ˚C; injection volume, 20 µL; detection was performed λ=254 nm.

The separation of digested soybean extract and VLIVP standard solution was performed on a modular capillary chromatographic system (Agilent Technologies, Pittsburgh, PA) consisting of a micro vacuum degasser, a capillary LC pump, a thermostatized autosampler (all model 1100) and a thermostatized column compartment (model 1200). Capillary chromatographic system was connected to the mass spectrometry detector. Separation was performed on a C18 Zorbax 300 SB (150 mm × 0.5 mm I.D., with 5 µm particle size) column from Agilent Technologies. The optimized chromatographic conditions for the separation of VLIVP peptide were: Milli-Q water/0.1% (v:v) AA (A) and ACN/0.1% (v:v) AA (B), binary gradient,18-30% B in 8 min, 30-95% B in 1 min, 95-5% B in 1 min, 5% B for 5 min; flow rate, 20 µL/min; temperature, 25˚C; injection volume, 2 µL.

2.6. MS/MS analysis

An ion trap (IT) mass spectrometer model amaZon SL (Bruker Daltonics, Bremen, Germany), equipped with an electrospray (ESI) source was employed. HyStar software was used to control HPLC and MS instruments. The mass spectrometer was operated in the positive ion mode, in the ultrascan mode, and with a mass scan range from 70 to 700 *m/z*. Optimization of IT conditions was performed by injecting two individual digested soybean extracts twice into the system. Optimized conditions were: capillary voltage, 5000 V; end plate voltage, -600 V; nebulizer pressure, 7 psi; dry gas flow, 2 L/min; dry gas temperature, 150°C; ion charge control (ICC) target, 150 000; collision energy, 0.5 amplitude. Extracted ion chromatogram (EIC) was obtained by the addition of the signals corresponding to six different transitions (540.4→ (425.3+397.3+326.2+281.1+215.0+213.0)) (extraction window ±0.5).

2.7. Characterization of the method and calibration

Both external standard and standard addition calibrations were used. The external calibration curve was obtained in the range from 5 to 100 ng/L. Each peptide calibration solution was obtained by the individual dilution of peptide stock solution to a desired concentration and injected three times. A commercial soybean was used for the development and characterization of the method. The same commercial soybean together with the soybean variety B, were

employed to evaluate the presence of the matrix interferences. The standard addition method was carried out by the addition of appropriate amounts of VLIVP standard solution to two individually digested soybean samples of the commercial soybean and the soybean variety B. Hereby, the concentration of VLIVP peptide increased in digested extract sample by 0%, 10%, 20%, and 30%. Each sample was injected in triplicate, EIC of peptide transition was extracted, averaged, and plotted against added amount of peptide standard. In order to evaluate the existence of matrix effects, comparison of external standard curve and standard addition curve was performed using Statgraphics Software Plus 5.1 (*Statpoint Technologies, Inc.,* Warrenton, VA, USA).

Limits of detection and quantification (LOD and LOQ) were calculated as the minimum concentration yielding a signal to noise ratio (SNR) equal to 3 and 10, respectively. For the evaluation of repeatability and inter-day and inter-sample precision, the relative standard deviation (%RSD) was calculated. Repeatability was estimated at two concentration levels (10 and 100 ng/L) by five repeated injections (n=5) of a peptide standard solution. The determination of inter-day precision was performed by the triplicate injection of a digested soybean protein extract in two consecutive days (n=6). The inter-sample precision was evaluated by the triplicate injection in the same day of five individual digested soybean protein extracts (n=15). Recovery was evaluated by spiking of soybean sample before protein extraction with known peptide amounts so that the final peptide concentration in the digested extract sample increased by 0%, 20%, 40%, and 60%.

3. Results and discussion

3.1. Extraction of soybean VLIVP peptide

VLIVP peptide is released during protease P digestion of 11S soybean globulin. Therefore, in order to obtain VLIVP peptide, soybean proteins must be extracted. Different methods have been published for this purpose being the methods of Nagano *et al.* [28] and Thanh and Shibaski [29] the most frequently reported. They enable both the extraction of soybean proteins and further isolation of 11S and 7S globulin fractions. Based on these two methods, an improved protocol taking 2 hours has lately been presented [27]. In order to reduce this extraction time, high intensity focused ultrasounds were applied. Different extraction times (1, 2, 5, 10, and 15 min) and ultrasonic amplitudes (20, 40, 60, 80, and 100%) were tried and peak areas corresponding to proteins were monitored by HPLC-UV. Results (not shown) demonstrated

that an extraction time of 2 min at a probe amplitude of 40% enabled a similar extraction to the observed with the original method taking 2 hours. The inter-sample precision (n=4) of two soybean protein extracts accelerated by the ultrasonic probe at optimal conditions (2 min, amplitude 40%) was 1.19% which suggested a high reproducibility.

A further fractionation of the protein extract was next proposed to reduce sample complexity. Protein fraction 11S, containing target peptide, was precipitated at its isoelectric point following the method of Liu *et.al* [27], redissolved, and injected into the HPLC. Comparison of the chromatogram corresponding to the whole protein extract and that of the 11S fraction (see Fig. 1) revealed that 11S globulins eluted at the end of the chromatogram (around 2.5-4 min).

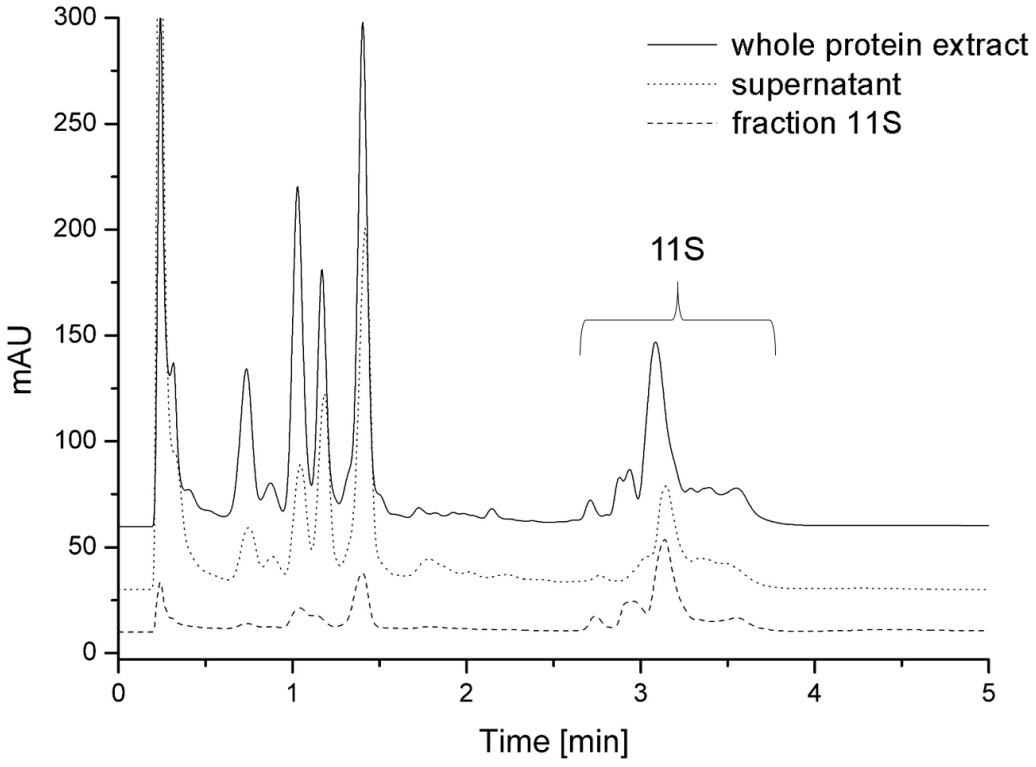

Fig. 1. Chromatograms obtained for the whole protein extract of soybean, for the 11S glycinin fraction, and for the remaining supernatant. Chromatographic conditions: column: POROS R2/10 (50 x 4.6 mm); mobile phases, Milli-Q water/0.1% (v:v) TFA (A); ACN/0.1% (v:v) TFA (B); binary gradient, 5-25% B in 1.7 min, 25-45% B in 1.3 min, 45-95% B in 1 min, 95-5% B in 1 min; flow rate, 3 mL/min; temperature, 60˚C; injection volume, 20 µL; and detection, λ=254 nm.

However, the intensity of signals obtained for this protein group was significantly lower than in the whole extract suggesting a non-quantitative precipitation. Indeed, the injection of

supernatant revealed that a significant part of this fraction remained in it. In order to improve 11S globulin recovery, it was reprecipited from the supernatant. This additional step improved slightly the recovery of 11S globulins but it did not provide its quantitative precipitation. Taking into account this result, this fractionation was discarded and the whole soybean protein extract was digested. Due to the high protein concentration of the extract and in order to avoid problems due to the complexity of sample, the protein extract was diluted five times before its digestion.

3.2. Identification of VLIVP in the soybean proteins digested extract

Soybean extract digestion with protease P was performed with the diluted extract using following starting conditions: pH 8.5, enzyme to substrate ratio 1:100 (w:w), 18 h, and 40°C.In order to identify the presence of target peptide in the soybean protein digested extract, a peptide standard solution (9 mg/L) was firstly injected into the capillary-HPLC-IT-MS system using the following conditions: chromatographic: Milli-Q water/0.3% (v:v) AA (A) and ACN/0.3% (v:v) AA (B), binary gradient, 5-40% B in 30 min, 40-95% B in 2 min, 95-5% B in 2 min, 5% B during 7 min; flow rate, 20 µL/min; temperature, 25 ˚C; injection volume, 2 µL; and MS: capillary voltage, 4500 V; end plate voltage, -500 V; nebulizer pressure, 6 psi; dry gas flow, 3 L/min; dry gas temperature, 200 °C; ion charge control (ICC) target, 100 000; collision energy, 0.5 amplitude. The MS spectrum of peptide VLIVP showed just single $[M+H]^+$ peptide ion with *m/z* value 540.4 (see Fig. 2.). The MS/MS spectrum of precursor ion (540.4 *m/z*) revealed that b ion series was mostly produced during the cleavage of target peptide. Some other minor ions like neutral loss of CO (-28 Da) from b_4^+ ion with 397.3 *m/z* (a_4^+ ion) and y_2^+ ion with 215.0 *m/z* were also present in the MS/MS spectra of VLIVP peptide. Next, the soybean proteins hydrolysate was injected into the capillary-HPLC-IT-MS system using previously employed MS and MS/MS conditions. Fig. 3.1 shows the EICs of ion 540.4 *m/z* obtained from the MS analysis of the digested extract and peptide standard solution. In both cases, an intense signal eluting around 19 min was detected. However, the MS spectrum of the digested extract also showed a strong signal at 540.5 *m/z* that was not well isolated from signal at 540.4 *m/z* corresponding to target peptide. In order to assure a high selectivity, MS/MS analysis of 540.4 *m/z* ion was required.

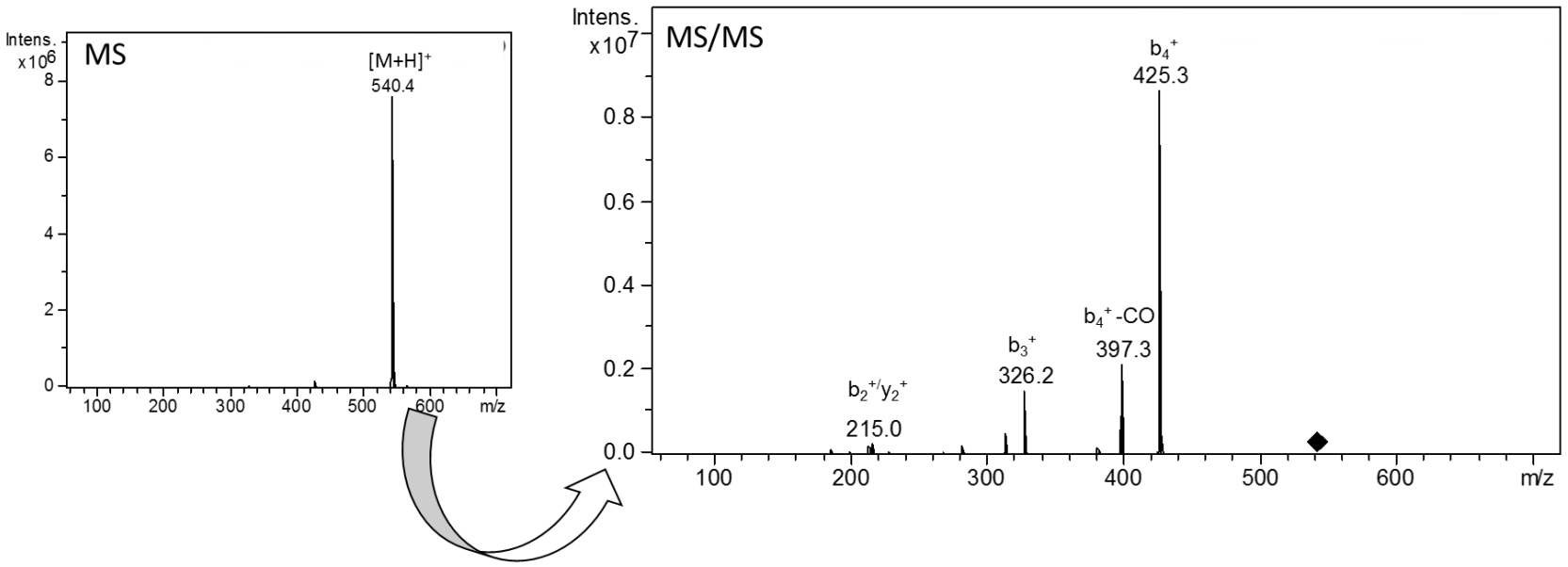

Fig. 2. MS and MS/MS spectra of VLIVP standard (9 mg/L) injected into the capillary-HPLC-IT-MS system. Chromatographic conditions: column, ZORBAX SB C18 (150x 0.5 mm);mobile phases, Milli-Q water/0.3% (v:v) AA (A) and ACN/0.3% (v:v) AA (B), binary gradient, 5-40% B in 30 min, 40-95% B in 2 min, 95-5% B in 2 min, 5% B during 7 min; flow rate, 20 µL/min; temperature, 25 ˚C; injection volume, 2 µL; and MS: capillary voltage, 4500 V; end plate voltage, -500 V; nebulizer pressure, 6 psi; dry gas flow, 3 L/min; dry gas temperature, 200 °C; ion charge control (ICC) target, 100 000; collision energy, 0.5 amplitude.

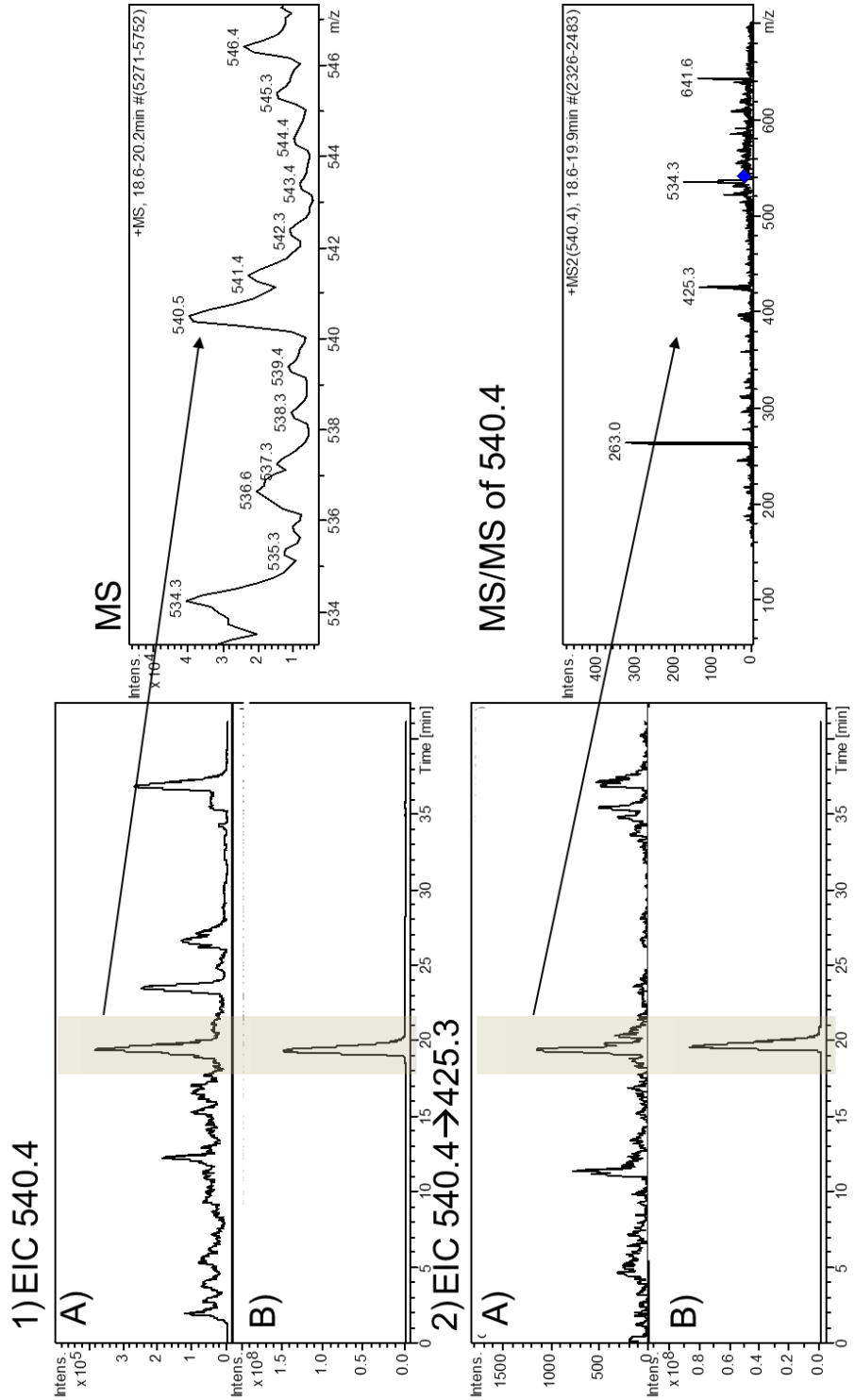

Fig. 3. 1) EIC of ion 540.4 *m/z* and 2) EIC of transition 540.4→ 425.3 of A) Protease P digested soybean protein extract and B) VLIVP peptide standard solution. MS and MS/MS spectra have also been included. Chromatographic and spectrometric conditions as in Fig 1.

The EIC of transition 540.4 *m/z*→425.3 *m/z* (see Fig. 3.2) for both digested extract and peptide standard solution were obtained. The presence of signal at 19 min in the digested soybean extract and the presence of ion 425.3 *m/z* in the MS/MS spectrum confirmed clearly the presence of peptide VLIVP in the digested soybean extract. However, it must be highlighted that the EIC intensity of transition 540.4 *m/z*→425.3 *m/z* in the digested extract (Fig. 3.2. A) was surprisingly low in comparison to the same transition in the peptide standard (Fig. 3.2. B). This could be explained taking into account the high complexity of the sample. Indeed, the signal intensity corresponding to ion 540.4 *m/z* in the EIC could derive from more than one molecule and not just from the target peptide while transition 540.4 *m/z*→425.3 *m/z* should derive just from the VLIVP peptide. In addition to transition 540.4 *m/z*→425.3*m/z*, other minor transitions were also observed (540.4 →(425.3+397.3+326.2+281.1+215.0+213.0)) and monitored for a more selective and sensitive determination of target peptide.

3.3. Optimization of the chromatographic separation of the soybean proteins digested extract

Once target peptide was identified in the protease P digested soybean protein extract, the chromatographic conditions were optimized. Since VLIVP peptide eluted at 19 min, the chromatographic gradient was changed in order to reduce analysis time. Five different chromatographic gradients were tried. The best separation was obtained with the following gradient: 18-30% of B in 8 min; 30-95% of B in 1 min; 95-5% of B in 1 min; 5% of B for 5 min. As a consequence, total analysis time was reduced from 41 min to just 15 min and VLIVP peptide eluted at 7 min. Ion-pairing reagent (AA) concentration was also optimized within the range 0.1-0.4% (v/v) observing the highest peak area for target peptide with 0.1% (v/v) of AA. Furthermore, several separation temperatures (25°C, 35°C, 45°C, 55°C, and 65°C) were also tried but no significant change in both separation time and EIC peak area was observed. Hence, 25°C was selected as optimal. Optimal conditions were employed in the analysis of a solution of standard peptide and a solution consisting of digested extract spiked with the same concentration of standard peptide. Comparison of EIC peak areas showed that just 32% of the peptide standard signal was observed when adding the digested extract under the optimized chromatographic conditions. This result could suggest the existence of strong peptide ionization suppression effects in the ESI source. In order to reduce ionization suppression, the dilution of digested extract in Tris-HCl buffer prior to the injection into the capillary-HPLC-IT-MS system was suggested. To study the effect of dilution, digested extracts were spiked with equal amounts of peptide standard and diluted at different levels (no dilution and 2, 5, 10, 20, and 50 times dilution). Surprisingly, the EIC peak areas of studied dilutions of digested extract increased.

The maximum peak area was observed when extract was diluted twenty times. Under these conditions, no ion suppression was observed.

3.4. Optimization of protease P digestion of soybean proteins extract

Soybean extract digestion with protease P was performed using some starting conditions chosen based on previous parameters used in bibliography. In order to obtain a better digestion performance and to reduce the digestion time, different parameters were optimized. Different buffer pHs were tested (7.0, 7.5, 8.0, 8.5, and 9.0). The highest signal intensity was obtained at pH 9.0 while for pH 8.5 the signal was lower and no peptide was detected from pH 7.0 to pH 8.0. In an attempt to accelerate the digestion using high intensity focused ultrasounds, different ultrasonic times (0.5, 1, 2, 5, 10, 15, 20 min) and ultrasonic amplitudes (20, 40, 60, and 100%) were tested. Unfortunately, no conditions enabled to observe target peptide and it was discarded. Moreover, different digestion times (0.5, 1, 3, 6, 12, 18, and 24 hours) and temperatures (room temperature, 30°C, 40°C, and 50°C) were tried. The highest signal intensity was detected at 18 h and 40°C, respectively. Other temperatures resulted in a lower or null peptide signal . Finally, the enzyme to substrate ratio (w:w) was optimized within the following values: 0.1:100, 0.5:100, 1:100, 5:100, 10:100, and 15:100 (w:w). The best enzyme to substrate ratio turned out to be 0.1:100. Rising up the enzyme amount resulted in a decreased signal intensity, which could suggest a strong autodigestion of the protease P enzyme. The blank digestion at selected enzyme to substrate ratio (0.1:100) did not show autodigestion. Furthermore, the effect of alkylation and reduction of soybean proteins before protease P digestion was also evaluated. The addition of these two steps led to the worst generation of target peptide and it was discarded.

3.5. Optimization of MS parameters

In order to increase sensitivity, the following IT parameters were optimized: capillary voltage, end plate voltage, nebulizer pressure, dry gas flow and temperature, ICC target, and collision energy. First parameters were related to the ESI ionization while the ICC and collision energy parameters were strictly connected to the IT performance.

Following capillary voltages were tried: 3000 V, 3500 V, 4000 V, 4500 V, 5000 V, 5500 V, and 6000 V. Maximum EIC peak area was obtained at 5000V. End plate voltage was evaluated in the range from -400 V to -650 V. Despite there was no significant signal variation, the use of -600 V yielded the most repeatable results and it was selected as optimal. Moreover,

the following nebulizer pressures were checked: 5 psi, 6 psi, 7 psi, and 8 psi. Also in this case, the influence of this parameter in the EIC peptide signal was not noticeable and the value giving the most repeatable results (7 psi) was chosen. Various dry gas flows (2, 3, 4, 5, and 6 L/min) were studied and 2 L/min resulted in the highest signal intensity. Regarding dry gas temperature (150°C, 200°C, 250°C, 300°C, 350°C), best results were observed at 150°C. Moreover, several ICC target values (50 000, 100 000, 150 000, and 200 000) were examined. EIC peak area increased together with ICC target until it reached 150 000, which was considered as optimal. Regarding collision energy, different amplitudes (0.1, 0.3, 0.5, 0.7, and 1.0) were tested. No peptide signal was detected with a collision energy amplitude of 0.1 while no significant differences were observed when collision energy amplitude was in the range from 0.3 to 0.7, and an amplitude of 1.0 showed no reproducible results. As a consequence, an amplitude of 0.5 was finally chosen.

3.6. Analytical characteristics of the developed methodology

Optimized methodology was characterized by the evaluation of the following parameters: linearity in the working concentration range, limits of detection and quantification (ng/mg dried soya), existence of matrix interferences, precision, and recovery. Table 1 summarizes obtained results.

Good linear correlation (R^2> 0.99) was observed between signal and concentration in the range from 5 to 100 ng/L of target peptide. The lowest concentration of target peptide detected by the method was 3.6 ng/g of soybean (as dry basis) (estimated as the minimum concentration yielding a SNR equal to 3) while the lowest concentration of target peptide that could be reliably determined was 12.1 ng/g of soybean (as dry basis) (estimated as the minimum concentration yielding a SNR equal to 10). The existence of matrix interferences was studied using two different soybean varieties. For that purpose, calibration plots obtained by the external and by the standard additions calibration methods were compared and no significant differences were observed (P > 0.05) showing that the proposed method did not suffer from matrix interferences.

Precision of the method was evaluated by the determination of instrumental repeatability, inter-day precision, and inter-sample precision. Instrumental repeatability, calculated by five consecutive injections of two VLIVP peptide solutions (10 and 100 ng/L), was always better than 3%. Inter-sample precision, determined by the consecutive injection of five individual soybean samples, was 6.35% while inter-day precision, calculated by the injection of one

sample solution in two consecutive days, was better than 4%.Recovery was determined by spiking soybean samples with known amounts of peptide standard, so peptide concentration after whole procedure increased by 20%, 40%, or 60%. Peptide recoveries were always very close to 100%.

Table 1. Analytical characteristics of the developed method.

Characterization parameter			**VLIVP**
Extraction window			±0.5
External calibration	**Range**		5-100 ppt
	Slope		821
	Linearity (R^2)		0.9983
Standard addition calibration	**Slope**	Commercial soya	862
		Variety B	848
	Linearity (R^2)	Commercial soya	0.9995
		Variety B	0.9998
LOD (ng/g dried soybean)[1]			3.6
LOQ (ng/g dried soybean)[2]			12.1
Repeatability % RSD (n=5)[3]	**at 10 ppt**		2.34
	at 100 ppt		0.76
Inter-day precision % (n=6)[4]			3.83
Inter-sample precision %RSD (n=15)[5]			6.35
Recovery (%)[6]	**20%**		102
	40%		97
	60%		102

3.7. Application of developed methodology to the analysis of soybean varieties

The developed method was finally applied to the determination of VLIVP peptide content in five different soybean varieties (Table 2).

[1]Determined as the minimum concentration which yielded an SNR equal to 3. Expressed in the ng/g units which was determined relative to 1 g of soybean sample.
[2]Determined as the minimum concentration which yielded an SNR equal to 10. Expressed in the ng/g units which was determined relative to 1 g of soybean sample.
[3]The repeatability was measured by five consecutive injection of peptide standard at two concentration levels.
[4]Precision determined in two consecutive days by the injection of soybean digested extract by triplicate.
[5]Determined as the RSD (%) value calculated for five individually prepared digested extracts of soybean proteins injected by triplicate.
[6]Percentage of peptide content added to the sample.

Table 2. Concentration of VLIVP peptide in different soybean varieties.[1]

Soybean	*Country of origin*	*Name*	*VLIVP [ng/g dried soybean]*
A[a]	Poland	Mazowiecka II	880 ±10
B[a]	Japan	Kachslung-4	798±8
C[a]	USA	Gieso	530±40
D[a]	USA	Davis	607 ±3
S[b]	Unknown	No information	562 ±7

[a] from CRF-INIA Soybean Germplasm collection
[b]commercial soybean

The content of the studied antihypertensive peptide ranged from 562 to 880 ng/g of soybean (as dry basis) being the variety from Poland that showing The highest VLIVP peptide yield These results demonstrated that the VLIVP peptide content varied among soybean genotypes and that not all soybean varieties present the same antihypertensive capacity.

4. Conclusions

An analytical methodology enabling the quantitation of the antihypertensive peptide VLIVP in different soybean varieties has been developed for the first time. The extraction of soybean proteins was carried out in just 2 minutes using a high intensity focused ultrasounds probe. A further fractionation of proteins was proposed to reduce sample complexity by the precipitation of 11S globulin but it resulted not quantitative. The optimization of the procedure for soybean proteins digestion with protease P revealed that it was very sensitive to variations of pH, temperature, and enzyme to substrate ratio and to the use of high intensity focused ultrasounds. A capillary-HPLC-MS/MS method was developed for the selective detection of the target peptide. Suitable optimization of chromatographic conditions and sample dilution was required to reduce peptide ionization suppression due to the matrix. The optimization of IT-MS parameters improved peptide signal intensity being the capillary voltage, dry gas flow and temperature, ICC target, and collision energy the parameters most affecting. The method enabled to detect up to 3.6 ng of peptide and to determine up to 12 ng of peptide in 1 g of soybean (as dry basis). Developed method showed to be precise, sensitive, and accurate and it was lack of matrix interferences. The method was applied to the quantitative determination of

[1]For every soybean variety two individually prepared extracts were obtained, and digested. Every sample was injected in triplicate.

VLVIP peptide in five different soybean varieties observing significant differences among peptide contents.

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References for Article 6:

[1] A. Chockalingam, World hypertension day and global awareness, Can. J. Cardiol. 24 (2008) 441-444.

[2] G. Li, G. Le, Y. Shi, S. Shrestha, Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, Nutr. Res. 24 (2004) 469-486.

[3] D. Martínez-Maqueda, B. Miralles, I. Recio, B. Hernández-Ledesma, Antihypertensive peptides from food proteins: a review, Food. Funct. 3 (2012) 350-361.

[4] B. Hernández-Ledesma, M. M. Contreras, I. Recio, Antihypertensive peptides: Production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23-35.

[5] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[6] R. López-Fañdino, J. Otte, J. van Camp, Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity, Int. Dairy J. 16 (2006) 1277-1293.

[7] A. Panchaud, M. Affolter, M. Kussmann, Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects, J. Proteomics 27 (2012) 3546-3559.

[8] H.D. Meiring, E. van der Heeft, G.J. ten Hove, A.P.J.M. de Jong, Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis, J. Sep. Sci. 25 (2002) 557-568.

[9] E. Domínguez-Vega, O. Kotkowska, M.C. García, A.L. Crego, M.L. Marina, Fast determination of the functional peptide soymetide in different soybean derived foods by capillary-high performance liquid chromatography, J. Chromatogr A 1218 (2011) 4928-4933.

[10] K. Inoue, T. Gotou, H. Kitajima, S. Mizuno, T. Nakazawa, N. Yamamoto, Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein, J. Biosci. Bioeng. 108 (2009) 111-115.

[11] H. Enari, Y. Takahashi, M. Kawarasaki, M. Tada, K. Tatsuta, Identification of angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their antihypertensive effect, Fisheries Sci. 74 (2008) 911-920.

[12] J.A. Gómez-Ruiz, M. Ramos, I. Recio, Identification and formation of angiotensin-converting enzyme-inhibitory peptides in Manchego cheese by high-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1054 (2004) 269-277.

[13] M. Sato, T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, T. Nakano, Angiotensin I-converting enzyme inhibitory peptides derived from wakame (Undaria pinnatifida) and their antihypertensive effect in spontaneously hypertensive rats, J. Agric. Food Chem. 50 (2002) 6245-6252.

[14] M. Guijarro-Diez, M.C. García, M.L. Marina, A.L. Crego, LC-ESI-TOF MS method for the evaluation of the immunostimulating activity of soybeans via the determination of the functional peptide soymetide, J. Agric. Food Chem. 61 (2013) 3611-3618.

[15] P. Puchalska, M.L. Marina, M.C. García, Development of a high-performance liquid chromatography-electrospray ionization- quadrupole- time- of- flight- mass spectrometry methodology for the determination of three highly antihypertensive peptides in maize crops, J. Chromatogr. A 1285 (2013) 69-77.

[16] T. Nakahara, A. Sano, H. Yamaguchi, K. Sugimoto, H. Chikata, E. Kinoshita, R. Uchida, Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin i-converting enzyme inhibitory substances, J. Agric. Food Chem. 58 (2010) 821-827.

[17] A. Quiros, M. Ramos, B. Muguerza, M.A. Delgado, P.J. Martín-Alvarez, A. Aleixandre, I. Recio, Determination of the antihypertensive peptide LHLPLP in fermented milk by high-performance liquid chromatography-mass spectrometry, J. Dairy Sci. 89 (2006) 4527-4535.

[18] Y. Hu, A. Stromeck, J. Loponen, D. Lopes-Lutz, A. Schieber, M.G. Gaenzle, LC-MS/MS quantification of bioactive angiotensin i-converting enzyme inhibitory peptides in rye malt sourdoughs, J. Agric. Food Chem. 59 (2011) 11983-11989.

[19] J.M. Curtis, D. Dennis, D.S. Waddell, T. MacGillivray, H.S. Ewart, Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS, J. Agric. Food Chem. 50 (2002) 3919-3925.

[20] U. Buetikofer, J. Meyer, R. Sieber, D. Wechsler, Quantification of the angiotensin-converting enzyme-inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in hard, semi-hard and soft cheeses, Int. Dairy J. 17 (2007) 968-975.

[21] J. Meyer, U. Buetikofer, B. Walther, D. Wechsler, R. Sieber, Hot topic: Changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese varieties, J. Dairy Sci. 92 (2009) 826-836.

[22] K.G.M. Gouda, L.R. Gowda, A.G.A. Rao, V. Prakash, Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max), J. Agric. Food Chem. 54 (2006) 4568-4573.

[23] M.C. García, M. Torre, M.L. Marina, A perfusion reversed-phase chromatographic method for ultrarapid determination of soybean proteins in soybean infant formulas and soybean milks: Method development and validation, J. Chromatogr. Sci. 36 (1998) 527-534.

[24] M.M. Contreras, I. López-Exposito, B. Hernández-Ledesma, M. Ramos, I. Recio, Application of mass spectrometry to the characterization and quantification of food-derived bioactive peptides, J. AOAC Int. 91 (2008) 981-94.

[25] C.S. Gilani, C. Xiao, N. Lee, Need for accurate and standardized determination of amino acids and bioactive peptides for evaluating protein quality and potential health effects of foods and dietary supplements, J. AOAC Int. 91 (2008) 894-900.

[26] B.O.E., Métodos Oficiales de análisis de aceites y grasas, cereales y derivados, productos lácteos y productos derivados de la uva, 19-07-1977, and 20-07-1977.

[27] C. Liu, H. Wang, Z. Cui, X. He, X. Wang, X. Zeng, H. Ma, Optimization of extraction and isolation for 11S and 7S globulins of soybean seed storage protein, Food Chem. 102 (2007) 1310-1316.

[28] T. Nagano, M. Hirotsuka, H. Mori, K. Kohyama, K. Nishinari, Dynamic viscoelastic study on the gelation of 7S globulin from soybeans, J. Agric. Food Chem. 40 (1992) 941-944.

[29] V. Thanh, K. Shibasak, Major proteins of soybean seeds - straightforward fractionation and their characterization, J. Agric. Food Chem. 24 (1976) 1117-1121.

III.1.3.

Development of an analytical methodology for the determination of protein kinase subunits by selected reaction monitoring assay in various rat tissues

III.1.3. Development of an analytical methodology for the determination of protein kinase subunits by selected reaction monitoring assay in various rat tissues.

Preface

Protein phosphorylation is one of the most important post-translational modifications playing a central role in cell regulation. Phosphorylation by protein kinases plays an essential role in signal transduction pathways transmitting stimulation by external signals from the membrane to the cell. Usually, an external impact (*e.g.* hormone) stimulates a receptor at the cell membrane which triggers the cascade of phosphorylation events within the cell [254, 255]. As previously mentioned, cAMP and cGMP are secondary messengers targeting PKA and PKG proteins, respectively. Upon activation of these two kinases, the phosphorylation occurs in the cell exerting important cardiovascular functions. PKA has an important role in cardiac contractility, while PKG is essential in BP regulation. CaMKII protein kinase, regulated by the Ca^{2+}/CAM level, is involved in many physiological responses. Increased activity of CaMKII protein was detected in failing heart of humans and animal models. Interestingly, all these proteins can be found as different variants [13]. PKA contains two regulatory and two catalytic subunits that are encoded in the genome as various isoforms (RIα, RIβ, RIIα, RIIβ and Cα, Cβ, and Cγ). On the other hand, PKG is expressed in variants Iα, Iβ, and II, while CaMKII as α, β, γ (A-C), and δ (1-7) isoforms. Although there are very small differences between these isoforms, it is suspected that they have very different specificity and funtions. Since differences among these isoforms used to be really small, there are no antibodies to distinguish among them. However, knowledge on how these specific isoforms work and are expressed is limited, let alone to understand their role in disease mechanisms. Therefore, the development of a method for the absolute quantification of these protein kinases isoforms in different tissues is of high interest.

SRM targeted proteomics is an important technique for unambiguous detection and quantification of a set of specific proteins in complex matrices. It consists of the selection of a group of proteotypic peptides for a specific protein or its isoforms and peptides fragments. Due to the unique triple quadrupole filtering feature, the appropriate selection of peptide to fragment (transition) ion settings enables to obtain extented dynamic range of the method. PKA, PKG, and CaMKII isoforms have highly similar sequences. Therefore, the appropriate selection of proteotypic peptides for every isoform could be a big challenge.

Objectives

The specific objectives of this work were:

- To select a set of proteotypic peptides that uniquely identify targeted PKA, PKG, and CaMKII isoforms.
- To select a set of highly abundant transitions for every proteotypic peptide.
- To validate proteotypic peptides and transitions using heavily labeled peptides.
- To increase the sensitivity of the method by its scheduling and the optimization of collision energy in the CID cell.
- To apply the SRM developed method to real samples.

Results

The results obtained in this research work will be included in the following scientific article:

- **Article 7:** *Nano-LC-QqQ-MS selected reaction monitoring (SRM) assay in the determination of PKA, PKG, and CaMKII isoforms in rat tissues.*
  P. Puchalska, G. Maddalo, P. Wijten, S. Soni, M. C. García, M. L. Marina, M. A. F. Altelaar, A. J. R. Heck, A. Scholten.

Article 7

Nano-LC-QqQ-MS selected reaction monitoring (SRM) assay in the determination of PKA, PKG and CaMKII isoforms in rat tissues

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Abstract

PKA, PKG and CaMKII proteins exert highly important cardiovascular functions in the organism. They are encoded in the genome as a set of various isoforms. High sequence similarity of these isoforms renders their detection using antibodies impossible. Despite their high sequence similarity, the different isoforms display huge variability in terms of representation in different tissues. In order to assess the distribution of all these kinase isoforms within specific tissues, a selected reaction monitoring (SRM) assay using triple quadrupole mass spectrometry (QqQ) was developed. Information from various sources (*in-silico* digestion simulation, previous results, Peptide Atlas, and BLAST) was used to set up a preliminary SRM assay. To facilitate the SRM development, digestions of the different kinases obtained by pull-downs performed on tissues were analyzed by Orbitrap Velos with HCD fragmentation. The application of this new approach enabled us to confirm the theoretical selection of proteotypic peptides and to define a list of possible transitions. Most of the transitions were confirmed on a QqQ instrument. Peptides and transitions were validated using isotopic heavily labeled peptides and possible interfering transitions were omitted. In order to gain sensitivity, the developed method was scheduled over the chromatographic run and the collision energy for every peptide was optimized. The developed assay was applied to digested lysates of heart, liver, and kidney. The high dynamic range of these samples prevented the determination of all isoforms. The application of SDS-PAGE to separate CaMKII isoforms enabled to reduce sample complexity from the heart lysate but, when it was applied to a big set of heart lysates, it showed high irreproducibility. Although the appropriate SRM assay has been set, additional studies are needed to reduce sample complexity and to remove the lack of reproducibility.

Keywords:

Protein kinases isoforms; SRM; Absolute quantification; Cardiovascular disease

1. Introduction

The proteome is a highly variable dynamic system which depends on various external factors, like state of development, metabolic state, tissue type, or interaction with other organisms or substances. Protein expression levels can change due to aging, influence of diseases, infections, *etc.* [1]. Advances in mass spectrometry (MS) instrumentation enabled to gain deep insight into signal transduction. However, in order to understand the dynamics nature of protein interaction networks from cell to cell and how they are influenced due to *i.e.* stimulation, the absolute quantification of selected sets of proteins at the exact cell/tissue state is crucial [2].

Signal transduction in the myocardium involves a broad range of multifunctional protein kinases like protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent kinase II (CaMKII) [3]. PKA and protein kinase G (PKG) are essential components in the cardiovascular system being involved in heart contractility and regulation of blood pressure [4-6]. They are activated through the signaling pathway of secondary messengers such as cyclic adenosine 3',5'-monophosphate (cAMP) and cyclic guanosine 3',5'-monophosphate (cGMP), respectively. Although cAMP and cGMP are involved in a variety of physiological responses, their major targets are, in fact, PKA and PKG protein kinases [4]. Other important roles in cardiac events is played by the CaMKII protein [7, 8], which is activated by a raised level of calcified calmodulin. In addition, this protein can be activated by autophosphorylation within the protein amino acid sequence [9]. Moreover, CaMKII levels have been shown to be overexpressed in heart pathological response mechanisms [10]. Mammalian PKA encompasses two regulatory (R) and two catalytic (C) subunits. Each subunit can be expressed as different isoforms: 4 regulatory (Iα, Iβ, Iiα, and IIβ) and 3 catalytic (α, β, and γ) [11]. PKG can be found as isoform type I with two versions (α and β) and as type II [6]. Finally, CaMKII presents 4 isoforms: α, β, γ, and δ [3, 9, 12, 13]. The differences between these protein kinases isoforms can be very small and, thus, their detection and quantification using antibodies is not possible. Despite these small differences, it has been shown that the actual function and distribution of these isoforms in organism tissues may be significantly different [7, 14]. Indeed, PKA RIα is mainly expressed in the heart and central nervous system, while PKA RIβ is the main isoform in nervous tissues such as spinal cord and brain. PKA RIIα and RIIβ were both determined in brain, when RIIα subunits is dominant in heart and RIIβ in liver and fat tissues [6]. PKG Iα was mainly detected in lung, heart, dorsal root gangalia, and cerebellum, while PKG Iβ in platelets, hippocampal

neurons, and olfactory bulb neurons [4]. On the other hand, CaMKII α and β are expressed in neuronal tissues [12] while CaMKII δ and γ are concentrated in myocardium [8]. Therefore, the information about the expression level of these isoforms in various tissues or in the same tissue at different disease levels, seems to be essential to deeply understand their function in the organism. Thus, a method that enables the absolute quantification of these isoforms in different tissues is highly desired.

Recently, selected (/multiple) reaction monitoring (SRM/MRM) has emerged as a promising technique for accurate quantification of low concentration targeted proteins [15-17]. SRM is carried out using a liquid chromatography (LC)/tandem MS/MS method in scan mode using a triple quadrupole (QqQ) MS instrument [18] that might enable rapid, sensitive, and specific quantification of proteins. SRM uses the unique capability of QqQ to act as a double filter. The first and third quadrupoles specifically select predefined mass per charge (*m/z)* values of peptide ion precursors and their fragmented ions (transitions), respectively, while the second quadrupole serves as a collision cell [15]. The combination of retention time, peptide mass, and its fragments practically eliminates ambiguities in peptide assignments [19]. SRM assay development consists of five general steps: biological question/definition of a set of proteins; determination of a set of representative peptides; selection of transitions (peptide/fragments pairs) that maximize sensitivity and selectivity; experimental validation of transitions; optimization of transitions [20]. Among these steps, the critical point is the selection of targeted peptides (commonly called proteotypic peptides (PTPs)) that need to fulfill very stringent criteria [15, 20]. Indeed, PTPs have to be uniquely associated with the protein of interest and routinely observed in LC-MS analysis [15]. SRM also requires to know the peptide fragmentation pattern by collision induced dissociation (CID). Commonly, the selection of transitions for SRM assays relies on shotgun proteomics from the discovery phase [18], mostly acquired using low energy ion-trap (IT) fragmentation. However, differences in terms of peptide fragmentation spectra observed in QqQ and IT might lead to erroneous and/or time consuming selection of inappropriate transitions. The latest development in LTQ-Orbitrap allows to perform peptide fragmentation in higher energy dissociation (HCD) that has shown to be highly correlated with conventional CID in QqQ [21]. Therefore, peptide fragmentation spectra obtained by HCD using LTQ-Orbitrap Velos have been proposed in this study to develop the SRM assay.

The aim of this work was to set up a SRM assay QqQ to assess the specific tissue distribution of PKA, PKG, and CaMKII kinase isoforms.

2. Materials and methods

2.1. Chemicals and samples

2.1.1. Materials

Water was freshly taken from a MilliQ system (Millipore, Bedford, MA, USA). Complete mini EDTA-free Cocktail and PhosSTOP phosphate inhibitor and enzyme Lys-C were from Roche (Disgnostics, Almere, Netherlands). Acetonitrile (ACN) was from Biosolve (Netherlands). Acetic acid (AA), formic acid (FA), KPO_4, NaCl, ammonium bicarbonate (ABC), sodium dodecyl sulfate (SDS), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Merck (Darmstadt, Germany). Adenosine-5'-diphosphate (ADP), guanosine-5'- diphosphate (GDP), Tween-20, dithiotreitol (DTT), iodoacetamide (IAA), and urea were supplied by Sigma (St.Louis, MO). XT-Mops and Laemmli buffer were from Bio-Rad (Hercules, USA). Trypsin was from Promega (Benelux, BV), 8-AHA-cAMP beads from BIOLOG (Bremen, Germany), BenchMark™ Protein Ladder from Life Technologies (Gaithersburg, MD, USA) and Page Ruler™ Prastained from Pierce (Rockford, IL USA). Heavily labeled peptides (C-terminal heavy Arginine (U- $^{13}C_6$, $^{15}N_4$/ +10 Da) or Lysine (U- $^{13}C_6$, $^{15}N_2$/ +8 Da)) were supplied by JPT (Berlin, Germany). Peptides have been dissolved in 80% ACN/ 1% FA (peptide IVVQGEPGDEFFIILEGTAAVLQR was in 40% ACN/ 1% FA) to different concentrations, frozen, and stored at -80°C until use.

2.1.2. Animal samples

All animal samples were provided by the Department of Medical Physiology, Division of Heart & Lungs, University Medical Centre in Utrecht (the Netherlands) (S. Soni). Animal care and handling was performed in accordance with the 'European Directive for the protection of Vertebrate animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE'. Experiments were approved by the committee for experiments on animals at Utrecht University. Male Wistar rats were housed in defined growing conditions (21°C, 60% humidity, 12:12 h light dark cycle) and fed with standard chow and water. Heart, kidney and liver were obtained from the rats. Some of the rats were subjected to transverse aortic constriction (TAC) or SHAM-12 surgeries. Rats were monitored and sacrificed at three different points. SHAM-12 (n=4) rats were sacryfied by blocking and unblocking aorta (control sample). TAC-4, TAC-12 and TAC-16 (n=4 in every group) rats were sacrified by blocking aorta during 4, 12, or 16 weeks, respectively.

2.2. Sample preparation methods

2.2.1. Tissue lysate preparation

Hearts, kidney and liver tissues were cut into small pieces and pulverized by pre-cooling with a liquid nitrogen pestle and mortar. Various lysis procedures were used. Lysis *procedure 1* was performed as previously [4] with modifications. *Procedure 1* consisted of mixing pulverized tissue with 500 µL of lysis buffer 1 (15 mL phosphate buffer solution (PBS) (50 mM KPO_4, 150 mM NaCl), 0.2% Tween 20, 1 tablet of Complete mini EDTA-free Cocktail and 1 tablet of PhosSTOP phosphate inhibitor) and keep at room temperature for 5 minutes. The sample was then vortex and centrifuged (14,000 g x 10 min, 4°C). The supernatant was collected. The lysis procedure was repeated twice and three supernatants were combined and kept on ice until further use. Lysates obtained from procedure 1 were exclusively used for the pull-down preparation. Lysis *procedure 2* was carried out like previously described [22] with some modifications. Pulverized tissue was mixed with 500 µL of lysis buffer 2 (50 mM ABC solution, 2% SDS, 1 tablet of Complete mini EDTA-free Cocktail, and 1 tablet of PhosSTOP phosphate inhibitor) and kept at room temperature for 5 minutes. Sample was then homogenized and centrifuged (14,000 g x 10 min, 4°C). The supernatant was collected, procedure repeated, and, the two supernatants combined. Third step of lysis *procedure 2* was enhanced by sonication with an ultrasound probe (0.5 cycle, 80% amplitude, three times 0.5 min with 0.5 min stops, sample placed on ice). After centrifugation (14,000 g x 10 min, 4°C), three supernatants were combined and analyzed.

SHAM and TAC hearts lysates (Lysis *procedure 3*) were provided by S. Soni (University Medical Centre in Utrecht). The procedure of lysis was similar to *procedure 1*. RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM Na_2HPO_4, 1% (v/v) Triton X-100, 1% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride, and 14 $\mu g \cdot mL^{-1}$ aprotinin) was used as lysis buffer [23].

The protein yield in the lysates obtained by the three procedures was determined using Bradford assay kit from Bio-Rad.

2.2.2. cAMP- pull-downs preparation

Pull-downs were prepared following a previously described procedure [4]. Prior to the pull-down procedure, around 50 µL of immobilized 8-AHA-cAMP beads were washed with 1 mL

of PBS buffer. Lysates obtained using *lysis procedure 1* (heart, kidney, and liver) were incubated with 10 mM ADP/GDP at 4°C for 15 min to reduce non-specific binding. Therefore, lysate was incubated with beads (lysate to beads ratio, 1:100) for 2 h at 4°C by rotary shaking. The supernatant was separated from beads (bound fraction). The bound fraction was washed several times (total volume 12 mL) with buffer (PBS, 0.2% Tween 20, 1 tablet of protease inhibitor mixture) to further reduce non-specific binding. The bound fraction was mixed with 90 µL of 8 M urea in order to elute proteins. Enriched proteins were digested by in-solution digestion.

2.2.3. In-solution digestion

This two-steps digestion was performed for all pull-downs as follow [4]. The reduction was performed by the addition of 200 mM DTT (reduction agent ratio to protein, 1:100 (v:w)) and samples were incubated for 15 min at 50°C in a shaker. Next, an alkylation step was executed by addition of 200 mM of IAA (alkylation agent to protein ratio, 1:200 (v:w)) and incubation for 20 min in the dark. The digestion with Lys-C enzyme (0.2 µg/µL) was performed for 4 h at 37°C (protein to enzyme ratio, 1:75 (v:w)). The concentration of urea was diluted till 2 M and lysates were digested overnight with trypsin enzyme (0.1 µg/µL) at 37°C (protein:enzyme ratio, 1:50 (v:w)). The digestion was stopped by the addition of 10% FA (FA solution to sample ratio, 1:100 (v:w)).

2.2.4. Filter aided sample preparation (FASP) digestion

Original FASP protocol [24] was modified and performed as follows: the filter with Mwco of 30 kDa (Amicon, Ultra, Millipore) was pre-rinsed with 0.25 mL of fresh MilliQ water and centrifuged for 15 min (14,000 x g). Around 200 µg of heart, kidney or liver protein lysates (*lysis procedure 2)* were taken, dissolved in FASP solution A (8 M urea in 0.1 M Tris buffer pH 8.5) to reach a volume of 230 µL, and reduced with 10 mM DTT in FASP solution B (8 M urea in 0.1 M Tris buffer pH 8.0) (reduction agent to protein ratio, 1:100 (v:w)) for 20 min at 56 °C in shaker. Reduced protein sample was introduced into the filters and centrifuged (14,000 g x 15 min, 20°C). Filter was washed with 200 µL of FASP solution A and centrifuged as previously. To alkylate proteins, 100 µL of 200 mM IAA in FASP solution A were added and filters were incubated in the thermomixer (600 g) for 20 min at room temperature in the dark. After the incubation, filters were centrifuged (14,000 g x 10 min, 20°C). To wash the filter, FASP solution B was added and centrifuged (14,000 g x 15 min, 20°C). This step was repeated and 40 µL of Lys-C enzyme (0.2 µg/µL) (enzyme to protein ratio, 1:50 (v:w)) in FASP solution

B was applied on the filter. Filter was incubated in hot air oven for 4 hours at 37°C. Then, 180 µL of trypsin solution (0.1 µg/µL) in ABC (enzyme to protein ratio, 1:50 (v:w)) was applied on the filter and left overnight in hot air oven at 37°C. Mwco filters were next transferred to a new collective tubes and obtained peptides centrifuged (14,000 g x 10 min, 20°C). Filter was washed with 50 µL of 0.5 M NaCl and, once again, centrifuged (14,000 g x 10 min, 20°C). Obtained eluates were acidified with 10% FA (FA solution to sample ratio, 1:100 (v:w)) to stop enzymatic reaction.

2.2.5. SDS-PAGE separation

A Bio-Rad system was used for the electrophoretic separation of proteins. Separation was carried out on Criterion XT precast gels 12% Bis-Tris (Bio-Rad) using XT -MOPS (diluted twenty times) as running buffer. Tissue lysate obtained with *lysis procedure 2* containing 30 µg of proteins was mixed with Laemmli buffer and DTT (5.4 mg/mL) so that volume ratios were 2:1:1 (v:v:v). Mixture was boiled at 95°C for 5 min in a thermostat and spin. Protein standards (BenchMark™ or Page Ruler^{TM} Prestained Protein Ladders) were injected in the first lane and used as molecular weight ladders. SDS-PAGE was performed at 20 mA for 30 min and 50 mA until the dye-front reached the bottom. Proteins were fixed by gentle agitation in 100 mL of 10% (v:v) glacial AA and 40% methanol for 30 min, stained with 50 mL of Bio-Safe Coomassie stain for 1 h, and washed with Milli-Q water. SDS-PAGE gels (n=12) of SHAM and TAC heart lysates were performed by S. Soni (University Medical Centre in Utrecht). Twelve heart lysates were divided into four groups, so that every group contained one SHAM-12, one TAC-4, one TAC-12, and one TAC-16 member. Separation of these lysates was performed by triplicate. In total, 12 gels containing 4 lysates were obtained and in-gel digestion was performed.

All gels were scanned on the GS-800 calibrated densitometer (Bio-Rad) using the highest resolution. The amount of loaded protein on SHAM and TAC gels was normalized by the comparison of the density of line with Mw around 17 kDa. The density of this line was corrected with the background and normalization was applied to the maximum value in the replicate.

2.2.6. In-gel digestion

Bands were cut from the gel under sterile conditions and washed with Milli-Q water. To shrink gel pieces, ACN was added for 15 min and removed. Therefore, 100 µL of 6.5 mM DTT in 50 mM ABC was added and samples were incubated for 1 h at 60 °C in shaker. After

removing DTT, gel pieces were shrunk once again (ACN, 15 min), 100 µL of 54 mM IAA was added, and samples were left at room temperature in the dark for 20 min. IAA was removed. Gel pieces were shrunk with ACN (15 min) and incubated for 15 min with 50 mM ABC (step repeated). Afterwards, gel pieces were shrunk once again, ACN removed, and 20 µL of cold trypsin solution (3 ng/µL) in 50 mM ABC was added to every sample to cover gel pieces. Samples were incubated for 90 min on ice. The excess of trypsin was removed and appropriate volume of 50 mM ABC was added to cover gel pieces. Samples were incubated overnight at 37°C in a stove. After digestion, peptides were extracted using an extended three step extraction procedure. Gel pieces were incubated for 20 min in the sonication bath in an appropriate volume (to cover gel pieces) of 5% (v/v) formic acid (FA), then 50% (v/v) ACN/5% (v/v) FA, and ACN, respectively. After each step, a fraction was collected and combined with the previous one. These three step extraction procedure was performed twice. Extracted peptides were dried in a *SpeedVac*™ system (Thermo, Bremen, Germany) and stored in -80°C until use. Prior to the analysis, samples were dissolved in 10% FA to attain appropriate concentration.

2.2.7. Solid phase extraction (SPE)

All digests were desalted by SPE using C18 Sep-Pak (Waters Associates Inc., Milford, Massachusetts) cartridges as previously described [21]. Briefly, acidified sample was loaded onto the SPE column, previously conditioned with 2 mL of ACN and 2 mL of 0.1 M AA (solvent A). Column with retained sample was washed twice with solvent A and eluted with 0.5 mL of 80% ACN in solvent A. Eluents were evaporated in a *SpeedVac*™ system and stored at -80°C till analysis. Prior to the analysis, samples were dissolved in 10% FA to attain appropriate concentration.

2.3. Nano-LC-MS

The same nano-LC configuration was performed on all used MS experiments. A 1100 series liquid chromatograph from Agilent Technologies (Pittsburg PA, USA) was used. Mobile phases consisted of water/0.1 M AA (mobile phase A) and ACN/water (80:20) containing 0.1 AA (mobile phase B). Prior to the separation, sample was trapped on a Reposil C18-AQ (Dr. Maisch, Ammerbuch, Germany), (100 µm x 20 mm, 3 µm, 120 Å double frit) trapping column at a flow rate of 5 µL/min for 10 min in mobile phase A. Afterwards, tryptic digests were separated on Reposil C18-AQ (Dr. Maisch, Ammerbuch, Germany) (50 µm x 400 mm, 3 µm, 120 Å) analytical column using the binary gradient: 0-24% B in 115 min, 24-50% B in 45 min, 50-100% B in 3 min, and 100% B during 17 min (total 180 min). For the optimization of the

collision energy, the following short gradient (total 95 min) was used: 0-8% B in 13 min, 8% B during 3 min, 8-15% B in 30 min, 15-63% B in 5 min, 63-84% B in 35 min, 84-100% B in 1 min, and 100% B during 1 min. In all cases, the flow rate was passively splitted to attain 600 nL/min during the analysis and the injected volume was 10 µL. The concentration of samples before injection was adjusted as follows: 5% of pull-downs, 200 ng of FASP digest, and around 10% of in-gel digests. Nanospray was achieved with in-house pulled and gold-coated fused silica capillaries (o.d. 360 µm; i.d. 20 µm; tip i.d. 10 µm) and an applied voltage of 1.7 kV. Heart, kidney, and liver digested pull-downs were analyzed on LTQ-Orbitrap Velos™, where peptide fragmentation involved Fourier Transform (FT) survey scan from 350-1500 *m/z* (resolution 30 000) and accumulation to a target value of 500,000 followed by HCD fragmentation (target value 30 000) of the 10 most intense peaks, and readout in the FT analyzer (resolution 7500). Normalized HCD collision energy was set following the equations: collision energy = 0.041**m/z*-0.573 and collision energy = 0.051**m/z* +0.095, for doubly and triply charged precursors, respectively. The rest of samples were analyzed on TSQ Vantage™ (QqQ) in data dependent tandem MS/MS mode. Q1 and Q3 window were set at 3.0 and 0.4 Da, respectively. To analyze all targeted proteins, 162 transitions corresponding to 27 PTPs with optimized collision energy were set up. In case of CaMKII, 40 transitions corresponding to 7 PTPs were selected. In both setups, analyzed transitions were scheduled in time with a time window of 12 min. Xcalibur™ software was used to control both MS equipments.

2.4. Data Analysis

Peptides from LTQ-Orbitrap Velos experiments were identified from raw files using Proteome Discoverer version 1.3. Peptide identification was performed by searching against IPI rat database 3.68. A maximum of two missed cleavages for trypsin digestion and carbamidomethylation (C) (fixed) and oxidation (M) (variable) modifications were set. Peptide and fragment tolerances were set at 0.6 Da. Further filtering was performed using the decoy search option utilizing percolator algorithm. Resulting data files were exported and filtered for <1% false discovery rate (FDR) at peptide level. SRM assay creation and analysis were performed using Skyline software (MacCoss Lab, Department of Genome Sciences, UW, USA).

3. Results and discussion

3.1. SRM method development

Investigation of proteins of interest. Basic information about studied proteins isoforms from rat (*Rattus norvegicus*) species have been searched and extracted from the Uniprot database (see supplemental material 1). Within all possible isoforms, PKA Cγ and PKG Iβ have not been yet sequenced in rat tissues, thus they had to be excluded from the list of studied protein isoforms. Uniprot alignment tool permitted to observe very high proteins similarities between PKA Cα-Cα2 (96.01%), PKA Cα-Cα1-Cβ (88.60%), CaMKII γ A-B (91.45%), and CaMKII δ 1-7 (89.31%) isoforms. The comparison of PKA R subunits (I α, I β, II α, II β) revealed a 26.44% of homology, while CaMKII isoforms (α, β, γA, δ1) showed 66.07% of similarity. Due to the high similarity of these protein isoforms, the selection of PTPs was challenging.

Selection of PTPs candidates. In order to choose a list of potential PTPs candidates, various information sources have been used. First, all targeted protein isoforms were digested *in-silico* with trypsin by Skyline software. The obtained peptides were refined, according to the following criteria: peptide length range from 8 to 25 amino acids, with a maximum of one missed cleavage within the sequence, 25 N-terminal amino acids, and exclusion of peptides containing methionine. Additionally, a vast set of mass spectrometry data obtained from rat tissues (13000 identified peptides) was also analyzed. Candidates fulfilling the following requirements were taken into account: unique peptides, without any observed modifications (carboamidomethylation, phosphorylation, oxidation, *etc.*) under the experimental conditions, with a maximum of one missed cleavage, and not present within other observed missed cleaved peptides. Subsequently, selected peptides were verified by 'Peptides Atlas' and using standard protein BLAST (*Basic Local Alignment Search Tool*) that evaluate these peptides were unique for every targeted protein isoform. Additionally, possible posttranslational modifications for each protein isoform were checked using Uniprot database and aligned with selected PTPs. Based on this information, it was possible to theoretically select 28 PTP candidates having 39 precursors (2+ and 3+ peptide ions) and 302 transitions for six PKA isoforms. Due to the high similarity of PKA Cα and Cα2, it was not possible to indicate any PTP to differentiate between them. For two isoforms of PKG, 9 PTPs candidates having 10 precursors and 71 transitions were selected. Interestingly, CaMKII α and β isoforms did not show any peptide enabling their differentiation. Therefore, PTPs for all CaMKII isoforms, without differentiation of them, were chosen. It was also not possible to indicate PTPs for γ (A-B) or δ (1-7) isoforms, thus they were

treated as one group of γ and δ isoforms. Consequently, 14 PTPs with 14 precursors and 99 transitions for three proteins (CaMKII as a whole and CaMKII γ and CaMKII δ groups) were obtained. In total, the preliminary method contained 11 protein isoforms with 51 PTPs candidates, 63 precursors, and 472 transitions.

Selection of transitions. Peptides predicted *in-silico* can facilitate SRM assay development, but they cannot constitute the unique criterium for the selection of PTPs for our SRM assay. Therefore, a novel approach based on LTQ-Orbitrap HCD measurements [21] was applied. Heart, kidney, and liver pull-downs, that contained high concetration of targeted kinases isoforms, were digested and analyzed. Due to the lower complexity of these samples, the confirmation or rejection of previously selected PTPs and transitions candidates could be performed. All isoforms of PKG and CaMKII proteins were identified in all digested pull-downs. In case of PKA, isoforms RIα, RIIα, RIIβ, and Cα were observed in all digested pull-downs. Isoform RIβ was presented just in the heart and kidney digested pull-downs, while Cβ was just detected in the kidney sample. Kidney digested pull-down was the only one that contained all targeted isoforms. Next step was the creation of a spectral library from LTQ-Orbitrap HCD digested pull-down experiments using Skyline. The highest ranking of *y*- and *b*-ions transitions (at least 4) for all PTPs precursors were selected. Elimination of some PTPs and transitions enabled the design of the SRM method that contained 6 PKA isoforms (20 peptides, 25 precursors, and 189 transitions), 2 PKG isoforms (7 peptides, 8 precursors, and 56 transitions), and 3 CaMKII isoforms (11 peptides, 11 precursors, and 76 transitions). In total, 38 peptides, 44 precursors, and 321 transitions were left. Although HCD and CID in QqQ (TSQ^{TM}) fragmentations are highly similar, the appropriate selection of PTPs and transitions need to be confirmed using a real SRM assay in the TSQ equipment. Since all studied isoforms have been previously detected in the digested kidney pull-down, this sample was analyzed on TSQ. The results showed that although most peptides and transitions had been confirmed, some of them had to be excluded. The final SRM assay contained 27 PTPs with 27 precursors and 162 transitions (see Table 1).

Table 1. SRM assay settings for the PKA, PKG and CaMKII isoforms (underline transitions were deleted after the validation step).

Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)	Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)
PKA RIα					**PKA RIβ**				
K.**HNIQALLK**.D [24, 31]	468,7849 ++	N [y7] - 799,5036+ L [y2] - 260,1969+ N [b2] - 252,1091+ I [b3] - 365,1932+ Q [b4] - 493,2518+ A [b5] - 564,2889+ L [b6] - 677,3729+ Q [b4] - 247,1295++	63	21.3	K.**HGIQQVLK**.E [24, 31]	461,7771 ++	G [y7] - 785,4880+ L [y2] - 260,1969+ **Q [b4] - 436,2303+** Q [b5] - 564,2889+ V [b6] - 663,3573+ Q [b4] - 218,6188++ Q [b5] - 282,6481++	41	21.0
K.**IVVQGEPGDEFFIILEGTAA-VLQR**.R [281, 304]	867,8023 +++	E [y9] - 944,5160+ G [y8] - 815,4734+ A [y6] - 657,4042+ A [y5] - 586,3671+ L [y3] - 416,2616+ F [b11] - 1171,5630+	139	37.5	K.**VSILESLEK**.W [252, 260]	509,2950 ++	S [y8] - 918,5142+ I [y7] - 831,4822+ L [y6] - 718,3981+ E [y5] - 605,3141+ S [y4] - 476,2715+ **L [y3] - 389,2395+**	131	19.6
PKA RIIα					**PKA RIIβ**				
R.**QQPPDLVDFAVEYFTR**.L [23, 38]	962,9756 ++	L [y11] - 1359,6943+ V [y10] - 1246,6103+ D [y9] - 1147,5419+ F [y8] - 1032,5149+ A [y7] - 885,4465+ Y [y4] - 586,2984+ F [y3] - 423,2350+ P [y14] - 834,9170++	166	32.1	R.**HQPADLLEFALQHFTR**.L [22, 37]	641,6672 +++	E [y9] - 1148,5847+ F [y8] - 1019,5421+ A [y7] - 872,4737+ L [y6] - 801,4366+ H [y4] - 560,2940+ Q [b2] - 266,1248+	155	27.5

Table 1. Continuation.

Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)	Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)
PKA RIα continuation					**PKA RIβ continuation**				
R.**AATIVATSDGSLWGLDR**.V [214, 230]	866,9469 ++	A [y12] - 1277,6121+ T [y11] - 1206,5749+ S [y10] - 1105,5273+ G [y8] - 903,4683+ T [b3] - 244,1292+ I [b4] - 357,2132+	139	29.8	R.**GTFDIYVK**.C [194, 201]	471,7502 ++	F [y6] - 784,4240+ D [y5] - 637,3556+ I [y4] - 522,3286+ Y [y3] - 409,2445+ V [y2] - 246,1812+ D [b4] - 421,1718+	108	15.4
R.**AASAYAVGDVK**.C [344, 354]	526,2746 ++	S [y9] - 909,4676+ Y [y7] - 751,3985+ A [y6] - 588,3352+ **V [y5] - 517,2980+** G [y4] - 418,2296+ V [y2] - 246,1812+	60	18.2	R.**AASAHAIGTVK**.C [359, 369]	513,2905 ++	A [y6] - 588,3715+ I [y5] - 517,3344+ G [y4] - 404,2504+ **H [y7] - 363,2189++** H [b5] - 438,2096+ A [b6] - 509,2467+	30	22.8
PKA Cα					**PKA Cβ**				
K.**AKEDFLK**.K [22, 28] (missed 1)	425,7371 ++	**K [y6] - 779,4298+** F [y3] - 407,2653+ L [y2] - 260,1969+ K [y6] - 390,2185++ **K [b2] - 200,1394+** D [b4] - 444,2089+	42	18.8	K.**AKEDFLR**.K [22, 28] (missed 1)	439,7402 ++	E [y5] - 679,3410+ D [y4] - 550,2984+ F [y3] - 435,2714+ **K [y6] - 404,2216++** K [b2] - 200,1394+ E [b3] - 329,1819+	51	18.3

Table 1. Continuation.

Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)	Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)
PKA Cα continuation					**PKA Cβ continuation**				
K.**TLGTGSFGR**.V [48, 56] (in common with Cβ)	448,2352 ++	L [y8] - 794,4155+ G [y7] - 681,3315+ T [y6] - 624,3100+ G [y5] - 523,2623+ S [y4] - 466,2409+ F [y3] - 379,2088+ G [b3] - 272,1605+	62	16.6	R.**LEYSFK**.D [106, 111]	393,7053 ++	E [y5] - 673,3192+ Y [y4] - 544,2766+ S [y3] - 381,2132+ F [y2] - 294,1812+ E [b2] - 243,1339+	78	15.7
K.**LEFSFK**.D [106, 111]	385,7078 ++	E [y5] - 657,3243+ F [y4] - 528,2817+ S [y3] - 381,2132+ F [y2] - 294,1812+ E [b2] - 243,1339+	124	13.4					
PKG I					**PKG II**				
K.**DSCIIK**.E [126, 131]	368,1889 ++	S [y5] - 620,3436+ C [y4] - 533,3116+ I [y3] - 373,2809+ I [y2] - 260,1969+ **C [b3] - 363,0969+**	40	13.8	R.**NYQQGSYIVK**.Q [187, 196]	600,3064 ++	Q [y7] - 794,4407+ G [y6] - 666,3821+ **I [y3] - 359,2653+** V [y2] - 246,1812+ Y [b2] - 278,1135+ **Q [b3] - 406,1721+**	60	20.7

Table 1. Continuation.

Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)	Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)
PKG I continuation					**PKG II continuation**				
K.**VFGELAILYNCTR**.T [164, 176]	778,4005 ++	G [y11] - 1309,6569+ **L [y9] - 1123,5928+** A [y8] - 1010,5088+ **I [y7] - 939,4717+** L [y6] - 826,3876+ Y [y5] - 713,3035+	145	25.8	K.**ALISDDVR**.S [358, 365]	444,7429 ++	I [y6] - 704,3573+ S [y5] - 591,2733+ D [y4] - 504,2413+ D [y3] - 389,2143+ V [y2] - 274,1874+	72	16.4
K.**TYNIILR**.G [565, 571]	446,7662 ++	Y [y6] - 791,4774+ N [y5] - 628,4141+ I [y4] - 514,3711+ I [y3] - 401,2871+ L [y2] - 288,2030+ Y [b2] - 265,1183+	99	15.5					
CaMKII all					**CaMKII γ**				
K.**GAFSVVR**.R [21, 27]	368,2110 ++	F [y5] - 607,3562+ S [y4] - 460,2878+ V [y3] - 373,2558+ V [y2] - 274,1874+ F [b3] – 363,0969+	74	14.8	R.**FTDDYQLFEELGK**.G [9, 21]	802,8776 ++	**L [y7] - 835,4560+** F [y6] - 722,3719+ E [y5] - 575,3035+ **L [y3] - 317,2183+** G [y2] - 204,1343+ **T [b2] - 249,1234+**	145	27.6

Table 1. Continuation.

Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)	Peptide [place within protein sequence]	Precursor and charge	Fragmented ion and charge	RT (min)	Coll. Energ. (V)
CaMKII all continuation					**CaMKII γ continuation**				
R.**DLKPENLLLASK**.L [134, 145]	447,5976 +++	L [y5] - 531,3501+ L [y4] - 418,2660+ A [y3] - 305,1819+ **S [y2] - 234,1448+** N [b6] - 697,3515+ L [b7] - 810,4356+	121	19	K.**FYFENLLSK**.N [445, 453]	580,8030 ++	Y [y8] - 1013,5302+ F [y7] - 850,4669+ E [y6] - 703,3985+ N [y5] - 574,3559+ S [y2] - 234,1448+ Y [b2] - 311,1390+	146	19.1
K.**AGAYDFPSPEWDTVTPEAK**.D [226, 244]	1040,9786 ++	P [y13] - 1456,6955+ P [y11] - 1272,6107+ T [y5] - 545,2930+ P [y4] - 444,2453+ A [y2] - 218,1499+ D [b5] - 478,1932+	140	37					
CaMKII δ									
R.**FTDEYQLFEELGK**.G [9, 21]	809,8854 ++	L [y7] - 835,4560+ F [y6] - 722,3719+ E [y5] - 575,3035+ E [y4] - 446,2609+ **L [y3] - 317,2183+** G [y2] - 204,1343+	145	26.9	K.**IPTGQEYAAK**.I [33, 42]	539,2824 ++	P [y9] - 964,4734+ T [y8] - 867,4207+ G [y7] - 766,3730+ E [y5] - 581,2930+ Y [y4] - 452,2504+ P [y9] - 482,7404++	47	21.6

Scheduling of transitions in time. Taking into account that in nano-LC peak width is around 30-40 seconds and 11 points is the minimum to quantify a targeted peptide, a cycle time of 3 seconds must be selected. Therefore, if 162 transitions are used, the transmission efficiency for every transition is 185 msec. However, an increase of the transmission efficiency can significantly rise ion signal sensitivity. Increased sensitivity for a set of ions can be achieved by scheduling transitions during LC run time. From previous digested pull-down experiments on TSQ, the retention times (RT) of all 27 PTPs were determined (see Table 1). Measured RT were correlated with results calculated by Sequence Specific Retention Calculator (*SSRCalc 3.0*) that predicts peptide retention time in the LC. Results showed a high correlation (data not shown) between measured and predicted RT (r=0.9402), that confirmed their proper identification. Afterwards, the transitions at a certain elution time were overlapped (2, 5, 10, and 12 minutes) (see Fig. 1). It was observed that scheduling with a 12 minute window decreased the concurrent transitions to 45 (66 msec/transition) which resulted in the raise of transmission efficiency and sensitivity by at least 2.8 times.

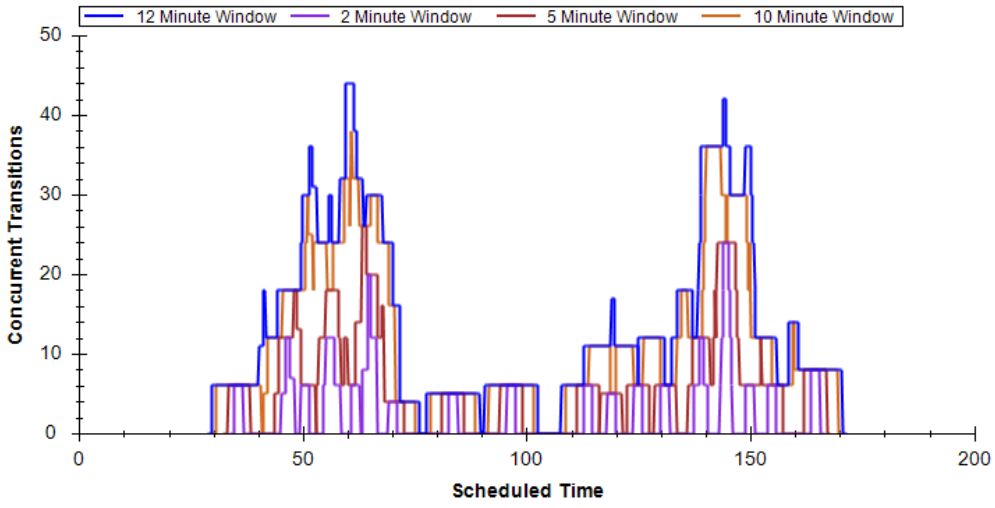

Fig. 1. Graph representing changes in concurrent transitions while various time windows (2, 5, 10, and 12 minutes) around the retention time of a peptide are set.

Validation of peptides using heavily labeled isotopic peptides. In order to validate PTPs and transitions, 27 heavily labeled counterparts were synthetized. Labeling of peptides was performed at the C-terminal arginine (U- $^{13}C_6$, $^{15}N_4$) and lysine (U- $^{13}C_6$, $^{15}N_2$) residues and their molecular masses were increased by 10 and 8 Da, respectively. A method containing both ordinary (light) and increased masses (heavy) of precursors and fragments for every peptide was created (overall 327 transitions). In order not to lose previously achieved sensitivity, the method was divided into three individual sub-methods, so as the maximum concurrent

transitions were 40. A kidney digested pull-down containing 1 pmol of every peptide was injected. In such a reduced background, the transitions should not show interferences. In all cases, heavily labeled peptides co-eluted with selected PTPs. Moreover, most of selected transitions could be validated (see examples on Fig. 2). Relative peak areas of all light and heavy transitions have been extracted and compared. The relative standard deviation (%RSD) between areas were calculated. For 15 transitions, the RSD was higher than 20% and, therefore, these transitions were removed from the SRM assay (see Table 1, deleted transitions underlined). Although HCD libraries have facilitated the development of the SRM assay, the ranks of some transitions varied when TSQ equipment was used.

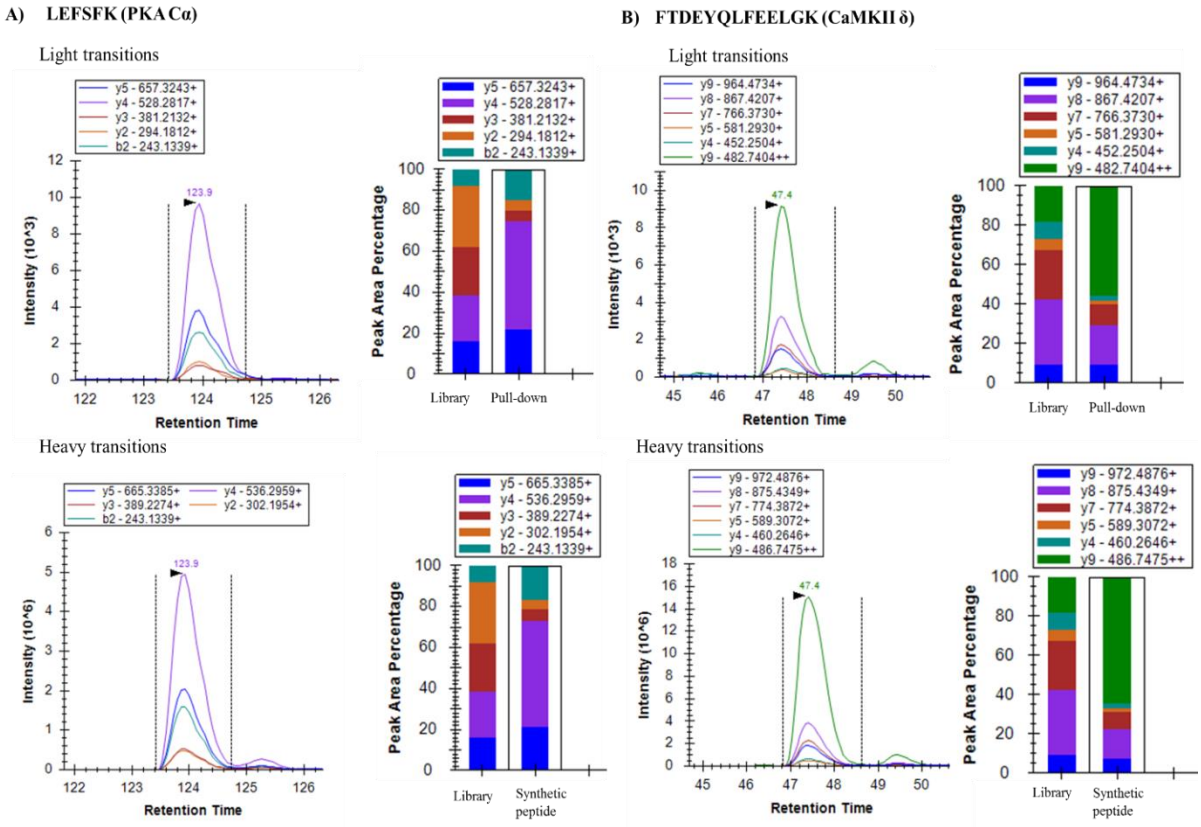

Fig. 2. Validation of peptides A) LEFSFK (from PKA Cα) and B) FTDEYQLFEELGK (from CaMKIIδ). Top graphs represent overlapped signals for light transitions (kidney digested pull-down) and histograms corresponding to their relative peak areas. Accordingly, bottom graphs correspond to heavy transitions (synthetic peptide) and their relative peak areas. Areas for library were obtained from the HCD measurements.

Optimization of the collision energy. In TSQ, applied collision energy for every peptide is calculated using standard equations (collision energy =0.03**m/z*+2.905, for doubly charged precursors, and collision energy =0.038**m/z*+2.281, for triply charged precursors). In order to

additionally increase peptide sensitivity, the collision energy was optimized using 10 values in a range from -5 to +5 V around the previously applied standard values. For this purpose just heavily labeled peptides were injected into the TSQ using shorter gradient. The areas of transitions were summarized and compared. The highest area was selected and used for further analysis (see Table 1). Although the optimization of the collision energy enabled to increase the transition signals, it did not vary significantly from the standard collision energy values. The correlation of standard and optimal collision energies for doubly and triply charged peptides is presented on Fig. 3.

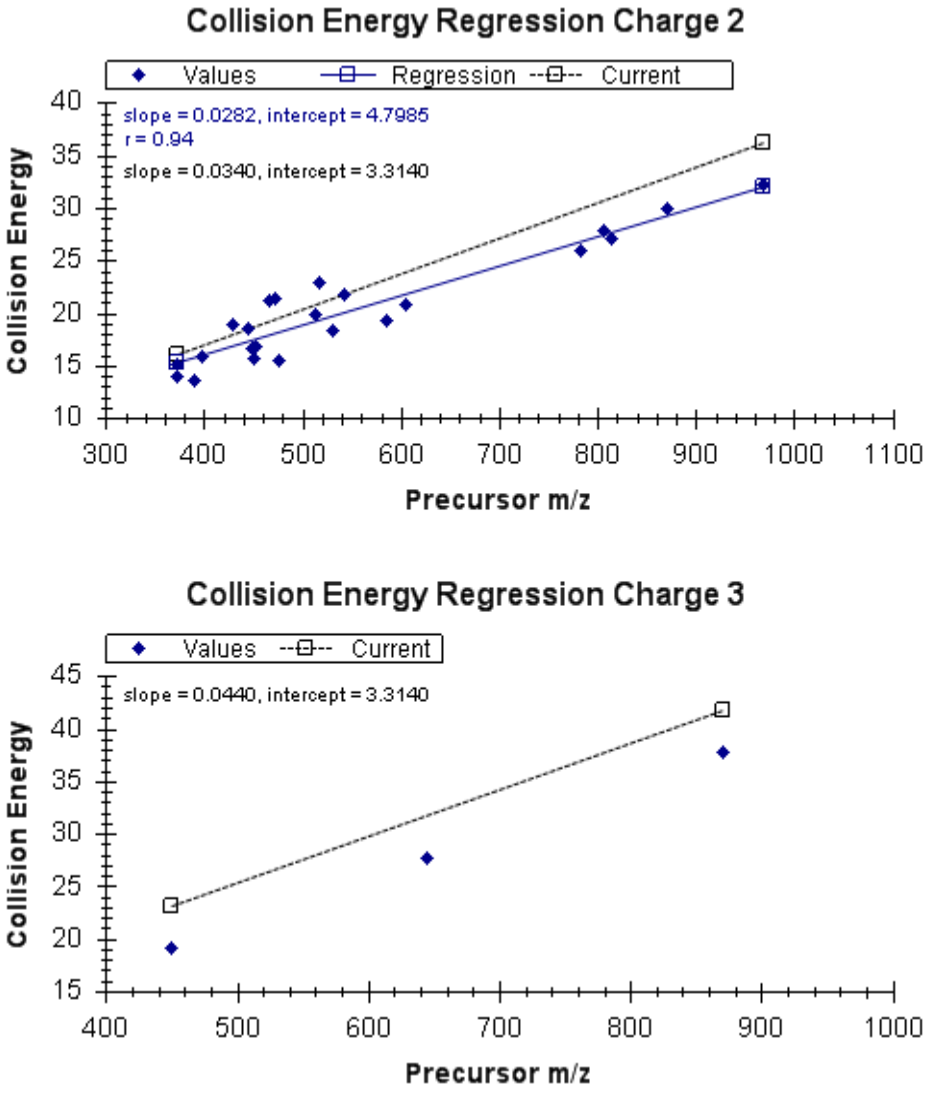

Fig. 3. Correlation of the selected collision energies using the supplier equations and optimal collision energies values for 27 selected PTPs precursor (doubly and triply charged).

3.2. Application of the developed method to heart, liver, and kidney lysates

In order to maximize protein extraction yield, heart, liver, and kidney lysates were obtained in harsh SDS conditions. To decrease the complexity of the sample, FASP digestion using a filter with 30 kDa Mwco was performed. All samples were injected twice in the TSQ equipment using the developed SRM assay. The results showed that just a few predicted PTPs and transitions could be detected during the analysis. The summary of all results is presented in supplemental material 2 (sample where PTP was found is marked with green box). The highest number of PTPs was determined in heart (7 peptides) and kidney (6 peptides), while in liver lysate just one PTP could be detected. This could be explained by the complexity of the sample. These results suggested that the most complex proteome was in the liver tissue or that, in this particular tissue, these isoforms were less abundant.

3.3. SDS-PAGE in-gel digestion SRM method

In order to decrease the complexity of lysates, a separation step using SDS-PAGE was applied. Therefore, the digestion with trypsin was accomplished using in-gel digestion. First, the lysate of kidney was separated into two independent wells. Therefore, from each separation, two individual bands A (90-50 kDa) and B (50-35 kDa) were cut and digested. After digestion, peptides were extracted using an extended extraction procedure. The application of SDS-PAGE before SRM assay did not enable to determine all expected PTPs (see supplemental material 2). Although two additional PTPs (9 in total) were detected, it was not enough to quantify targeted isoforms. The same procedure was tried for the heart lysate. In this case, 13 out of all 27 PTPs were detected. These results suggested that the complexity of the sample was the biggest issue hindering the application of the designed SRM assay. Interestingly, 7 PTPs for CaMKII isoforms were determined in the heart lysate previously separated by SDS-PAGE. To study whether this methodology enabled the quantification of CaMKII isoforms in various heart lysates, the SRM method with previous SDS-PAGE separation was applied to a rat heart model with progression to heart failure.

3.4. Application of the SDS-PAGE in-gel digestion SRM method to a rat heart model with progression to heart failure

A heart model with TAC was the experimental portrait for pressure overloaded (hypertension) induced cardiac hypertrophy and heart failure. Heart contractility was

temporary enhanced, which led to myocardium hypertrophy and, over the time, to heart dilation and failure [25]. Therefore, in this study three groups of rats (each group n=4) with blocked aorta (TAC) during different periods of time (4, 12 and 16) were chosen for investigation. SHAM-12 (n=4) was a control group where the aorta was blocked and unblocked. For all rats lysates (n=16), the SDS-PAGE separation was performed. Rat lysates were divided into 4 series where in every gel, one member of every group (SHAM-12, TAC-4, TAC-12 and TAC-16) was separated. Three technical replicates for every gel were performed. Moreover, one test gel for the preliminary studies was done. All gels were digested using the same procedure. SRM method was reduced to 7 PTPs and 40 transitions corresponding to just CaMKII isoforms. Firstly, four digests (representing every group member), obtained from test gels, were mixed. In this way, sample background that could influence the detection of PTPs was obtained. Various percentages of gels were evaluated (5%, 10%, and 20%). To such mixtures, heavily labeled counterparts of PTPs were added (10 pmol), so that possible interferences from the background could be determined. The relative areas of transitions for light and heavy peptides were extracted and compared. All transitions were detected in the gel mixture sample. Results showed that when 5% and 10% of gel was injected, areas of 6 transitions were slightly influenced by the background interferences, while when 20% of gel was injected, areas of 9 transitions were interfered. Finally, 48 samples of heart lysates prepared by the SDS-PAGE in-gel digestion were spiked with various amounts of heavily labeled peptides (see supplemental material 3) and injected in the TSQ equipment using the developed SRM assay. Interestingly, just two PTPs corresponding to the CaMKII δ isoforms were determined (FTDEYQLFEELGK and IPTGQEYAAK). For these peptides, the areas of transitions for light and heavily labeled peptides were extracted and summarized. Based on this information, the amount of FTDEYQLFEELGK and IPTGQEYAAK peptides in every sample was calculated. Results showed that quantification of peptides was highly irreproducible. The RSD (%) between technical SDS-PAGE replicates varied significantly. Combined RSD (%) for every rat group (SHAM-12, TAC-4, TAC-12 and TAC-16) yielded a RSD in range 18-90%. Combined results showed in Fig. 4, suggested that developed procedure was not reproducible at some stage during the sample preparation.

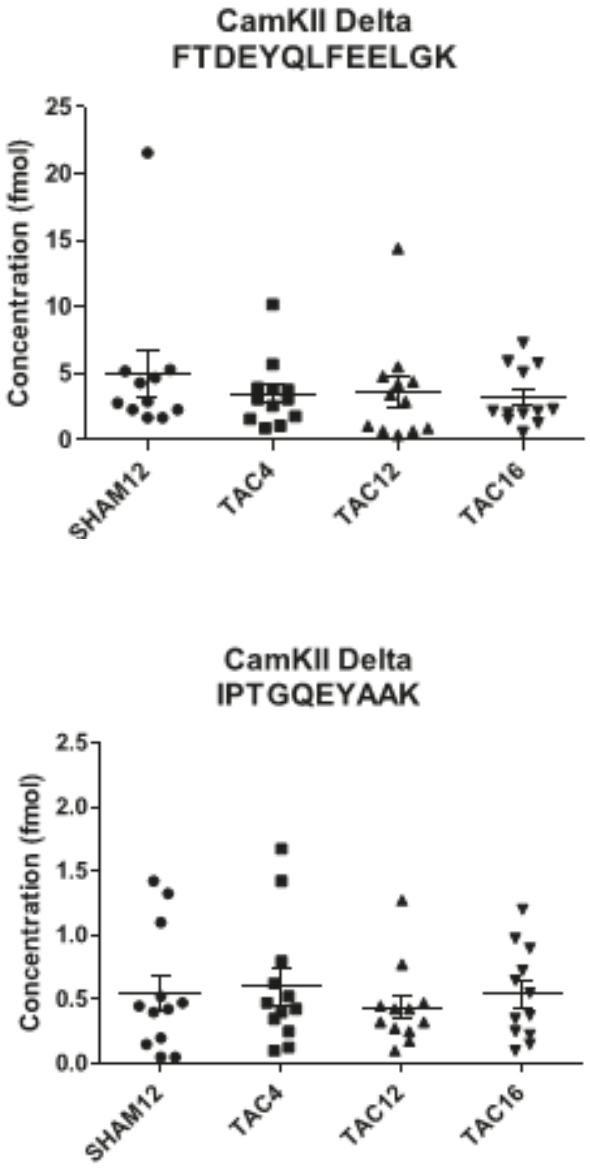

Fig. 4. Quantification of FTDEYQLFEELGK and IPTGQEYAAK peptides for the CaMKII δ isoform obtained by SDS-PAGE in-gel digestion SRM approach.

4. Conclusions

A SRM assay for PKA, PKG, and CaMKII isoforms determination has been established in this work. PTPs and transitions for every isoform were set up using the information collected by a vast set of MS data acquired previously, *in-silico* prediction, the Peptide Atlas database information, and experiments performed on an Orbitrap Velos with HCD. High similarity between targeted isoforms was detected which hindered the selection of PTPs. Due to this high

similarity, it was not possible to select any PTP to differentiate PKA Cα from $C\alpha_2$, CaMKII α, and β, from other CaMKII isoforms, and among CaMKII isoforms groups γ (A-C) and δ (1-7). The final SRM assay design for 11 isoforms consisted of 27 PTPs with 27 precursors and 162 transitions. Selected transitions were verified on the TSQ showing that most of the HCD spectra were correctly translated to TSQ. The method was also scheduled in time over the LC-run and sensitivity increased at least 2.8 times. The collision energy for every 27 PTPs was optimized in the range from -5 to +5V, showing not significant differences between standard and optimized collision energy values. All PTPs were validated using heavily labeled peptide counterparts leading to exclusion of some interfered transitions. Optimized SRM assay was applied to heart, kidney, and liver lysates. However, due to the complexity of the sample, just some transitions were detected. In order to reduce complexity, tissue lysates were separated by SDS-PAGE. Although the complexity of samples decreased, still several peptides were not detected. For heart lysates previously separated by SDS-PAGE, 13 PTPs were detected, where 6 were for the CaMKII isoforms. SRM assay for just CaMKII isoforms was applied to a set of rat hearts with progression to heart failure. Transitions were validated in such a matrix and appropriate percentage of sample amount and heavily labeled peptides was selected. However, due to the high sample complexity, just two peptides for the CaMKII δ isoform were detected. Overall, this approach was highly irreproducible which could be caused by the SDS-PAGE procedure (separation, digestion, or extraction of peptides from gel). As alternative, implementation of different separation techniques (*e.g.* enrichment using agarose beads) can be proposed for the reduction of sample complexity.

Appendix A. Supplementary data

Supplemental material 1. List of studied proteins.

Protein name	Uniprot accession	Amino acids length	Protein molecular weight (Da)
PKA RI α	P09456	381	43.095
PKA RI β	P81377	381	43.282
PKA RII α	P12368	401	45.540
PKA RII β	P12369	416	46.123
PKA Cα	P27791	351	40.620
PKA Cα2	P27791	343	39.879
PKA Cβ	P68182	351	39.870
PKA Cγ		not sequenced	
PKG Iα	A9LNM8	671	76.369
PKG Iβ		not sequenced	
PKGII	Q64595	762	87.182
CaMKII α	P11275	478	54.115
CaMKII β	P08413	542	60.402
CaMKII γA	P11730	527	59.038
CaMKII γB	P11730	517	58.493
CaMKII γC	P11730	533	55.961
CaMKII δ1	P15791	533	60.081
CaMKII δ2	P15791	499	56.447
CaMKII δ3	P15791	510	57.709
CaMKII δ4	P15791	519	58.549
CaMKII δ5	P15791	478	54.191
CaMKII δ6	P15791	512	57.825
CaMKII δ7	P15791	498	56.293

Supplemental material 2. Summary of results obtained using the developed SRM assay in heart, kidney, and liver lysates

Protein isoform	Kidney	Heart	Liver	SDS-PAGE Kidney	SDS-PAGE Heart
PKA RI α					
K.HNIQALLK.D [24. 31]	■	■		■	■
K.IVVQGEPGDEFFIILEGTAAVLQR.R [281. 304]					
PKA RI β					
K.HGIQQVLK.E [24. 31]				■	
K.VSILESLEK.W [252. 260]					
PKA RII α					
R.QQPPDLVDFAVEYFTR.L [23. 38]					
R.AATIVATSDGSLWGLDR.V [214. 230]	■		■	■	■
R.AASAYAVGDVK.C [344. 354]					
PKA RII β					
R.HQPADLLEFALQHFTR.L [22. 37]					
R.GTFDIYVK.C [194. 201]					
R.AASAHAIGTVK.C [359. 369]					
PKA C α					
K.AKEDFLK.K [22. 28] (missed 1)					■
K.TLGTGSFGR.V [48. 56] (in common with Cβ)	■	■		■	■
K.LEFSFK.D [106. 111]	■	■		■	■
PKA C β					
K.AKEDFLR.K [22. 28] (missed 1)	■				
R.LEYSFK.D [106. 111]		■		■	
PKG I					
K.DSCIIK.E [126. 131]					
K.VFGELAILYNCTR.T [164. 176]					
K.TYNIILR.G [565. 571]					■
PKG II					
R.NYQQGSYIVK.Q [187. 196]				■	
K.ALISDDVR.S [358. 365]					■
CaMKII all					
K.GAFSVVR.R [21. 27]		■			■
R.DLKPENLLLASK.L [134. 145]	■	■		■	■
K.AGAYDFPSPEWDTVTPEAK.D [226. 244]	■			■	■
CaMKII γ					
R.FTDDYQLFEELGK.G [9. 21]					■
K.FYFENLLSK.N [445. 453]					■
CaMKII δ					
R.FTDEYQLFEELGK.G [9. 21]					■
K.IPTGQEYAAK.I [33, 42]					

Supplemental material 3. Amount of spiked heavily labeled peptides into the SDS-PAGE in-gel digested of SHAM-12, TAC-4, TAC-12, and TAC-16 heart lysates.

Protein	Sequence*	Spiked amount
CamKII all	GAFSVVX	20 fmol
CamKII all	DLKPENLLLASB	5 fmol
CamKII all	AGAYDFPSPEWDTVTPEAB	10 fmol
CamKII γ	FTDDYQLFEELGB	10 fmol
CamKII γ	FYFENLLSB	2.5 fmol
CamKII δ	FTDEYQLFEELGB	10 fmol
CamKII δ	IPTGQEYAAB	2.5 fmol

*(X= heavily labeled arginine; B=heavily labeled lysine)

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References for Article 7:

[1] M. Pischetsrieder, R. Baeuerlein, Proteome research in food science, Chem. Soc. Rev. 38 (2009) 2600-2608.

[2] N. Bisson, D.A. James, G. Ivosev, S.A. Tate, R. Bonner, L. Taylor, T. Pawson, Selected reaction monitoring mass spectrometry reveals the dynamics of signaling through the GRB2 adaptor, Nat. Biotechnol. 29 (2011) 653-U138.

[3] H. Schulman, M. Anderson, Ca2+/Calmodulin-dependent protein kinase ii in heart failure. Drug Discov. Today 7 (2010) 117-122.

[4] A. Scholten, A Multi-Angular mass spectrometric view at cyclic nucleatide signaling proteins, Doctoral Thesis (2006).

[5] T.T. Aye, Application of chemical proteomics to biomarker discovery in cardiac research, Doctoral Thesis (2010).

[6] A. Scholten, T. Aye, A.J.R. Heck, A multi-angular mass spectrometric view at cyclic nucleotide dependent protein kinases: In vivo characterization and structure/function relationships, Mass Spectrom. Rev. 27 (2008) 331-353.

[7] M.E. Anderson, J.H. Brown, D.M. Bers, CaMKII in myocardial hypertrophy and heart failure, J. Mol. Cell. Cardiol. 51 (2011) 468-473.

[8] P.D. Swaminathan, A. Purohit, T.J. Hund, M.E. Anderson, Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias, Circ. Res. 8 (2012) 1661-77.

[9] J.R. Erickson, B.J. He, I.M. Grumbach, M.E. Anderson, CaMKII in the cardiovascular system: sensing redox states, Physiol. Rev. 91 (2011) 889-915.

[10] M.E. Anderson, CaMKII and a failing strategy for growth in heart, J. Clin. Invest. 119 (2009) 1082-1085.

[11] K. Tasken, E. Aandahl, Localized effects of cAMP mediated by distinct routes of protein kinase A, Physiol. Rev. 84 (2004) 137-167.

[12] L. Griffith, Calcium/calmodulin-dependent protein kinase II: An unforgettable kinase, J. Neurosci. 24 (2004) 8391-8393.

[13] A. Hudmon, H. Schulman, Neuronal Ca2+/calmodulin-dependent protein kinase II: The role of structure and autoregulation in cellular function, Annu. Rev. Biochem. 71 (2002) 473-510.

[14] T.T. Aye, A. Scholten, N. Taouatas, A. Varro, T.A.B. Van Veen, M.A. Vos, A.J.R. Heck, Proteome-wide protein concentrations in the human heart, Mol. Biosyst. 6 (2010) 1917-1927.

[15] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial. Mol. Syst. Biol. 4 (2008).doi: 10.1038/msb.2008.61.

[16] P. Picotti, R. Aebersold, Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions, Nat. Methods 30 (2012) 555-566.

[17] P. Picotti, O. Rinner, R. Stallmach, F. Dautel, T. Farrah, B. Domon, H. Wenschuh, R. Aebersold, High-throughput generation of selected reaction-monitoring assays for proteins and proteomes, Nat. Methods 7 (2010) 43-46.

[18] C.M. Colangelo, L. Chung, C. Bruce, K. Cheung, Review of software tools for design and analysis of large scale MRM proteomic datasets, Methods 61 (2013) 287-298.

[19] M. Bantscheff, M. Schirle, G. Sweetman, J. Rick, B. Kuster, Quantitative mass spectrometry in proteomics: a critical review, Anal. Bioanal. Chem. 389 (2007) 1017-1031.

[20] S. Gallien, E. Duriez, B. Domon, Selected reaction monitoring applied to proteomics, J. Mass Spectrom. 46 (2011) 298-312.

[21] E.L. de Graaf, A.F.M. Altelaar, B. van Breukelen, S. Mohammed, A.J.R. Heck, Improving SRM assay development: a global comparison between triple quadrupole, ion trap, and higher energy CID peptide fragmentation spectra, J. Proteome Res. 10 (2011) 4334-4341.

[22] A. Scholten, S. Mohammed, T.Y. Low, S. Zanivan, T.A. van Veen, B. Delanghe, A.J.R. Heck, In-depth quantitative cardiac proteomics combining electron transfer dissociation and the metalloendopeptidase Lys-N with the SILAC mouse, Mol. Cell. Proteomics. 10 (2011) 10.1074/mcp.O111.008474.

[23] T.P. de Boer, L. Nalos, A. Stary, B. Kok, M.J.C. Houtman, G. Antoons, T.A.B. van Veen, J.D.M. Beekman, B.L. de Groot, T. Opthof, M.B. Rook, M.A. Vos, M.A.G. van der Heyden, The anti-protozoal drug pentamidine blocks K(IR)2.x-mediated inward rectifier current by entering the cytoplasmic pore region of the channel, Br. J. Pharmacol. 159 (2010) 1532-1541.

[24] J.R. Wiśniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359-U60.

[25] A. de Almeida, R. van Oort, X. Wehrens, Transverse aortic constriction in mice, J. Vis. Exp. 21 (2010) 1729.

III.2.
Global discussion of results

III.2. Global discussion of results

The main goal of this doctoral thesis was the characterization, identification, and quantification of peptides and proteins having significant influence on the prevention and understanding of hypertension. For that purpose, different analytical strategies were developed and applied to the determination and characterization of peptides and proteins in plant (vegetables and foods) and animal (rat organs) tissues.

The identification by mass spectrometry of native bioactive peptides in SBIFs required their previous extraction, characterization. The determination of peptides with significant antihypertensive activity in soybean (peptide VLIVP from the 11S globulin fraction) and maize (LRP, LQP, and LSP peptides from the α-zein fraction) required the previous extraction of proteins containing those peptides, their digestion using suitable enzymes and conditions, and their quantification by mass spectrometry. The determination of proteins involved in the regulation of blood pressure (PKA, PKG, and CaMKII) in different animal tissues required their previous extraction followed by tryptic digestion and quantification by mass spectrometry.

New approaches for the extraction, purification, and digestion of all these peptides and proteins were proposed and optimized. Regarding extraction, high intensity focused ultrasounds were applied in all cases for the acceleration of extraction procedures. In addition, a wide variety of novel separation, purification, and detection techniques of peptides and proteins were also implemented. In all cases, mass spectrometry using different mass analyzers (IT, Q-TOF, QqQ, and LTQ-Orbitrap) were employed.

This part of the thesis globally discusses all results obtained and compares the techniques and methodologies used in every case for the extraction, purification, isolation, characterization, detection, and determination of peptides and proteins of interest in the prevention and understanding of hypertension.

Development of methods for the extraction of proteins with significant influence in the prevention and understanding of hypertension from different plant and animal tissues

Different methods were developed for the extraction of proteins containing highly antihypertensive peptides in plant tissues (soybean and maize) and proteins involved in the regulation of hypertension in animal tissues (heart, kidney, and liver). For that purpose, different buffers were selected depending on the nature of targeted proteins and further

analytical workflows. In all cases, high intensity focused ultrasounds were employed to accelerate extraction process.

Regarding plant tissues, different methods were developed for the extraction of 11S globulins from soybean and of α-zeins from maize since they are important sources of antihypertensive peptides (LRP, LSP, and LQP in maize α-zeins and VLIVP in soybean proteins). Existing methods enabling the extraction of 11S globulins from soybean seeds were very time consuming (extraction times of 2 h) while there was no method suitable for the extraction of α-zeins from whole maize kernels. According to their different nature, globulins are extractable in salty solutions while zeins (prolamins) are soluble in alcohol solutions. This fact was reflected when extraction buffers were proposed. Soybean globulins were easily extracted with a 0.03 M Tris-HCl buffer at pH 8.5, while maize α-zeins in 70% of EtOH. Nevertheless, under these conditions, various protein families of soybean globulins (mainly 11S and 7S globulins) were extracted at the same time, while for maize proteins almost exclusively α-zeins were obtained. The use of an ultrasound probe enabled to reduce the extraction time: the highest protein yield of soybean globulins was obtained within 2 min while 10 min were needed for the extraction of maize α-zeins. In the case of globulins, this constituted a significant improved with respect to a previously reported method (reduction of the extraction time from 2 h to 2 min). Additional differences between these plant proteins were observed in ultrasound power being necessary a higher probe amplitude in the case of maize α-zeins (a 40% of probe amplitude for soybean globulins and a 90% of probe amplitude for maize α-zeins).

On the other hand, the selection of a extraction procedure for proteins from animal tissues highly depended on the further step in the workflow. Targeted proteins (PKA, PKG, and CaMKII) were extractable in PBS, ABC or RIPA buffers using both mild (Tween 20) or harsh (SDS) conditions. When the interest was to keep proteins assemblies together, milder conditions were preferred. When the interest was to extract all proteins with a high protein yield, harsh conditions were applied. While this step allowed to obtain the highest possible protein yield, it also produced the highest sample complexity. In comparison to plant proteins, during the extraction of animal tissues proteins, various enzyme inhibitors had to be added to buffer solutions. This step was necessary in order to avoid targeted protein digestion by co-extracted enzymes in the lysis buffer.

Assessment of clean up strategies for the purification of extracted proteins

Clean-up procedures were employed for the purification of proteins extracted from plant and animal tissues. In the case of soybean globulins and proteins extracted from animal tissues, this step enabled to reduce sample complexity for its further study. In the case of maize α-zeins, a purification step was necessary to remove interferences and to change the surrounding environment by one suitable for the digesting enzyme employed afterwards.

Various strategies like precipitation at pI, precipitation with acetone, enrichment of targeted proteins or separation by SDS-PAGE were carried out for this purification. For soybean globulins and maize α-zeins, that are major proteins in these plant tissues, simple purification by precipitation was employed. Nevertheless, the selective precipitation of 11S globulins from the previously obtained soybean globulin extract at its pI (pH 6.4) did not show to be quantitative. Therefore, this step was rejected and the whole globulin extract was used in further studies for the determination of the antihypertensive peptide VLIVP. Unlike 11S globulins, the use of a non-selective precipitation of α-zeins with acetone enabled its quantitative precipitation.

In the case of targeted PKA, PKG, and CaMKII isoforms, that are low abundant proteins in animal tissues, a more sophisticated enrichment using agarose beads with attached secondary messengers (cAMP) or SDS-PAGE was addressed. Agarose beads were effective for the purification of PKA, PKG, and CaMKII isoforms from the protein extracts obtained from animal tissues (liver, kidney, and heart). On the other hand, SDS-PAGE enabled the reduction of sample complexity in these extracts but recovery of proteins from gels was limited.

Development of approaches to obtain bioactive and tryptic peptides

In this work, peptides were obtained and employed with different purposes. In some cases, peptides were obtained for their characterization and the evaluation of their bioactive properties (bioactive peptides from SBIFs). In other cases, targeted peptides were obtained for their quantification (antihypertensive peptides from maize and soybean). A final purpose for the extraction of peptides was their further use for the quantification of parent proteins (PKA, PKG, and CaMKII).

Two different approaches were used to obtain peptides depending whether these peptides were naturally present in the sample or they were within a parent protein. When peptides were

naturally occurring, such as native peptides in SBIFs, it was not necessary the previous extraction of proteins. In this case, a direct extraction of peptides by UF of SBIFs through 10 kDa Mwco filters resulted suitable for this purpose. When peptides were within the sequence of protein, such as antihypertensive peptides in soybean and maize and peptides from PKA, PKG, and CaMKII, extracted and purified proteins had to be hydrolyzed with suitable enzymes in order to release peptides. The enzyme employed in every case was selected according to the aim of the work.

Antihypertensive peptides were released from 11S soybean globulin by the action of protease P (peptide VLIVP) and from maize α-zeins by the action of thermolysin (peptides LRP, LSP, and LQP). Protease P and thermolysin are both enzymes from bacterial origin and for both of them the enzymatic digestion was performed *in-solution*. Protease P required a low enzyme to substrate ratio (0.1:100 (w:w)) and a long digestion time (18 h) while thermolysin digestion required a higher enzyme to substrate ratio (5:100 (w:w)) but a shorter digestion time (6 h). These bacterial enzymes could not withstand the high energy produced by the high intensity focused ultrasounds and they inactivate. Additionally, the enzymatic digestion with both enzymes either was worse or did not occur when including alkylation and reduction steps previous to the digestion.

The thermolysin digestion of maize α-zeins to obtain antihypertensive peptides LRP, LQP, and LSP was also performed on a membrane (FASP). FASP approach was, to the best of our knowledge, implemented for the first time in this work into food matrices. FASP approach separates proteins from smaller interferences previously to their digestion. Nevertheless, the analysis of the sample using ESI-Q-ToF-MS revealed that targeted peptides were not present in the digested extract probably because peptides could not be released from intact proteins or may be retained on the membrane itself.

Digestion with trypsin enzyme was employed to release proteotypic peptides from PKA, PKG, and CaMKII. These tryptic peptides were used for the quantification of these proteins in animal tissues (heart, kidneys, and liver) by tandem mass spectrometry. *In-solution*, FASP, and *in-gel* digestions were implemented in this case. Unlike maize proteins, FASP approach resulted useful for the digestion of PKA, PKG, and CaMKII from animal tissues. Moreover, previous alkylation and reduction of proteins were, in this case, crucial steps within the digestion procedure. While the peptide digest obtained by *in-solution* and FASP digestions showed a high

complexity, peptide extraction from gels in the *in-gel* digestion strategy resulted non reproducible.

Purification strategies were also applied to remove interferences from peptide extracts. In some cases, these interferences disturbed the assays conducted for the evaluation of peptide bioactivity (such as in the evaluation of bioactive peptides in SBIFs) while, in other cases, they interfered the peptide ionization in the ESI source (such as in the quantification of peptides). SPE was used to clean tryptic digest and to remove urea (ionization inhibitor in ESI) from the digested extract of maize α-zeins. In the case of maize α-zeins, two different SPE cartridges (Sep-Pak and Supelco) containing different amounts of beads were employed to remove ionization suppressors in the quantification of LRP, LSP, and LQP peptides. The use of Sep-Pak cartridges enabled the removal of ionization interferences. Peptide ionization suppression was also observed in the quantification of VLIVP peptide obtained by the digestion of soybean proteins with protease P. Indeed, just a 32% of peptide standard signal was detected when it was added to the digested extract. In this case, the ionization suppression effect was reduced just by the simple dilution of the digested extract. In fact, when the digested extract was diluted twenty times, ion suppression was not observed.

Development of analytical methodologies for the separation and identification of targeted and non-targeted peptides

Peptide separations were performed using nano-, micro- and conventional RP–HPLC columns with new fused-core stationary phases. Ion-pairing reagent type and concentration, elution gradient, and column temperature were carefully optimized to obtain the best separation of peptides in every case. In general, the ion-pairing reagent enabling the most suitable peptide separation by RP-LC and ionization by ESI was AA. Indeed, separation of VLIVP bioactive peptide from soybean 11S globulin with suitable ESI ionization was obtained with a 0.1% (v/v) AA while smaller peptides (LRP, LSP, and LQP) from maize α-zeins preferred 0.3% (v/v) of AA. Regarding the elution gradients, VLIVP peptide was retained quite well on the stationary phase and, therefore, a gradient starting with a 18% mobile phase B (containing ACN) was designed. Unlike VLIVP from soybean, smaller peptides from maize were not much retained on the stationary phase and the initial mobile phase composition was decreased to the minimum (3% ACN).

UV-Vis detection of peptides was carried out at 210 nm. Moreover, different MS spectrometers with ESI ionization were also used, all working in the positive ion mode. While for tryptic peptides, standard ESI source parameters were applied, for bioactive peptides, these parameters had to be optimized. Indeed, as presented before, for small bioactive peptides, an appropriate selection of ESI parameters was essential to obtain an abundant peptide signal. This fact was especially important when there was no basic amino acid within the peptide sequence such as in the case of peptides LSP and LQP from maize α-zeins. For these two peptides, a strong fragmentation was observed in the ESI source. In order to reduce spontaneous fragmentation of these peptides and to increase peptide signal intensity, ESI parameters were carefully investigated. The use of a lower fragmentator voltage removed almost all spontaneous fragmentation and improved signal intensities. Moreover, other parameters also affecting peptides signal intensities were nebulizer pressure and sheath gas flow and temperature.

Antihypertensive peptide VLIVP in soybean was also analysed by ESI-MS. In this case, no in-source peptide fragmentation was observed, probably due to its longer peptide sequence. A suitable optimization of parameters enabled, again, to increase method sensitivity. In this case, parameters mostly affecting the MS signal were capillary voltage and, like previously for maize peptides, dry gas flow and temperature.

Different types of MS and MS/MS analyzers were employed. High resolution MS systems were selected for the identification of non-targeted peptides: a Q-ToF for non-targeted identification of bioactive peptides and a LTQ-Orbitrap Velos for non-targeted identification of tryptic peptides. Non-targeted identification of native bioactive peptides obtained from SBIFs required the use of PEAKS software that enables to carry out both *de novo* sequencing and database searching. The application of both procedures in parallel for the identification of these native peptides yielded more confidence results. Therefore, several potential antioxidant and antihypertensive peptides were identified in SBIFs. On the other hand, non-targeted identification of tryptic peptides from animal tissues was performed using Proteome Discoverer software. Digested pull-downs of liver, kidney and heart were successfully analyzed and these proteotypic peptides most commonly observed and corresponding to PKA, PKG, and CaMKII isoforms were detected and next used for the quantification of these parent proteins.

Targeted bioactive peptides (LRP, LSP, and LQP from maize and VLIVP from soybean) were analyzed with ToF and IT mass spectrometers while for targeted tryptic peptides, a QqQ was employed. All targeted peptides were identified by the comparison of MS/MS or/and MS

spectra of peptide standards and studied samples, by comparison of their elution time, and by spiking studied samples with targeted peptide standards.

Quantification of small peptides LRP, LSP, and LQP was carried out from the MS spectrum since their MS/MS spectrum was too poor for their quantification. Therefore, the identification of these peptides in highly complex food matrices required high resolution MS analyzer as ToF. For the longer bioactive peptide VLIVP, the IT analyzer in the pseudo-SRM mode was used, while for tryptic peptides the QqQ equipment in SRM mode was applied. Both IT and QqQ are low resolution mass spectrometers, thus, the MS/MS mode was necessary. Higher sensitivities were obtained when coupling nano- and micro-LC separations to QqQ and IT analyzers, respectively.

Assessment of sensitivity improvement strategies

It is important to highlight some approaches to significantly improve method sensitivity. Few of them, like nano- and capillary-LC or the optimization of ESI source parameters, were already mentioned. For IT and QqQ, method sensitivity is very affected by the amount of ions that can be analyzed at any instant. In the case of the IT, the ICC (ion charge control) target is the parameter controlling this feature being necessary its optimization to obtain a suitable signal intensity for the detection of the antihypertensive peptide VLIVP in soybean digested extracts. On the other hand, when analyzing prototypic peptides by QqQ, the amount of ions analyzed at every instant was controlled by scheduling the number of transitions (peptide→fragment ions) over the LC-run. Therefore, a lower number of transitions resulted in a higher number of ions and a higher sensitivity. Thanks to this strategy, the sensitivity of the developed method to quantify PKA, PKG, and CaMKII isoforms was increased at least 2.8 times.

Another important parameter affecting method sensitivity in both IT and QqQ was the applied collision energy. These two analyzers work in different manner and obtained mass spectra can be significantly different. Nevertheless, in both cases, the optimization of analyzed the collision energy (in the case of IT) or their previous estimation using theoretical equations (in the case of QqQ) was essential to obtain a high sensitivity.

Critical evaluation of the analytical characteristics of the developed methods for the quantification of bioactive peptides

The methods developed for the quantification of bioactive peptides were characterized by the evaluation of several parameters like linearity in the working concentration range, limits of detection and quantification, existence of matrix interferences, precision, and recovery. The quantification of LRP, LSP, and LQP antihypertensive peptides from maize α-zeins was performed by conventional HPLC using a fused-core column connected to a Q-ToF MS system. The quantification of VLIVP antihypertensive peptide in the protease P digested extract of soybean proteins was carried out by micro-HPLC connected to an IT analyzer. Maize antihypertensive peptides were analyzed using just the MS mode, while the soybean peptide was quantified using the pseudo-SRM mode.

Due to the high mass accuracy of the ToF analyzer, some parameters (slope of the calibration curve, selectivity, and LOD or LOQ) highly depended on the applied EW value. Hence, EW for every peptide was carefully examined and selected. For LRP, the EW was 200 ppm while for LSP and LQP was 100 ppm. On the other hand, a standard EW of ±0.5 was selected in the case of the IT analyzer. For both methods, LOD and LOQ were calculated as the minimum concentration yielding a SNR equal to 3 and 10 times, respectively. The comparison of the values obtained for the two quantification methods showed that micro-HPLC-IT using pseudo-SRM mode provided around 1000 times lower LOD/LOQ than HPLC-Q-ToF. In fact, the calibration curve was at the ppt level in micro-HPLC-IT and at ppb level in conventional HPLC-Q-ToF. It can be concluded that micro-HPLC-IT represented much more sensitive quantification than the method where conventional HPLC-Q-ToF system was used.

Matrix effects were evaluated in both methods by the comparison of slopes obtained by the external standards and standard additions calibrations. For the conventional HPLC-Q-ToF method, the effect of the matrix was observed for almost all peptides, while in the micro-HPLC-IT method, matrix effects were absent.

Application of the developed methods to the quantification of targeted peptides and proteins in plant and animal tissues

The quantification of targeted proteins and peptides can be carried out using label free or label based methods. Bioactive antihypertensive peptides (LRP, LSP, and LQP in maize and VLIVP in soybean) were quantified using the label free approach. For that purpose, the standard

additions method was used to quantify LRP, LSP, and LQP peptides by HPLC-Q-ToF and the external standard calibration method was employed in the quantification of VLIVP peptide by micro-HPLC-IT. Previous to the quantification, a stability study of the peptide standards over time was conducted. This study reported, for both soybean and maize peptides, that the best conditions for storing peptides were 10% AA at temperatures from 4 °C to RT.

The VLIVP peptide was quantified in soybean protein extracts digested with protease P using micro-HPLC-IT in pseudo-SRM mode. Five different soybean crops were analyzed and significant differences (562-880 ng/g) among peptide contents were observed. These results suggested that not all soybean crops presented the same antihypertensive properties. On the other hand, LRP, LSP, and LQP antihypertensive peptides were quantified in the thermolysin digests of maize α-zeins. Results firstly obtained by relative quantification using UV detection were next confirmed by the absolute quantification of these peptides by mass spectrometry (ToF). In general, the most abundant peptide was LSP, followed by LQP and LRP. In order to facilitate the comparison of the antihypertensive capacity of maize varieties containing these three antihypertensive peptides, the activity of LSP and LQP was expressed in terms of equivalent concentration of the most active peptide LRP. These results demonstrated that the antihypertensive properties of maize crops varied with the variety. In addition to the maize crops included in the manuscripts, the content in antihypertensive peptides in a genetically modified maize variety and in its isogenic non-transgenic line were also estimated. Comparison of genetically modified and its non-transgenic lines showed just slight differences in the level of the antihypertensive peptide LSP while LQP and LRP contents were identical.

Proteotypic peptides from CaMKII isoforms were quantified using heavily labeled peptide counterparts on a nano-LC-QqQ system using the SRM mode. However, due to the sample complexity, just two out of seven CaMKII proteotypic peptides were identified using this approach. Moreover, the results obtained for these peptides varied significantly, which suggested that some stage of the sample preparation procedure was poorly repeatable.

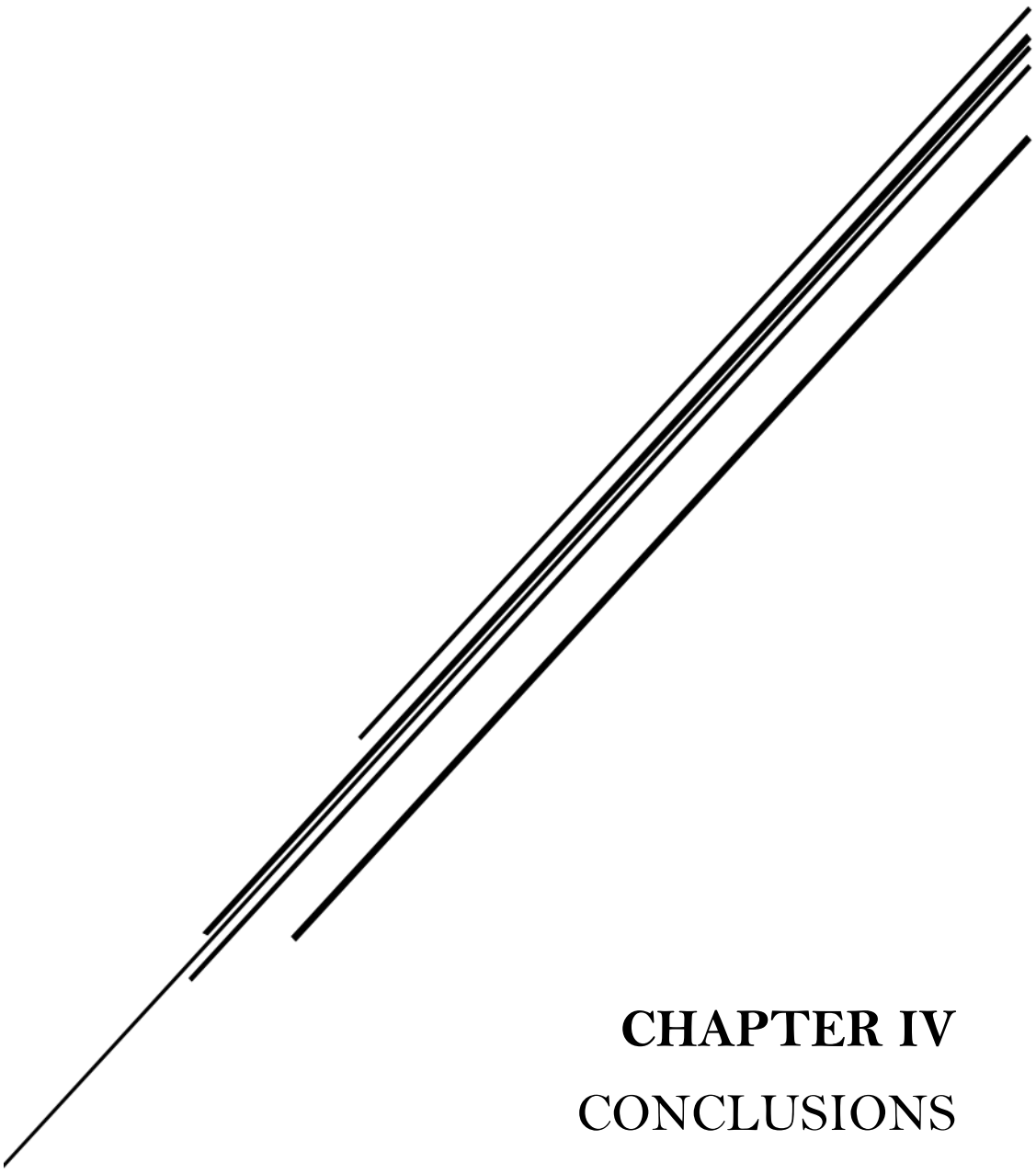
Isolation and identification of antioxidant and antihypertensive peptides in SBIFs

Fractionation of antioxidant and antihypertensive peptides from SBIFs has been performed according to their molecular weights by UF and according to their pI by OFFGEL isoelectrofocusing. UF was employed as a first fractionation step. The identification of peptides by MS after this fractionation showed the limited resolution of the technique when it was used

for the separation of molecules with similar molecular weights. Nevertheless, UF resulted suitable when separating molecules presented more different molecular weights (*e.g.* UF with Mwco filters of 3 kDa was successfully employed to separate α-zeins (19 and 22 kDa) and polyamine conjugates (440 and 410 Da)). In order to overcome the limited resolution of UF, a further fractionation by OFFGEL isoelectrofocusing was proposed for the separation of antioxidant peptides. Nevertheless, ampholytes needed to establish the pH gradient in isoelectrofocusing significantly interfered on following assays. Several approaches were tried for the removal of these ampholytes (Zip-tips, spin-columns, UF, and monolithic columns). Zip-tips, spin-columns, and UF failed in the attempt to remove ampholytes due to the similar size of peptides and ampholytes. The proper optimization of the chromatographic separation conditions on a monolithic column enabled to clean the peptide fraction from closely related ampholytes.

Extracted native peptides from five different SBIFs have been explored for their antioxidant and antihypertensive capacities. The antioxidant capacity in all SBIFs was high and very similar. This behavior contrasted with the observed in the case of the ACE inhibitor capacity that varied significantly among SBIFs (IC_{50} values from 2.45 μg/mL to 63.90 μg/mL). The highest antioxidant capacity was always observed in the fraction containing peptides from 5 to 10 kDa while this was the fraction exerting the lowest antihypertensive capacity.

Moreover, antioxidant capacity of this fraction did not vary significantly after gastrointestinal digestion observing the opposite behavior in the fractions exerting the highest ACE inhibitor capacity (fractions from 3 to 5 kDa and < 3 kDa). In both cases, an HPLC-Q-ToF system and the PEAKS program enabled to identify more than 100 different peptides in every fraction. It is remarkable the presence of RPSYT peptide since it was present in all SBIFs and yielded both antioxidant and antihypertensive activities. Additionally, this peptide could withstand gastrointestinal digestion and resisted high processing temperatures.



CHAPTER IV

CONCLUSIONS

IV. Conclusions

From the results obtained in this doctoral thesis, it can be concluded that:

- The investigation on the presence of native ACE inhibitory and antioxidant peptides in different soybean commercial infant formulas showed that, from the four proposed peptide isolation methods, ultrafiltration was the simplest and the most adequate. In fact, the use of 12% TCA resulted in the co-precipitation of peptides together with proteins, while slow heating with 5% TCA did not allow to completely precipitate proteins.

- Extracts obtained from soybean infant formulas showed both antioxidant and ACE inhibitory capacities. The antioxidant capacity was high and very similar for all the samples analyzed. The highest ACE inhibitory capacity was observed for those soybean based infant formulas with the highest degree of hydrolysis.

- Fractionation of peptide extracts from commercial soybean based infant formulas revealed the highest antioxidant capacity for peptide fractions from 5 to 10 kDa. The highest ACE inhibitory capacities were found in peptide fractions below 5 kDa. Peptide fractions below 3 kDa from soybean infant formulas 4 and 5 showed exceptionally high ACE inhibitory capacities exerting IC_{50} values (1.20 ± 0.05 and 0.57 ± 0.04 μg/ mL) higher than those corresponding to known antihypertensive milk peptides VPP (2.80 μg/mL) and IPP (1.67 μg/mL).

- Although the use of OFFGEL isoelectrofocusing to fractionate bioactive peptides from food matrices was proposed for the first time in this doctoral thesis, individual fractions obtained by OFFGEL showed lower antioxidant capacities than whole peptide fractions from 5 to 10 kDa suggesting a synergic effect among antioxidant peptides. Thus, the OFFGEL separation step was omitted.

- Fractions showing the highest antioxidant or antihypertensive capacities were submitted to simulated gastrointestinal digestion observing no significant change in the antioxidant capacity and a slight reduction in the ACE inhibitory capacity. Nevertheless, ACE inhibitory capacity was still maintained at a high level.

- Peptides present in the fractions with the highest antioxidant and ACE inhibitory capacities were identified by HPLC-Q-ToF and PEAKS software. Poor ultrafiltration performance was found especially for peptides with low molecular weights. Comparison of identified peptides before and/or after the gastrointestinal digestion enabled the selection of various potential antioxidant and ACE inhibitory peptides that could resist the action of gastrointestinal enzymes.

- The peptide with the sequence RPSYT showed moderate ACE inhibitory activity and high antioxidant activity being resistant to simulated gastrointestinal digestion and high temperatures.

- Soybean based infant formulas were proposed, for the first time, as a source of ACE inhibitory and antioxidant peptides which could have a huge impact on the pediatric and medicinal areas taking into account the poor literature dealing with these foods.

- A first rapid analytical methodology for the estimation of the content of LRP, LSP, and LQP antihypertensive peptides in maize crops was developed by RP-HPLC with UV detection. The accelerated extraction using high intensity focused ultrasounds and the purification of α-zeins from whole maize kernels were optimized. An adequate solvent enabling the solubilization of precipited α-zeins and their suitable thermolysin digestion was selected. Peptides were separated in the protein hydrolysate in less than 5 min and identified by HPLC-Q-ToF.

- The application of the developed HPLC-UV methodology enabled to estimate the relative content of the three highly antihypertensive peptides in various maize crops. The LRP content was very low in all maize crops, while LQP (IC_{50} = 2.0 μM) and LSP (IC_{50} = 1.7 μM) peptides, presenting an activity more than twice that of the known VPP and IPP peptides (IC_{50} = 9.13 and 5.15 μM, respectively) from milk, were present in much higher concentrations. It was possible to observe significant differences among maize crops suggesting different antihypertensive capacities of maize lines.

- In order to obtain better selectivity and sensitivity and to perform an absolute quantification of LRP, LSP, and LQP peptides in digested α-zeins extracts, an HPLC-Q-ToF methodology was developed. Optimization of ESI parameters enabled to remove in-source peptide fragmentation and significantly increase peptide signal intensities. Parameters mostly affecting peptides signal intensities were fragmentator voltage, nebulizer pressure, and sheath gas flow and temperature. Two different SPE (Sep-Pak and Supelco) cartridges were compared to remove ionization suppression effects. The Sep-Pak cartridge provided an appropriate selectivity for all three targeted antihypertensive peptides.

- The developed HPLC-Q-ToF method was characterized by the evaluation of various parameters. Some parameters highly depended on the applied extraction window and optimum extraction windows had to be selected for every antihypertensive peptide. Since the method suffered from matrix interferences, the standard additions calibration method was used for the quantification of the three peptides in maize crops.

- The application of the developed HPLC-ESI-Q-ToF methodology enabled to determine the concentration of the three antihypertensive peptides in six maize crops. The highest LRP yield was found in A632 and EZ11A lines. The content of LSP was the highest in EZ11A and MC lines, while LQP peptide was more concentrated in the EZ11A variety. In order to comprehensively assess, the antihypertensive capacity of maize lines, a new parameter (*antihypertensive equivalent factor*) taking into account

the antihypertensive activity of every peptide and its content in every maize line was proposed and estimated. According to these antihypertensive equivalent factors, the highest antihypertensive capacity corresponded to the EZ11A line.

- The comparison of the results obtained using UV and Q-ToF detectors showed, in general, the same concentration order for the three antihypertensive peptides determined in the maize lines. Nevertheless, for the LQP peptide, strong interferences resulted in an overestimation of its content when using the UV detector.

- A first analytical methodology for the estimation of VLIVP peptide in soybean genotypes has been developed by μHPLC-IT. A soybean protein extraction method was improved by the application of high intensity focused ultrasounds resulting in the significant reduction of the extraction time from 120 min to just 2 min. The attempt to purify just 11S globulins from the whole protein extract showed to be not quantitative and was rejected.

- Optimization of conditions used in the digestion of the whole soybean protein extract with protease P revealed their strong sensitivity to pH, temperature, and enzyme to substrate ratio.

- Peptide VLIVP was identified in digested soybean proteins extracts by μHPLC-IT using both MS and MS/MS modes. The appropriate optimization of chromatographic parameters enabled the separation of the peptide from the rest of the extract in an analysis time of 7 min. Matrix effects were reduced by diluting twenty times the digested extracts. ESI and IT parameters were optimized. The parameters most affecting peptide signal intensity were the capillary voltage, dry gas flow and temperature, ion charge control target, and collision energy.

- The μHPLC-IT method was characterized by the evaluation of its analytical characteristics. The results showed that the method was very sensitive, precise, and free of matrix interferences which permitted the use of the external standard calibration method. The method enabled to detect up to 3.6 ng of peptide and to determine up to 12 ng of peptide in 1 g of soybean (as dry basis).

- The developed μHPLC-IT methodology was applied to the quantification the antihypertensive VLIVP peptide in five soybean crops. The highest VLIVP peptide content was found in the variety Mazowiecka II from Poland. The significant differences in VLIVP peptide content observed among soybean varieties suggested different antihypertensive capacity of these soybean crops.

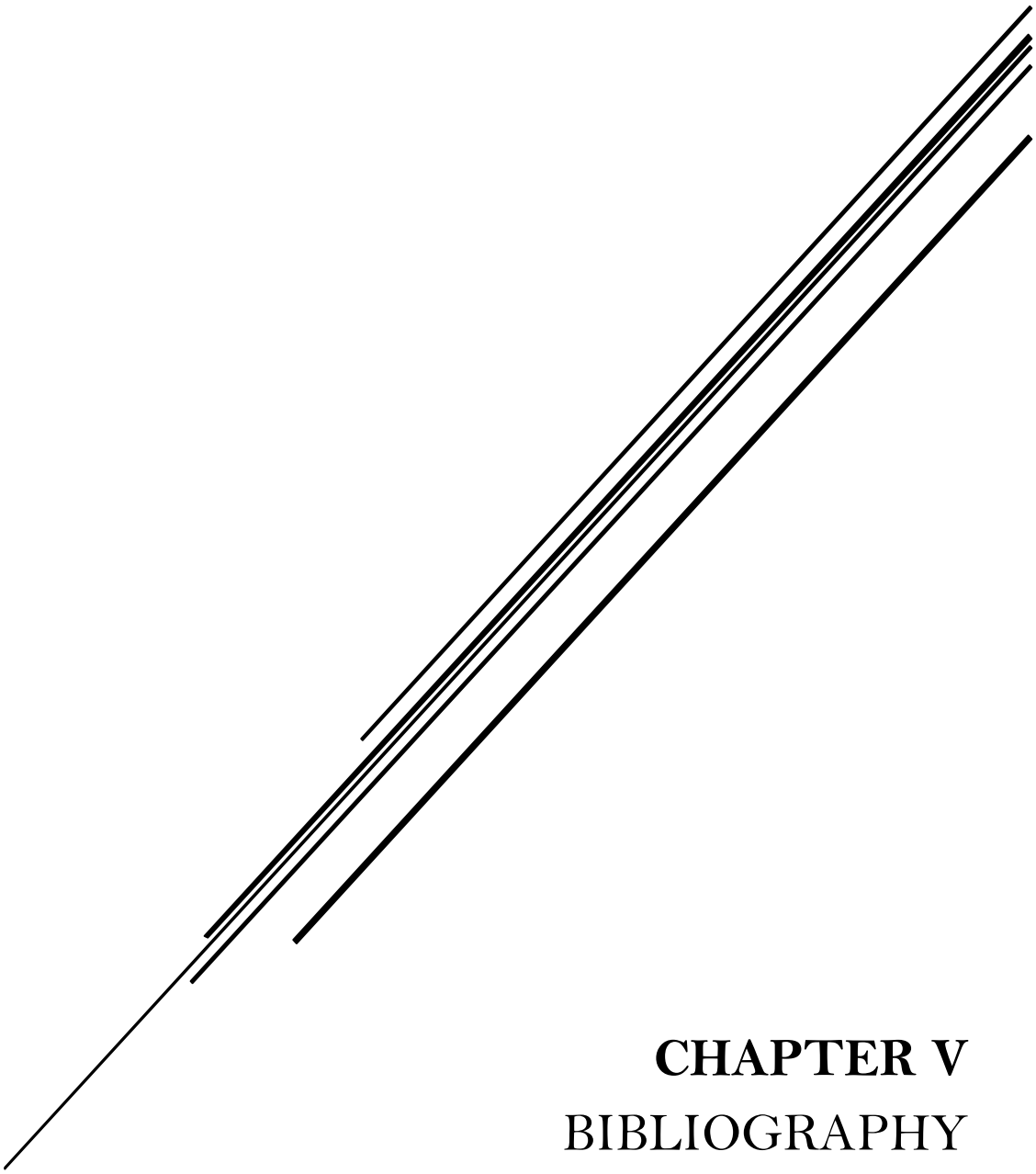
- The comparison of the protein extraction methods showed that soybean globulins are much easily extracted by high intensity focused ultrasounds than maize α-zeins proteins.

- The comparison of bacterial enzymes showed that thermolysin required higher enzyme to substrate ratio and shorter digestion times than protease P. At the same time, both of these proteases were inactivated when high intensity focused ultrasounds were applied. Additionally, the introduction of reduction and alkylation steps previous to the digestion resulted in the reduction of enzymes activities.

- Quantification studies presented in this thesis confirmed significant differences in antihypertensive capacities of plant crops. In addition, they highlight the importance of the development of analytical methodologies for the absolute quantification of antihypertensive peptides in highly complex food matrices.

- To assess the distribution of PKA, PKG, and CaMKII isoforms in specific tissues, an SRM assay using nano-LC-QqQ was developed. Final SRM assay consisted of 27 PTPs with 27 precursors and 162 transitions. The scheduling of the transitions over the LC run increased sensitivity. Selected PTPs and most of the transitions were validated by the analysis of digested kidney pull-down spiked with heavily labeled isotopic peptides counterparts. The optimization of collision energy for all PTPs revealed that it strongly influenced peptide signal intensities but it did not vary significantly from the collision energy values theoretically calculated using standard equations.

- Application of the developed SRM assay did not enable to quantify selected PKA, PKG, and CaMKII isoforms in heart, liver, and kidney lysates. In order to decrease sample complexity, kidney and liver lysates were previously separated using SDS-PAGE. Nevertheless, the application of the SDS-PAGE in-gel digestion SRM method to a rat heart model enabled to determine just two PTPs for the δ isoform of CaMKII. Moreover, the quantification of these two peptides showed significant differences among technical replicates. Results suggested a lack of reproducibility at some stage of the procedure used for sample preparation. To solve this issue, an appropriate selection of separation techniques to reduce the sample complexity seems to be crucial.



CHAPTER V
BIBLIOGRAPHY

V. Bibliography

References for Introduction and Prefaces parts:

[1] S. Paszun, B. Stanisz , Rys historyczny- nadciśnienie tętnicze, układ renina-angiotensyna i synteza pierwszego inhibitora enzymu konwertującego angiotensynę, Nowiny Lekarskie 77 (2008) 392-398.

[2] Anonymous, European society of hypertension-european society of cardiology guidelines for the management of arterial hypertension, J. Hypertens. 21 (2003) 1011-1053.

[3] World Health Organization, A global brief on hypertension; Silent killer, global public health crisis, WHO/DCO/WHD/2013.2 (2013).

[4] A. Chockalingam, World hypertension day and global awareness, Can. J. Cardiol. 24 (2008) 441-444.

[5] P.K. Whelton, Epidemiology and the prevention of hypertension, J. Clin. Hypertens. 6 (2004) 636-642.

[6] P. August, Initial treatment of hypertension, N. Engl. J. Med. 348 (2003) 610-617.

[7] P.M. Kearney, M. Whelton, K. Reynolds, P. Muntner, P.K. Whelton, J. He, Global burden of hypertension: analysis of worldwide data, Lancet 365 (2005) 217-223.

[8] V. Fuster, A. King, The dual challenge of BP control, Nat. Rev. Cardiol. 6 (2009) 729-730.

[9] M. Pereira, N. Lunet, A. Azevedo, H. Barros, Differences in prevalence, awareness, treatment and control of hypertension between developing and developed countries, J. Hypertens. 27 (2009) 963-975.

[10] V. Mohanlal, A. Parsa, M.R. Weir, Role of dietary therapies in the prevention and treatment of hypertension, Nat. Rev. Nephrol. 8 (2012) 413-422.

[11] M. Ezzati, S. Vander Hoorn, A. Rodgers, A. López, C. Mathers, C. Murray, Comparative Risk Assessment Collab, Estimates of global and regional potential health gains from reducing multiple major risk factors, Lancet 362 (2003) 271-280.

[12] K. Lock, J. Pomerleau, L. Causer, D.R. Altmann, M. McKee, The global burden of disease attributable to low consumption of fruit and vegetables: implications for the global strategy on diet, Bull. World Health Organ. 83 (2005) 100-108.

[13] A. Scholten, A Multi-Angular mass spectrometric view at cyclic nucleatide signaling proteins, Doctoral Thesis in Proteomics at Utrecht University (2006).

[14] R. FitzGerald, B. Murray, D. Walsh, Hypotensive peptides from milk proteins, J. Nutr. 134 (2004) 980S-988S.

[15] G.F. DiBona, U.C. Kopp, Neural control of renal function, Physiol. Rev. 77 (1997) 75-197.

[16] Q. Meng, S. Oparil, Purification and assay methods for angiotensin-converting enzyme, J. Chromatogr. A 743 (1996) 105-122.

[17] M. Volpe, C. Savoia, P. De Paolis, B. Ostrowska, D. Tarasi, S. Rubattu, The renin-angiotensin system as a risk factor and therapeutic target for cardiovascular and renal disease, J. Am. Soc. Nephrol. 13 (2002) S173-S178.

[18] B.A. Murray, R.J. FitzGerald, Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production, Curr. Pharm. Des. 13 (2007) 773-791.

[19] Z. Chen, C. Peng, R. Jiao, Y.M. Wong, N. Yang, Y. Huang, Anti-hypertensive nutraceuticals and functional foods, J. Agric. Food Chem. 57 (2009) 4485-4499.

[20] B. Sarmadi, A. Ismail, M. Hamid, Antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of cocoa (Theobroma cacao L.) autolysates, Food Res. Int. 44 (2011) 290-296.

[21] M.V. Singh, M.E. Anderson, Is CaMKII a link between inflammation and hypertrophy in heart?, J. Mol. Med. 89 (2011) 537-543.

[22] T.T. Aye , Application of chemical proteomics to biomarker discovery in cardiac research, Doctoral Thesis in Proteomics at Utrecht University (2010).

[23] J.R. Erickson, B.J. He, I.M. Grumbach, M.E. Anderson, CaMKII in the cardiovascular system: sensing redox states, Physiol. Rev. 91 (2011) 889-915.

[24] L. Stryer, J.M. Berg, J.L. Tymoczko, Biochemistry, New York; W. H. Freeman (2002).

[25] T.T. Aye, A. Scholten, N. Taouatas, A. Varro, T.A.B. Van Veen, M.A. Vos, A.J.R. Heck, Proteome-wide protein concentrations in the human heart, Mol. Biosys. 6 (2010) 1917-1927.

[26] P.A. Daly, M.J. Sole, Myocardial catecholamines and the pathophysiology of heart failure. Circulation 82 (1990) I35-I43.

[27] A. Scholten, T. Aye, A.J.R. Heck, A multi-angular mass spectrometric view at cyclic nucleotide dependent protein kinases: In vivo characterization and structure/function relationships, Mass Spectrom. Rev. 27 (2008) 331-353.

[28] P.D. Swaminathan, A. Purohit, T.J. Hund, M.E. Anderson , Calmodulin-dependent protein kinase II: linking heart failure and arrhythmias, Circ. Res. 8 (2012) 1661-1677.

[29] M.E. Anderson, CaMKII and a failing strategy for growth in heart, J. Clin. Invest. 119 (2009) 1082-1085.

[30] M.E. Anderson, J.H. Brown, D.M. Bers, CaMKII in myocardial hypertrophy and heart failure, J. Mol. Cell. Cardiol. 51 (2011) 468-473.

[31] M.I. Pérez, V.M. Musini, Pharmacological interventions for hypertensive emergencies: a Cochrane systematic review, J. Hum. Hypertens. 22 (2008) 596-607.

[32] S. Taddei, L. Ghiadoni, A. Salvetti, Current treatment of patients with hypertension - Therapeutic implications of insight, Drugs 63 (2003) 1435-1444.

[33] D. Hering, M.D. Esler, H. Krum, F. Mahfoud, M. Bohm, P.A. Sobotka, M.P. Schlaich, Recent advances in the treatment of hypertension, Expert Rev. Cardiovasc. Ther. 9 (2011) 729-44.

[34] L.M. Souza, R. Riera, H. Saconato, A. Demathe, A.N. Atallah, Oral drugs for hypertensive urgencies: systematic review and meta-analysis, Sao Paulo Med. J. 127 (2009) 366-372.

[35] S. Ferreira, D. Bartelt, L. Greene, Isolation of bradykinin-potentiating peptides from bothrops-jararaca venom, Biochem. 9 (1970) 2583- 2593.

[36] D. Cushman, M. Ondetti, History of the design of captopril and related inhibitors of angiotensin converting enzyme, Hypertension 17 (1991) 589-592.

[37] A.J. Turner, N.M. Hooper, The angiotensin-converting enzyme gene family: genomics and pharmacology, Trends Pharmacol. Sci. 23 (2002) 177-183.

[38] A.B. Atkinson, J.I.S. Robertson, captopril in the treatment of clinical hypertension and cardiac-failure, Lancet 2 (1979) 836-839.

[39] S. Lee, Z. Qian, S. Kim, A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats, Food Chem. 118 (2010) 96-102.

[40] T. Nakahara, A. Sano, H. Yamaguchi, K. Sugimoto, H. Chikata, E. Kinoshita, R. Uchida, Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin i-converting enzyme inhibitory substances, J. Agric. Food Chem. 58 (2010) 821-827.

[41] V. Perkovic, R. Huxley, Y. Wu, D. Prabhakaran, S. MacMahon, The burden of blood pressure-related disease - A neglected priority for global health, Hypertension 50 (2007) 991-997.

[42] L. Lindholm, The problem of uncontrolled hypertension, J. Hum. Hypertens. 16 (2002) S3-S8.

[43] L.J. Appel, F.M. Sacks, V.J. Carey, E. Obarzanek, J.F. Swain, E.R. Miller, P.R. Conlin, T.P. Erlinger, B.A. Rosner, N.M. Laranjo, J. Charleston, P. McCarron, L.M. Bishop, Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids - Results of the OmniHeart randomized trial, J. Am. Med. Assoc. 294 (2005) 2455-2464.

[44] J. He, M.R. Wofford, K. Reynolds, J. Chen, C. Chen, L. Myers, D.L. Minor, P.J. Elmer, D.W. Jones, P.K. Whelton, Effect of dietary protein supplementation on blood pressure a randomized, controlled trial, Circulation 124 (2011) 589-595.

[45] F. Shahidi, Y. Zhong, Bioactive peptides, J. AOAC Int. 91 (2008) 914-931.

[46] K. Erdmann, W.Y. Cheung Belinda, H. Schroder, The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease, J. Nutr. Biochem. 19 (2008) 643-654.

[47] M. Kussmann, A. Panchaud, M. Affolter, Proteomics in nutrition: status quo and outlook for biomarkers and bioactives, J. Proteome Res. 9 (2010) 4876-4887.

[48] A. Schieber, Functional foods and nutraceuticals, Food Res. Int. 46 (2012) 437.

[49] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[50] F. De Leo, S. Panarese, R. Gallerani, L.R. Ceci, Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food, Curr. Pharm. Des. 15 (2009) 3622-3643.

[51] F. Lalor, P.G. Wall, Health claims regulations. Comparison between USA, Japan and European Union, Br. Food J. 113 (2011) 298-313.

[52] B. Walther, R. Sieber, Bioactive proteins and peptides in foods, Int. J. Vitam. Nutr. Res. 81 (2011) 181-192.

[53] J. Schrezenmeir, H. Korhonen, C. Williams, H. Gill, N. Shah, Beneficial natural bioactive substances in milk and colostrum - Occurrence, biochemical and technological characteristics of bioactive substances - Physiological effects and potential health benefits - Foreword, Br. J. Nutr. 84 (2000) S1-S1.

[54] N.P. Moller, K.E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, Bioactive peptides and proteins from foods: indication for health effects, Eur. J. Nutr. 47 (2008) 171-182.

[55] H. Korhonen, A. Pihlanto, Bioactive peptides: Production and functionality, Int. Dairy J. 16 (2006) 945-960.

[56] D.D. Kitts, K. Weiler, Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery, Curr. Pharm. Des. 9 (2003) 1309-1323.

[57] M. Hayes, R.P. Ross, G.F. Fitzgerald, C. Stanton, Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview, Biotechnol. J. 2 (2007) 426-434.

[58] P. Minkiewicz, J. Dziuba, A. Iwaniak, M. Dziuba, M. Darewicz, BIOPEP database and other programs for processing bioactive peptide sequences, J. AOAC Int. 91 (2008) 965-980.

[59] R. Hartmann, H. Meisel, Food-derived peptides with biological activity: from research to food applications, Curr. Opin. Biotechnol. 18 (2007) 163-169.

[60] D. Martínez-Maqueda, B. Miralles, I. Recio, B. Hernández-Ledesma, Antihypertensive peptides from food proteins: a review, Food Funct. 3 (2012) 350-361.

[61] M. Phelan, D. Kerins, The potential role of milk-derived peptides in cardiovascular disease, Food Funct. 2 (2011) 153-167.

[62] S. Sharma, R. Singh, S. Rana, Bioactive peptides: a review, Int. J. Bioautomation 15 (2011) 223-250.

[63] M. Danquah, D. Agyei, Pharmaceutical applications of bioactive peptides OA Biotechnology 29 (2012) 1-7.

[64] B. Hernández-Ledesma, M. M. Contreras, I. Recio, Antihypertensive peptides: Production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23-35.

[65] H. Korhonen, A. Pihlanto, Food-derived bioactive peptides - Opportunities for designing future foods, Curr. Pharm. Des. 9 (2003) 1297-1308.

[66] M.C. García, P. Puchalska, C. Esteve, M.L. Marina, Vegetable foods: A cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities, Talanta 106 (2013) 328-349.

[67] G. Li, G. Le, Y. Shi, S. Shrestha, Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects, Nutr. Res. 24 (2004) 469-486.

[68] S. Maruyama, H. Suzuki, A Peptide Inhibitor of Angiotensin-I Converting Enzyme in the tryptic hydrolysate of casein, Agric. Biol. Chem. 46 (1982) 1393-1394.

[69] R. López-Fañdino, J. Otte, J. van Camp, Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity, Int. Dairy J. 16 (2006) 1277-1293.

[70] M. Hayes, C. Stanton, G.F. Fitzgerald, R.P. Ross, Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part II: bioactive peptide functions, Biotechnol. J. 2 (2007) 435-449.

[71] C. Guang, R.D. Phillips, Plant food-derived angiotensin I converting enzyme inhibitory peptides, J. Agric. Food Chem. 57 (2009) 5113-5120.

[72] K. Katayama, Jamhari, T. Mori, S. Kawahara, K. Miake, Y. Kodama, M. Sugiyama, Y. Kawamura, T. Nakayama, M. Muruyama, M. Muguruma, Angiotensin-I converting enzyme inhibitory peptide derived from porcine skeletal muscle myosin and its antihypertensive activity in spontaneously hypertensive rats, J. Food Sci. 72 (2007) S702-S706.

[73] E. Escudero, L. Mora, P.D. Fraser, M. Aristoy, K. Arihara, F. Toldrá, Purification and Identification of antihypertensive peptides in Spanish dry-cured ham, J. Proteomics 78 (2013) 499-507.

[74] K. Nakade, R. Kamishima, Y. Inoue, A. Ahhmed, S. Kawahara, T. Nakayama, M. Maruyama, M. Numata, K. Ohta, T. Aoki, M. Muguruma, Identification of an antihypertensive peptide derived from chicken bone extract, Anim. Sci. J. 79 (2008) 710-715.

[75] H. Byun, S. Kim, Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (Theragra chalcogramma) skin, Process Biochem. 36 (2001) 1155-1162.

[76] Y. Wang, H. He, X. Chen, C. Sun, Y. Zhang, B. Zhou, Production of novel angiotensin I-converting enzyme inhibitory peptides by fermentation of marine shrimp Acetes chinensis with Lactobacillus fermentum SM 605, Appl. Microbiol. Biotechnol. 79 (2008) 785-791.

[77] X. Liu, M. Zhang, A. Jia, Y. Zhang, H. Zhu, C. Zhang, Z. Sun, C. Liu, Purification and characterization of angiotensin I converting enzyme inhibitory peptides from jellyfish Rhopilema esculentum, Food Res. Int. 50 (2013) 339-343.

[78] S. Lee, Y. Kim, S. Kim, E. Kim, J. Hwang, T. Park, B.K. Kim, S.H. Moon, B. Jeon, Y. Jeon, C. Ahn, J. Je, P. Park, Purification and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from an enzymatic hydrolysate of duck skin byproducts, J. Agric. Food Chem. 60 (2012) 10035-10040.

[79] C. Ahn, Y. Jeon, Y. Kim, J. Je, Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis, Process Biochem. 47 (2012) 2240-2245.

[80] D. Pan, Y. Luo, M. Tanokura, Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of Lactobacillus helveticus JCM1004, Food Chem. 91 (2005) 123-129.

[81] H. Tonouchi, M. Suzuki, M. Uchida, M. Oda, Antihypertensive effect of an angiotensin converting enzyme inhibitory peptide from enzyme modified cheese, J. Dairy Res. 75 (2008) 284-290.

[82] Y. Nogata, T. Nagamine, M. Yanaka, H. Ohta, Angiotensin I converting enzyme inhibitory peptides produced by autolysis reactions from wheat bran, J. Agric. Food Chem. 57 (2009) 6618-6622.

[83] Y. Yang, G. Tao, P. Liu, J. Liu, Peptide with anglotensin I-converting enzyme inhibitory activity from hydrolyzed corn gluten meal, J. Agric. Food Chem. 55 (2007) 7891-7895.

[84] S. Yano, K. Suzuki, G. Funatsu, Isolation from alpha-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity, Biosci. Biotechnol. Biochem. 60 (1996) 661-663.

[85] K.G.M. Gouda, L.R. Gowda, A.G.A. Rao, V. Prakash, Angiotensin I-converting enzyme inhibitory peptide derived from glycinin, the 11S globulin of soybean (Glycine max), J. Agric. Food Chem. 54 (2006) 4568-4573.

[86] M. Tomatsu, A. Shimakage, M. Shinbo, S. Yamada, S. Takahashi, Novel angiotensin I-converting enzyme inhibitory peptides derived from soya milk, Food Chem. 136 (2013) 612-616.

[87] B.H. Sarmadi, A. Ismail, Antioxidative peptides from food proteins: A review, Peptides 31 (2010) 1949-1956.

[88] A. Pihlanto, Antioxidative peptides derived from milk proteins, Int. Dairy J. 16 (2006) 1306-1314.

[89] A.G.P. Samaranayaka, E.C.Y. Li-Chan, Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications, J. Funct. Foods 3 (2011) 229-254.

[90] O. Power, P. Jakeman, R.J. FitzGerald, Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides, Amino Acids 44 (2013) 797-820.

[91] A. Ghiselli, M. Serafini, F. Natella, C. Scaccini, Total antioxidant capacity as a tool to assess redox status: Critical view and experimental data, Free Radic. Biol. Med. 29 (2000) 1106-1114.

[92] Z. Qian, W. Jung, S. Kim, Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, Rana catesbeiana Shaw, Bioresour. Technol. 99 (2008) 1690-1698.

[93] C. Jao, W. Ko, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging by protein hydrolyzates from tuna cooking juice, Fisheries Sci. 68 (2002) 430-435.

[94] A.G.P. Samaranayaka, E.C.Y. Li-Chan, Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (Merluccius productus), Food Chem. 107 (2008) 768-776.

[95] X. Dong, B. Zhu, H. Zhao, D. Zhou, H. Wu, J. Yang, D. Li, Y. Murata, Preparation and in vitro antioxidant activity of enzymatic hydrolysates from oyster (Crassostrea talienwhannensis) meat, Int. J. Food Sci. Technol. 45 (2010) 978-984.

[96] M. Salami, A.A. Moosavi-Movahedi, M.R. Ehsani, R. Yousefi, T. Haertle, J. Chobert, S.H. Razovi, R. Henrich, S. Balalaie, S.A. Ebadi, S. Pourtakdoost, A. Niasari-Naslaji, Improvement of the antimicrobial and antioxidant activities of camel and bovine whey proteins by limited proteolysis, J. Agric. Food Chem. 58 (2010) 3297-3302.

[97] L. Zhu, J. Chen, X. Tang, Y.L. Xiong, Reducing, radical scavenging, and chelation properties of in vitro digests of alcalase-treated zein hydrolysate, J. Agric. Food Chem. 56 (2008) 2714-2721.

[98] E. Pena-Ramos, Y. Xiong, Antioxidant activity of soy protein hydrolysates in a liposomal system, J. Food Sci. 67 (2002) 2952-2956.

[99] S. Mandal, R.K. Mandal, Seed storage proteins and approaches for improvement of their nutritional quality by genetic engineering, Curr. Sci. 79 (2000) 576-589.

[100] T.A.B. Osborne , The proteins of the wheat kernel, Carnegie Institution of Washington, Washington D.C. (1907) 1-119.

[101] T.J. Anderson, B.P. Lamsal, Zein extraction from corn, corn products, and co-products and modifications for various applications: a review, Cereal Chem. 88 (2011) 159-173.

[102] B.M. Prasanna, S.K. Vasal, B. Kassahun, N.N. Singh, Quality protein maize, Curr. Sci. 81 (2001) 1308-1319.

[103] R. Adeyemo, Maize- Consumption expenditure of rural and urban workers - implications for Nigeria, JMAS. 22 (1984) 163-166.

[104] R. Shukla, M. Cheryan, Zein: the industrial protein from corn, Ind. Crops Prod. 13 (2001) 171-192.

[105] C.M. Wilson, A nomenclature for zein polypeptides based on isoelectric-focusing and sodium dodecyl-sulfate polyacrylamide-gel electrophoresis, Cereal Chem. 62 (1985) 361-365.

[106] A. Esen, An immunodominant site of gamma-zein1 is in the region of tandem hexapeptide repeats, J. Protein Chem. 9 (1990) 453-460.

[107] A. Esen, A proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (Zea-Mays-L), J. Cereal Sci. 5 (1987) 117-128.

[108] A.S. Tatham, J.M. Field, V.J. Morris, K.J. Ianson, L. Cardle, M.J. Dufton, P.R. Shewry, Solution conformational-analysis of the alpha-zein proteins of maize, J. Biol. Chem. 268 (1993) 26253-26259.

[109] W.R. Adams, S.S. Huang, A.L. Kriz, M.H. Luethy, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of zeins in mature maize kernels, J. Agric. Food Chem. 52 (2004) 1842-1849.

[110] J.F. Wang, P.H. Geil, D.R.J. Kolling, G.W. Padua, Analysis of zein by matrix-assisted laser desorption/ionization mass spectrometry, J. Agric. Food Chem. 51 (2003) 5849-5854.

[111] D. Fukushima, Recent progress of soybean protein foods: Chemistry, technology, and nutrition, Food Rev. Int. 7 (1991) 323-351.

[112] M. Friedman, D. Brandon, Nutritional and health benefits of soy proteins, J. Agric. Food Chem. 49 (2001) 1069-1086.

[113] M.C García, M. Torre, M.L Marina, F. Laborda, Composition and characterization of soyabean and related products, Crit. Rev. Food Sci. Nutr. 37 (1997) 361-391.

[114] W. Wang, E. De Mejia, A new frontier in soy bioactive peptides that may prevent age-related chronic diseases, Compr. Rev. Food Sci. 4 (2005) 63-78.

[115] R. Mujoo, D.T. Trinh, P.K.W. Ng, Characterization of storage proteins in different soybean varieties and their relationship to tofu yield and texture, Food Chem. 82 (2003) 265-273.

[116] M. Pischetsrieder, R. Baeuerlein, Proteome research in food science, Chem. Soc. Rev. 38 (2009) 2600-2608.

[117] J.M. Gallardo, I. Ortea, M. Carrera, Proteomics and its applications for food authentication and food-technology research, TrAC Trend Anal. Chem. 52 (2013) 135-141.

[118] J. Carrasco-Castilla, A. J. Hernández-Alvarez, C. Jimenez-Martínez, G. F. Gutierrez-López, G. Davila-Ortiz, Use of proteomics and peptidomics methods in food bioactive peptide science and engineering, Food Eng. Rev. 4 (2012) 224-243.

[119] M. Carbonaro, Proteomics: present and future in food quality evaluation, Trends Food Sci. Technol. 15 (2004) 209-216.

[120] H. Steen, M. Mann, The ABC's (and XYZ's) of peptide sequencing, Nat. Rev. Mol. Cell Biol. 5 (2004) 699-711.

[121] G. Maddalo, Strategies to explore the membrane proteome of a cell, Doctoral thesis in Analytical Chemistry at Stockholm University (2011).

[122] C. Esteve Gil, Separación y caracterización de las proteínas de la aceituna y aceite de oliva. Identificación mediante técnicas proteómicas y differencación varietal del olivo en base a perfiles proteícos, Doctoral thesis in Analytical Chemistry at University of Alcala (2013).

[123] M. Kussmann, M. Affolter, Proteomics at the center of nutrigenomics: Comprehensive molecular understanding of dietary health effects, Nutrition 25 (2009) 1085-1093.

[124] P. Minkiewicz, J. Dziuba, M. Darewicz, A. Iwaniak, M. Dziuba, D. Nałęcz, Food peptidomics, Food Technol. Biotech. 46 (2008) 1-10.

[125] A. Panchaud, M. Affolter, M. Kussmann, Mass spectrometry for nutritional peptidomics: How to analyze food bioactives and their health effects, J. Proteomics 27 (2012) 3546-3559.

[126] A.C. Grabski, Chapter 18 Advances in Preparation of Biological Extracts for Protein Purification, in: Methods in Enzymology, Academic Press, pp. 285-303.

[127] U. Michelsen, J. von Hagen, Chapter 19: Isolation of subcellular organelles and structures, in: methods in enzymology, Academic Press, pp. 305-328.

[128] B. Cañas, C. Piñeiro, E. Calvo, D. López-Ferrer, J. Manuel Gallardo, Trends in sample preparation for classical and second generation proteomics, J. Chrotmatogr. A 1153 (2007) 235-258.

[129] M. Peach, N. Marsh, D.J. MacPhee, Protein solubilization: attend to the choice of lysis buffer, in: B.T. Kurien, H.R. Scofield (Eds.), Protein Electrophoresis, Humana Press, 2012, pp. 37-47.

[130] D. Martínez-Maqueda, B. Hernández-Ledesma, L. Amigo, B. Miralles, J. Gómez-Ruiz, Extraction/fractionation techniques for proteins and peptides and protein digestion, in: proteomics in foods, Springer US, 2013, pp. 21-50.

[131] Y. Zhang, B.R. Fonslow, B. Shan, M. Baek, J.R. Yates, Protein analysis by shotgun/bottom-up proteomics, Chem. Rev. 10 (2013) 2343-2394.

[132] T. Yang, S. Da-Wen, Enhancement of food processes by ultrasound: a review, Crit. Rev. Food Sci. Nutr. DOI: 10.1080/10408398.2012.667849 (2013).

[133] H.M. Santos, J.L. Capelo, Trends in ultrasonic-based equipment for analytical sample treatment, Talanta 31 (2007) 795-802.

[134] E. Domínguez-Vega, Estrategias analíticas innovadoras utilizando técnicas electroforéticas y cromatográficas capilares para la determinación de aminoácidos y péptidos en alimentos, Doctoral thesis in Analytical Chemistry at University of Alcala (2011).

[135] R.R. Burgess, Protein precipitation techniques, Methods Enzymol. 463 (2009) 331-342.

[136] A. Poliwoda, P.P. Wieczorek, Sample pretreatment techniques for oligopeptide analysis from natural sources, Anal. Bioanal. Chem. 393 (2009) 885-897.

[137] J.W. Lawton, Zein: A history of processing and use, Cereal Chem. 79 (2002) 1-18.

[138] A. Kale, F. Zhu, M. Cheryan, Separation of high-value products from ethanol extracts of corn by chromatography, Ind. Crop. Prod. 26 (2007) 44-53.

[139] F. Zhu, A.V. Kale, M. Cheryan, Fractionation of zein by size exclusion chromatography, J. Agric. Food Chem. 55 (2007) 3843-3849.

[140] M.P. Hojilla-Evangelista, L.A. Johnson, Optimizing extraction of zein and glutelin-rich fraction during sequential extraction processing of corn, Cereal Chem. 80 (2003) 481-484.

[141] S.R. Bean, G.L. Lookhart, J.A. Bietz, Acetonitrile as a buffer additive for free zone capillary electrophoresis separation and characterization of maize (Zea mays L.) and sorghum (Sorghum bicolor L. Moench) storage proteins, J. Agric. Food Chem. 48 (2000) 318-327.

[142] J.M. Rodríguez-Nogales, M.C. García, M.L. Marina, Development of a perfusion reversed-phase high performance liquid chromatography method for the characterisation of maize products using multivariate analysis, J. Chrotmatogr. A 1104 (2006) 91-99.

[143] G.L. Erny, M. L. Marina, A. Cifuentes, CE-MS of zein proteins from conventional and transgenic maize, Electrophoresis 28 (2007) 4192-4201.

[144] J. Landry, S. Delhaye, C. Damerval, Comparative efficiencies of isopropyl and tert-butyl alcohols for extracting zeins from maize endosperm, J. Agric. Food Chem. 50 (2002) 4131-4134.

[145] N. Parris, L.C. Dickey, Extraction and solubility characteristics of zein proteins from dry-milled corn, J. Agric. Food Chem. 49 (2001) 3757-3760.

[146] L.C. Swallen, Zein - A new industrial protein, Ind. Eng. Chem. 33 (1941) 394-398.

[147] L. Anta, M. L. Marina, M. C. García, Simultaneous and rapid determination of the anticarcinogenic proteins Bowman-Birk inhibitor and lectin in soybean crops by perfusion RP-HPLC, J. Chrotmatogr. A 1217 (2010) 7138-7143.

[148] C. Liu, H. Wang, Z. Cui, X. He, X. Wang, X. Zeng, H. Ma, Optimization of extraction and isolation for 11S and 7S globulins of soybean seed storage protein, Food Chem. 102 (2007) 1310-1316.

[149] A. Sussulini, J.S. García, M.F. Mesko, D.P. Moraes, A.M.M. Flores, C.A. Pérez, M.A.Z. Arruda, Evaluation of soybean seed protein extraction focusing on metalloprotein analysis. Microchim. Acta 158 (2007) 173-180.

[150] V. Thanh, K. Shibasaki, Major proteins of soybean seeds - straightforward fractionation and their characterization, J. Agric. Food Chem. 24 (1976) 1117-1121.

[151] T. Nagano, M. Hirotsuka, H. Mori, K. Kohyama, K. Nishinari, Dynamic viscoelastic study on the gelation of 7S globulin from soybeans, J. Agric. Food Chem. 40 (1992) 941-944.

[152] A. Castro-Rubio, F. Castro-Rubio, M.C. García, M.L. Marina, Development of a perfusion reversed-phase HPLC method for the separation of soybean and cereal (wheat, corn, and rice) proteins in binary mixtures. Application to the detection of soybean proteins in commercial bakery products, J. Sep. Sci. 28 (2005) 996-1004.

[153] F. Castro-Rubio, M.L. Marina, M.C. García, Perfusion reversed-phase high-performance liquid chromatography/mass spectrometry analysis of intact soybean proteins for the characterization of soybean cultivars, J. Chrotmatogr. A 1170 (2007) 34-43.

[154] E. Domínguez-Vega, O. Kotkowska, M. C. García, A.L. Crego, M. L. Marina, Fast determination of the functional peptide soymetide in different soybean derived foods by capillary-high performance liquid chromatography, J. Chrotmatogr. A 1218 (2011) 4928-4933.

[155] E. Domínguez-Vega, M. C. García, A.L. Crego, M. L. Marina, First approach based on direct ultrasonic assisted enzymatic digestion and capillary-high performance liquid chromatography for the peptide mapping of soybean proteins, J. Chrotmatogr. A 1217 (2010) 6443-6448.

[156] A, Scholten, S. Mohammed, T.Y. Low, S. Zanivan, T.A. van Veen, B. Delanghe, A.J.R. Heck, In-depth quantitative cardiac proteomics combining electron transfer dissociation and the metalloendopeptidase Lys-N with the SILAC mouse, Mol. Cell. Proteomics. 10 (2011) O111.008474.

[157] A. Scholten, M.K. Poh, T.A. van Veen, B. van Breukelen, M.A. Vos, A.J.R. Heck, Analysis of the cGMP/cAMP interactome using a chemical proteomics approach in mammalian heart tissue validates sphingosine kinase type 1-interacting protein as a genuine and highly abundant AKAP, J. Proteome Res. 5 (2006) 1435-1447.

[158] T.T. Aye, S. Mohammed, H.W. van den Toorn, T.A. van Veen, M.A. van der Heyden, A. Scholten, A.J.R. Heck, Selectivity in enrichment of cAMP-dependent protein kinase regulatory subunits type I and type II and their interactors using modified cAMP affinity resins, Mol. Cell Proteomics 8 (2009) 1016-1028.

[159] A. Scholten, T.A. van Veen, M.A. Vos, A.J.R. Heck, Diversity of cAMP-dependent protein kinase isoforms and their anchoring proteins in mouse ventricular tissue, J. Proteome Res. 6 (2007) 1705-1717.

[160] D. Hyoung Lee, J. Ho Kim, J. Sik Park, Y. Jun Choi, J. Soo Lee, Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom Tricholoma giganteum, Peptides 25 (2004) 621-627.

[161] J.H. Jang, S.C. Jeong, J.H. Kim, Y.H. Lee, Y.C. Ju, J.S. Lee, Characterisation of a new antihypertensive angiotensin I-converting enzyme inhibitory peptide from Pleurotus cornucopiae, Food Chem. 15 (2011) 412-418.

[162] Y. Kokean, T. Nishii, H. Sakakura, Y. Furuichi, Effect of frying with edible oil on antihypertensive properties of Hatakeshimeji (*Lyophyllum decastes* Sing.) mushroom, Food Sci. Technol. Res. 11 (2005) 339-343.

[163] T. Saito, T. Nakamura, H. Kitazawa, Y. Kawai, T. Itoh, Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda cheese, J. Dairy Sci. 83 (2000) 1434-1440.

[164] J.A. Gómez-Ruiz, G. Taborda, L. Amigo, I. Recio, M. Ramos, Identification of ACE-inhibitory peptides in different Spanish cheeses by tandem mass spectrometry, Eur. Food Res. Technol. 223 (2006) 595-601.

[165] C.G. Papadimitriou, A. Vafopoulou-Mastrojiannaki, S.V. Silva, A. Gomes, F.X. Malcata, E. Alichanidis, Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity, Food Chem. 105 (2007) 647-656.

[166] A. Quiros, B. Hernández-Ledesma, M. Ramos, L. Amigo, I. Recio, Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine kefir, J. Dairy Sci. 88 (2005) 3480-3487.

[167] J. Zhang, E. Tatsumi, C. Ding, L. Li, Angiotensin I-converting enzyme inhibitory peptides in douchi, a Chinese traditional fermented soybean product, Food Chem. 98 (2006) 551-557.

[168] Z. Shin, R. Yu, S. Park, D. Chung, C. Ahn, H. Nam, K. Kim, H. Lee, His-His-Leu, An angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo, J. Agric. Food Chem. 49 (2001) 3004-3009.

[169] X. Zhu, K. Watanabe, K. Shiraishi, T. Ueki, Y. Noda, T. Matsui, K. Matsumoto, Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers, Peptides 29 (2008) 338-344.

[170] M. Kuba, K. Tanaka, S. Tawata, Y. Takeda, M. Yasuda, Angiotensin I-converting enzyme inhibitory peptides isolated from tofuyo fermented soybean food, Biosci. Biotechnol. Biochem. 67 (2003) 1278-1283.

[171] B.F. Gibbs, A. Zougman, R. Masse, C. Mulligan, Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food, Food Res. Int. 37 (2004) 123-131.

[172] D. Wang, L. Wang, F. Zhu, J. Zhu, X.D. Chen, L. Zou, M. Saito, L. Li, In vitro and in vivo studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food), Food Chem. 107 (2008) 1421-1428.

[173] A. Gupta, B. Mann, R. Kumar, R.B. Sangwan, Antioxidant activity of Cheddar cheeses at different stages of ripening, Int. J. Dairy Technol. 62 (2009) 339-347.

[174] K. Suetsuna, Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from Allium sativum L (garlic), J. Nutr. Biochem. 9 (1998) 415-419.

[175] K. Suetsuna, K. Maekawa, J. Chen, Antihypertensive effects of Undaria pinnatifida (wakame) peptide on blood pressure in spontaneously hypertensive rats, J. Nutr. Biochem. 15 (2004) 267-272.

[176] M. Ma, I.Y. Bae, H.G. Lee, C. Yang, Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (Fagopyrum esculentum Moench), Food Chem. 96 (2006) 36-42.

[177] O.N. Donkor, A. Henriksson, T.K. Singh, T. Vasiljevic, N.P. Shah, ACE-inhibitory activity of probiotic yoghurt, Int. Dairy J. 17 (2007) 1321-1331.

[178] M.Q. Ling, A. Yili, H.A. Aisa, Z. Bo, O.N. Veshkurova, S.I. Salikhov, Isolation of a new peptide from seeds of Apium graveolens indigenous to China, Chem. Nat. Compd. 46 (2011) 932-934.

[179] J. Je, J. Park, W. Jung, P. Park, S. Kim, Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce, Crassostrea gigas, Food Chem. 90 (2005) 809-814.

[180] J. Je, P. Park, H. Byun, W. Jung, S. Kim, Angiotensin I converting enzyme (ACE) inhibitory peptide derived from the sauce of fermented blue mussel, Mytilus edulis, Bioresour. Technol. 96 (2005) 1624-1629.

[181] J. Chobert, K. El-Zahar, M. Sitohy, M. Dalgalarrondo, F. Metro, Y. Choiset, T. Haertle, Angiotensin I-converting-enzyme (ACE)-inhibitory activity of tryptic peptides of ovine beta-lactoglobulin and of milk yoghurts obtained by using different starters, Lait 85 (2005) 141-152.

[182] A. Zambrowicz, M. Timmer, A. Polanowski, G. Lubec, T. Trziszka, Manufacturing of peptides exhibiting biological activity, Amino Acids 44 (2013) 315-320.

[183] Z. Qian, W. Jung, S. Lee, H. Byun, S. Kim, Antihypertensive effect of an angiotensin I-converting enzyme inhibitory peptide from bullfrog (Rana catesbeiana Shaw) muscle protein in spontaneously hypertensive rats, Process Biochem. 42 (2007) 1443-1448.

[184] K. Arihara, Y. Nakashima, T. Mukai, S. Ishikawa, M. Itoh, Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins, Meat Sci. 57 (2001) 319-324.

[185] K. Yokoyama, H. Chiba, M. Yoshikawa, Peptide inhibitors for angiotensin-i-converting enzyme from thermolysin digest of dried bonito, Biosci. Biotech. Bioch. 56 (1992) 1541-1545.

[186] W. Chiang, M. Tsou, Z. Tsai, T. Tsai, Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor, Food Chem. 98 (2006) 725-732.

[187] J. Otte, S.M. Shalaby, M. Zakora, A.H. Pripp, S.A. EI-Shabrawy, Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme and time of hydrolysis, Int. Dairy J. 17 (2007) 488-503.

[188] E. Mendis, N. Rajapakse, H. Byun, S. Kim, Investigation of jumbo squid (Dosidicus gigas) skin gelatin peptides for their in vitro antioxidant effects, Life Sci. 77 (2005) 2166-2178.

[189] E. Mendis, N. Rajapakse, S.K. Kim, Antioxidant properties of a radical-scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate, J. Agric. Food Chem. 53 (2005) 581-587.

[190] N. Rajapakse, E. Mendis, H. Byun, S. Kim, Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems, J. Nutr. Biochem. 16 (2005) 562-569.

[191] P. Park, W. Jung, K. Nam, F. Shahidi, S. Kim, Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk, J. Am. Oil Chem. Soc. 78 (2001) 651-656.

[192] Y. Li, B. Jiang, T. Zhang, W. Mu, J. Liu, Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH), Food Chem. 106 (2008) 444-450.

[193] X. Tang, Z. He, Y. Dai, Y.L. Xiong, M. Xie, J. Chen, Peptide fractionation and free radical scavenging activity of zein hydrolysate, J. Agric. Food Chem. 58 (2010) 587-593.

[194] N. Yamamoto, M. Ejiri, S. Mizuno, Biogenic peptides and their potential use, Curr. Pharm. Des. 9 (2003) 1345-1355.

[195] J.R. Wisniewski, A. Zougman, N. Nagaraj, M. Mann, Universal sample preparation method for proteome analysis, Nat. Methods 6 (2009) 359-U60.

[196] H.J. Issaq, T.P. Conrads, G.M. Janini, T.D. Veenstra, Methods for fractionation, separation and profiling of proteins and peptides, Electrophoresis 23 (2002) 3048-3061.

[197] Z. Witkiewicz, J. Hetpera, Słownik chromatografii i elektroforezy, Wydawnictwo Naukowe PWN (2004).

[198] J.M. Berg, J.L. Tymoczko, L. Stryer, The purification of proteins is an essential first step in understanding their function, IN: Biochemistry. 5th edition. New York: W H Freeman; (2002).

[199] K. Sandra, M. Moshir, F. D'hondt, K. Verleysen, K. Kas, P. Sandra P, Highly efficient peptide separations in proteomics Part 1. Unidimensional high performance liquid chromatography, J. Chromatogr. B 15 (2008) 48-63.

[200] H.J. Issaq, K.C. Chan, J. Blonder, X. Ye, T.D. Veenstra, Separation, detection and quantitation of peptides by liquid chromatography and capillary electrochromatography, J. Chrotmatogr. A 1216 (2009) 1825-1837.

[201] M.C. García, A.C. Hogenboom, H. Zappey, H. Irth, Effect of the mobile phase composition on the separation and detection of intact proteins by reversed-phase liquid chromatography - electro spray mass spectrometry, J. Chrotmatogr. A 957 (2002) 187-199.

[202] T. Herraiz, Sample preparation and reversed phase-high performance liquid chromatography analysis of food-derived peptides, Anal. Chim. Acta 352 (1997) 119-139.

[203] W.A. Neville, Reversed-phase chromatography of proteins, IN: P. Cutler (Ed.), Protein Purification Protocols, Humana Press, 2003, pp. 259-281.

[204] I. Neverova, J. Van Eyk, Role of chromatographic techniques in proteomic analysis, J. Chromatogr. B 815 (2005) 51-63.

[205] M. Rodriíguez-Aller, R. Gurny, J. Veuthey, D. Guillarme, Coupling ultra high-pressure liquid chromatography with mass spectrometry: Constraints and possible applications, J. Chrotmatogr. A 1292 (2013) 2-18.

[206] D. Guillarme, J. Schappler, S. Rudaz, J. Veuthey, Coupling ultra-high-pressure liquid chromatography with mass spectrometry, TrAC Trend Anal. Chem. 29 (2010) 15-27.

[207] N. Wu, A.M. Clausen, Fundamental and practical aspects of ultrahigh pressure liquid chromatography for fast separations, J. Sep. Sci. 30 (2007) 1167-1182.

[208] M.C. García, M.L. Marina, M. Torre, Perfusion chromatography: an emergent technique for the analysis of food proteins, J. Chrotmatogr. A 880 (2000) 169-187.

[209] Ö. Bahadir Acikara, G. Saltan Çitoğlu, S. Özbilgin, B. Ergene, Affinity Chromatography and Importance in Drug Discovery, Column Chromatography, http://www.intechopen.com/books/column-chromatography/affinity-chromatography-and-importance-in-drug-discovery (2013).

[210] K. Cabrera, Applications of silica-based monolithic HPLC columns, J. Sep. Sci. 27 (2004) 843-852.

[211] M.E. Swartz, UPLC™: an introduction and review, J. Liq. Chromatogr. Rel. Technol. 28 (2005) 1253-1263.

[212] A. Abrahim, M. Al-Sayah, P. Skrdla, Y. Bereznitski, Y. Chen, N. Wu, Practical comparison of 2.7 mu m fused-core silica particles and porous sub-2 mu m particles for fast separations in pharmaceutical process development, J. Pharm. Biomed. Anal. 51 (2010) 131-137.

[213] L. Novakova, H. Vlckova, A review of current trends and advances in modern bio-analytical methods: Chromatography and sample preparation, Anal. Chim. Acta 656 (2009) 8-35.

[214] H.D. Meiring, E. van der Heeft, G.J. Hove, A.P.J.M. de Jong, Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis, J. Sep. Sci. 25 (2002) 557-568.

[215] J.M. Saz, M.L. Marina, Application of micro- and nano-HPLC to the determination and characterization of bioactive and biomarker peptides, J. Sep. Sci. 31 (2008) 446-458.

[216] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali, Recent applications in nanoliquid chromatography, J. Sep. Sci. 30 (2007) 1589-1610.

[217] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, Nature 422 (2003) 198-207.

[218] S. Gedela, N.R. Medicherla, Chromatographic techniques for the separation of peptides: Application to proteomics, Chromatographia 65 (2007) 511-518.

[219] V. Wysocki, K. Resing, Q. Zhang, G. Cheng, Mass spectrometry of peptides and proteins, Methods 35 (2005) 211-222.

[220] P. Picotti, R. Aebersold, Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions, Nat. Methods 30 (2012) 555-566.

[221] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, Mol. Syst. Biol. 4 (2008).

[222] J.V. Olsen, J.C. Schwartz, J. Griep-Raming, M.L. Nielsen, E. Damoc, E. Denisov, O. Lange, P. Remes, D. Taylor, M. Splendore, E.R. Wouters, M. Senko, A. Makarov, M. Mann, S. Horning, A dual pressure linear ion trap Orbitrap instrument with very high sequencing speed, Mol. Cell Proteomics 8 (2009) 2759-2769.

[223] M. Scigelova, A. Makarov, Orbitrap mass analyzer-overview and applications in proteomics, Proteomics 6 (2006) 16-21.

[224] M. Holcapek, R. Jirasko, M. Lisa, Recent developments in liquid chromatography-mass spectrometry and related techniques, J. Chrotmatogr. A 1259 (2012) 3-15.

[225] L. Quan, M. Liu, CID, ETD and HCD fragmentation to study protein post-translational modifications, Mod. Chem. Appl. 1 (2013) e102.

[226] E.L. de Graaf, A.F.M. Altelaar, B. van Breukelen, S. Mohammed, A.J.R. Heck, Improving SRM assay development: a global comparison between triple quadrupole, ion trap, and higher energy CID peptide fragmentation spectra, J. Proteome Res. 10 (2011) 4334-4341.

[227] M. M. Contreras, I. López-Exposito, B. Hernández-Ledesma, M. Ramos, I. Recio, Application of mass spectrometry to the characterization and quantification of food-derived bioactive peptides, J. AOAC Int. 91 (2008) 981-94.

[228] C.S. Gilani, C. Xiao, N. Lee, Need for accurate and standardized determination of amino acids and bioactive peptides for evaluating protein quality and potential health effects of foods and dietary supplements, J. AOAC Int. 91 (2008) 894-900.

[229] N. Yamamoto, M. Maeno, T. Takano, Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by Lactobacillus helveticus CPN4, J. Dairy Sci. 82 (1999) 1388-1393.

[230] K.H. Tokunaga, C. Yoshida, K.M. Suzuki, H. Maruyama, Y. Futamura, Y. Araki, S. Mishima, Antihypertensive effect of peptides from Royal Jelly in spontaneously hypertensive rats, Biol. Pharm. Bull. 27 (2004) 189-192.

[231] M. Sato, T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, T. Nakano, Angiotensin I-converting enzyme inhibitory peptides derived from wakame (Undaria pinnatifida) and their antihypertensive effect in spontaneously hypertensive rats, J. Agric. Food Chem. 50 (2002) 6245-6252.

[232] H. Enari, Y. Takahashi, M. Kawarasaki, M. Tada, K. Tatsuta, Identification of angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their antihypertensive effect, Fisheries Sci. 74 (2008) 911-920.

[233] A. Geerlings, I.C. Villar, F. Hidalgo Zarco, M. Sánchez, R. Vera, A. Zafra Gómez, J. Boza, J. Duarte, Identification and characterization of novel angiotensin-converting enzyme inhibitors obtained from goat milk, J. Dairy Sci. 89 (2006) 3326-3335.

[234] K. Inoue, T. Gotou, H. Kitajima, S. Mizuno, T. Nakazawa, N. Yamamoto, Release of antihypertensive peptides in miso paste during its fermentation, by the addition of casein, J. Biosci. Bioeng. 108 (2009) 111-115.

[235] A. Quiros, M. Ramos, B. Muguerza, M.A. Delgado, P.J. Martín-Alvarez, A. Aleixandre, I. Recio, Determination of the antihypertensive peptide LHLPLP in fermented milk by high-performance liquid chromatography-mass spectrometry, J. Dairy Sci. 89 (2006) 4527-4535.

[236] J.M. Curtis, D. Dennis, D.S. Waddell, T. MacGillivray, H.S. Ewart, Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC-MS/MS, J. Agric. Food Chem. 50 (2002) 3919-3925.

[237] U. Butikofer, J. Meyer, R. Sieber, B. Walther, D. Wechsler, Occurrence of the angiotensin-converting enzyme inhibiting tripeptides Val-Pro-Pro and Ile-Pro-Pro in different cheese varieties of Swiss origin, J. Dairy Sci. 91 (2008) 29-38.

[238] J. Meyer, U. Buetikofer, B. Walther, D. Wechsler, R. Sieber, Hot topic: Changes in angiotensin-converting enzyme inhibition and concentrations of the tripeptides Val-Pro-Pro and Ile-Pro-Pro during ripening of different Swiss cheese varieties, J. Dairy Sci. 92 (2009) 826-836.

[239] M. Guijarro-Diez, M. C. García, M. L. Marina, A. L. Crego, LC-ESI-TOF MS method for the evaluation of the immunostimulating activity of soybeans via the determination of the functional peptide soymetide, J. Agric. Food Chem. 61 (2013) 3611-3618.

[240] Y. Hu, A. Stromeck, J. Loponen, D. Lopes-Lutz, A. Schieber, M.G. Gaenzle, LC-MS/MS quantification of bioactive angiotensin i-converting enzyme inhibitory peptides in rye malt sourdoughs, J. Agric. Food Chem. 59 (2011) 11983-11989.

[241] P. Picotti, O. Rinner, R. Stallmach, F. Dautel, T. Farrah, B. Domon, H. Wenschuh, R. Aebersold, High-throughput generation of selected reaction-monitoring assays for proteins and proteomes, Nat. Methods 7 (2010) 43-46.

[242] D. Cushman, H. Cheung, Spectrophotometric assay and properties of angiotensin-converting enzyme of rabbit lung, Biochem. Pharmacol. 20 (1971) 1637-1648.

[243] F. Geng, Y. He, L. Yang, Z. Wang, A rapid assay for angiotensin-converting enzyme activity using ultra-performance liquid chromatography-mass spectrometry, Biomed. Chromatogr. 24 (2010) 312-317.

[244] D. Huang, B. Ou, R. Prior, The chemistry behind antioxidant capacity assays, J. Agric. Food Chem. 53 (2005) 1841-1856.

[245] D. Garrett, M. Failla, R. Sarama, Development of an in vitro digestion method to assess carotenoid bioavailability from meals, J. Agric. Food Chem. 47 (1999) 4301-4309.

[246] A. Cantani, P. Lucenti, Natural history of soy allergy and/or intolerance in children, and clinical use of soy-protein formulas, Pediatr. Allergy Immu. 8 (1997) 59-74.

[247] R. Merritt, B. Jenks, Safety of soy-based infant formulas containing isoflavones: The clinical evidence, J. Nutr. 134 (2004) 1220S-1224S.

[248] M.C. García, M. Torre, M.L. Marina, A perfusion reversed-phase chromatographic method for ultrarapid determination of soybean proteins in soybean infant formulas and soybean milks: Method development and validation, J. Chromatogr. Sci. 36 (1998) 527-534.

[249] E. Escudero, M. Aristoy, H. Nishimura, K. Arihara, F. Toldrá, Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham, Meat Sci. 91 (2012) 306-311.

[250] N. Matoba, Y. Yamada, M. Yoshikawa, Design of a genetically modified soybean protein preventing hypertension based on an anti-hypertensive peptide derived from ovalbumin, Curr. Med. Chem. Cardiovasc. Hematol. Agents. 1 (2003) 197-202.

[251] K. Nishizawa, A. Kita, C. Doi, Y. Yamada, K. Ohinata, M. Yoshikawa, M. Ishimoto, Accumulation of the bioactive peptides, novokinin, LPYPR and rubiscolin, in seeds of genetically modified soybean, Biosci. Biotech. Bioch. 72 (2008) 3301-3305.

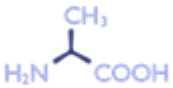
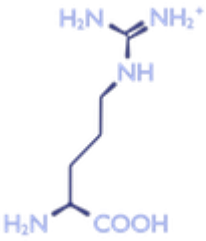
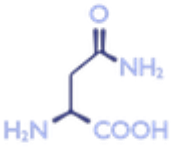
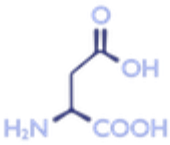
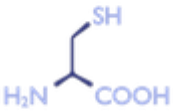
[252] K. Prak, Y. Maruyama, N. Maruyama, S. Utsumi, Design of genetically modified soybean proglycinin A1aB1b with multiple copies of bioactive peptide sequences, Peptides 27 (2006) 1179-1186.

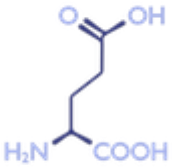
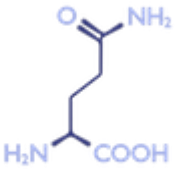
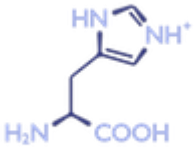
[253] N. Maruyama, B. Mikami, S. Utsumi, The development of transgenic crops to improve human health by advanced utilization of seed storage proteins, Biosci. Biotech. Bioch. 75 (2011) 823-828.

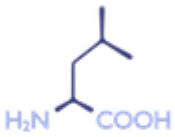
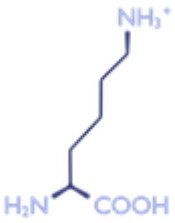
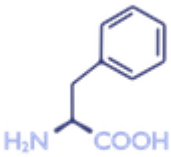
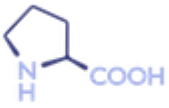
[254] M. Jia, K.W. Lin, S. Souchelnytskyi, Phosphoproteomics: Detection, identification and importance of protein phosphorylation, integrative proteomics (2012), Dr. Hon-Chiu Leung (Ed.), ISBN: 978-953-51-0070-6, InTech, DOI: 10.5772/31305.

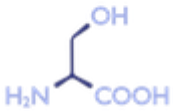
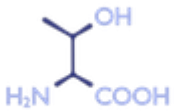
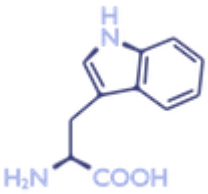
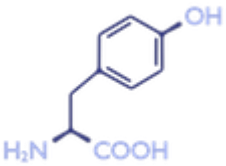
[255] C. Schaab, Analysis of phosphoproteomics data, IN: M. Hamacher, M. Eisenacher, C. Stephan (Eds.), Humana Press, 2011, pp. 41-57.

APPENDIX I

Amino acid	Three letter abbreviation		One letter abbreviation	Polarity
Alanine	Ala		A	Weak Polar
Arginine	Arg		R	Polar
Asparagine	Asn		N	Polar
Aspartic acid	Asp		D	Polar
Cysteine	Cys		C	Non-Polar

Amino acid	Three letter abbreviation		One letter abbreviation	Polarity
Glutamic acid	Glu		E	Polar
Glutamine	Gln		Q	Polar
Glycine	Gly		G	Weak Polar
Histidine	His		H	Polar
Isoleucine	Ile		I	Non-Polar

Amino acid	Three letter abbreviation		One letter abbreviation	Polarity
Leucine	Leu		L	Non-Polar
Lysine	Lys		K	Polar
Methionine	Met		M	Non-Polar
Phenylalanine	Phe		F	Non-Polar
Proline	Pro		P	Weak Polar

Amino acid	Three letter abbreviation		One letter abbreviation	Polarity
Serine	Ser		S	Weak Polar
Threonine	Thr		T	Weak Polar
Tryptophan	Trp		W	Non-Polar
Tyrosine	Tyr		Y	Non-Polar
Valine	Val		V	Non-Polar

LIST OF PUBLICATIONS

1. P. Puchalska, M. C. García, M. L. Marina.
   Development of capillary HPLC-IT-MS method for the determination of VLIVP antihypertensive peptide in soybean crops.
   J. Chromatogr. A, in press.

2. P. Puchalska, M. C. García, M. L. Marina.
   Identification of native angiotensin-I converting enzyme inhibitory peptides in commercial soybean based infant formulas using HPLC-Q-ToF-MS.
   Food Chem., in press.

3. P. Puchalska, C. Esteve, M. L. Marina, M. C. García.
   Chapter 17-Peptides.
   IN: *"Handbook of Food Analysis", 3rd Edition, Chapter 17. CRC Press, Taylor& Francis, USA, 2014, ISBN: 978-1-46655-654-6.*

4. P. Puchalska, M. L. Marina, M. C. García.
   Isolation and identification of antioxidant peptides from commercial soybean-based infant formulas.
   Food Chem., 2014, 148, 147-154

5. M. C. García, P. Puchalska, C. Esteve, M. L. Marina.
   Vegetable foods: a cheap source of proteins and peptides with antihypertensive, antioxidant, and other less occurrence bioactivities.
   Talanta, 2013, 106, 328-349.

6. P. Puchalska, M. L. Marina, M. C. García.
   Development of an HPLC-ESI-Q-ToF-MS methodology for the determination of three highly antihypertensive peptides in maize crops.
   J. Chromatogr. A, 2013, 1285, 69-77.

7. P. Puchalska, M. L. Marina, M. C. García.
   Development of a RP-HPLC analytical methodology for the determination of antihypertensive peptides in maize crops.
   J. Chromatogr. A, 2012, 1234, 64-71.

8. P. Puchalska, M. L. Marina, M. C. García.
   Isolation and characterization of peptides with antihypertensive activity in foodstuffs.
   Crit. Rev. Food Sci. Nutr., in press

9. L. Sánchez-Hernández, P. Puchalska, C. García-Ruiz, A. L. Crego, M. L. Marina
   Determination of trigonelline in seeds and vegetable oils by capillary electrophoresis as a novel marker for the detection of adulterations in olive oils.
   J. Agric. Food Chem., 2010, 58, 7489-7496.

10. P. Puchalska, E. Pittler, M. Trojanowicz, G. Gübitz, M. G. Schmid
    Enantioseparation of amino acids and α-hydroxy acids on ligand-exchange continuous beds by capillary electrochromatography.
    Electrophoresis, 2010, 31, 1517-1520.

SUMMARY OF CURRICULUM VITAE

The author of this thesis was born on 22nd August 1985 in Mińsk Mazowiecki, Poland. During her Master thesis, she had the opportunity to complete three internships (in total 8 months) in various European Investigation Centers (Austria, Hungary, and Spain). Her master project was devoted to the application of capillary electrophoresis to chiral analysis, monitoring of food adulteration, and identification of genetically modified foods. She graduated M.Sc. (Master of Science) from University of Warsaw (Poland) under the supervision of Prof. M. Trojanowicz in 2009. Few months after the graduation, she was granted by the University of Alcalá to join the group of Prof. M. L. Marina. She developed methods for the identification and determination of bioactive peptides in foods using novel chromatographic and mass spectrometric approaches under the supervision of Dr. M.C. García and Prof. M. L. Marina. During her PhD training, she completed two internships (in total 5 months), both held in the University of Utrecht (The Netherlands).

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