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ISOLATION AND IDENTIFICATION BY HIGH RESOLUTION LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY OF NOVEL PEPTIDES WITH MULTIFUNCTIONAL LIPID-LOWERING CAPACITY

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ABSTRACT

This work describes the isolation, characterization, and identification by RP-HPLC-ESI-Q-TOF of novel peptides that interfere in the fat digestion and absorption mechanisms by multiple pathways. Peptides were ultrafiltrated and peptides in the most active fraction were further separated by semipreparative RP-HPLC. Nine different subfractions were obtained observing a high amount of peptides in subfraction F3. Peptides in subfraction F3 could simultaneously reduce the solubility of cholesterol in micelles and inhibit pancreatic cholesterol esterase and pancreatic lipase, even after a simulated gastrointestinal digestion. The identification of lipid-lowering peptides has been scarcely performed and when done, low selectivity or sensitivity of employed identification techniques or conditions did not yield reliable results. Separation and detection of peptides by RP-HPLC-ESI-Q-TOF-MS was optimized and most favorable conditions were employed for the identification of peptides using *de novo* sequencing. Ten different peptides with 4-9 amino acids were identified. Main feature of identified peptides was the high acidity derived from a high presence of amino acids glutamic acid and aspartic acid in their sequences.

Keywords: HPLC-MS/MS, hypolipidemia, peptides, olive by-product.

1. Introduction

Hypercholesterolemia and hyperglycemia are metabolic disorders characterized by high levels of cholesterol and triglycerides in blood. Hyperlipidemia has increased worldwide due to the popularity of high-fat diets and non healthy lifestyles (Jacobson, Miller, & Schaefer, 2007). Prevalence of hyperlipidemia is a risk factor in cardiovascular diseases such as coronary heart disease and atherosclerosis. Different synthetic drugs for treating this disease have been developed such as inhibitors of cholesterol endogenous synthesis, inhibitors of membrane proteins that promote the intestinal absorption of cholesterol, bile acid sequestrants, etc (Descamps, De Sutter, Guillaume, & Missault, 2011). Nevertheless, diverse side effects (increase of hepatic transaminases and creatine kinase, muscle weakness, headache, sleep disorders, etc.) have been described derived from the long-term consumption of these synthetic drugs (Heidrich, Contos, Hunsaker, Deck, & Vander Jagt, 2004). Moreover, these synthetic drugs are usually limited to fight hyperlipidemia by following a single mechanism. Therefore, and especially in cases where cholesterol and triglycerides levels are moderate, the consumption of foods that contain or are enriched with lipid-lowering substances is advisable.

Bioactive peptides are natural molecules that have demonstrated a wide range of activities although hypolipidemic peptides have been much less reported than others. Peptides with capacity to reduce cholesterol and/or triglyceride levels have been obtained from different foods (Alhaj, Kanekanian, Peters, & Tatham, 2010; Rho, Park, Ahn, Shin, & Lee, 2007; Zhang, Yokoyama, & Zhang, 2012; Yust, Millán-Linares, Alcaide-Hidalgo, Milán, & Pedroche, 2012; Lammi et al., 2016; Marques, Fontanari, Pimenta, Soares-Freitas, & Areas, 2015; Liyanage et al., 2010). In addition to these products, some byproducts derived from the food industry are also considered sources

of bioactive peptides although, again, not much attention has been paid to hypolipidemic peptides (García, Orellana, & Marina, 2016). Nakade *et al.* (2009) described the hypocholesterolemic capacity of a protein hydrolysate obtained from a meat industry byproduct that enabled the reduction of the micellar cholesterol solubility, the suppression of the cholesterol uptake by Caco-2 cells, and the cholesterol reduction in the serum of rats fed with the hydrolysate. More recently, our research group has published a work describing antihypertensive, antioxidant, and hypocholesterolemic activities in seed hydrolysates obtained from *Prunus* genus fruits and olives. We observed that the hydrolysate obtained with Alcalase enzyme from the *Picual* olive seeds showed a high capability to reduce micellar cholesterol solubility (García, González-García, Vásquez-Villanueva, & Marina, 2016). Nevertheless, further studies are required to confirm these first results and to characterize these peptides.

Identification of peptides and proteins is mainly performed using reversed-phase (RP)-HPLC coupled to tandem mass spectrometry. Despite there is a wide bibliography devoted to the characterization and identification of bioactive peptides (García, Orellana, & Marina, 2016), a few works have been addressed in the case of lipid-lowering peptides. Moreover, in most cases no peptide identification was carried out or only amino acid composition was detailed (Zhang, Yokoyama, & Zhang, 2012). The first identification of an hypolipidemic peptide was carried out using a protein sequencer which enabled to identify the peptide IIAEK in bovine milk β-lactoglobulin (Nagaoka, 2001). Few years later, Zhong et al. (2007) could identify the hypolipidemic peptide WGAPSL in soy using RP-HPLC-MS. Alhaj et al. (2010) used RP-HPLC-MS/MS with mobile phases containing 0.1% (v/v) trifluoroacetic acid (TFA) for the detection of peptides with hypocholesterolemic activity but, as expected, no sequencing and only information on molecular weight of peptides was obtained. Marques and co-

workers (2015) also identified hypocholesterolemic peptides from cowpea using RP-HPLC-MS/MS but reliability of data is questioned since only peptides with ALC between 50-80% were identified.

The aim of this work was to evaluate the capacity of olive seed peptides to reduce the amount of exogenous cholesterol and triglycerides, to isolate peptides with lipid-lowering capacity from the olive seed, and to optimize a RP-HPLC-tandem mass spectrometry (MS/MS) method enabling the reliable identification of novel peptides.

2. Materials and methods

2.1 Chemical and samples

All chemicals and reagents were of analytical grade. Water was daily obtained from a Milli-Q system from Millipore (Bedford, MA, USA). Supergradient HPLC grade acetonitrile (ACN), acetic acid (AA), acetone, methanol (MeOH), and hexane were purchased from Scharlau Chemie (Barcelona, Spain). Tris (hydroxymethyl) aminomethane (Tris), hydrochloric acid (HCl), sodium dihydrogen phosphate, di-sodium tetraborate, and sodium dodecyl sulphate (SDS) were from Merck (Darmstadt, Germany). Sodium hydroxide, dithiothreitol (DTT), trifluoroacetic acid (TFA), bovine pancreatic cholesterol esterase, porcine pancreatic lipase, *p*-nitrophenylbutylrate (*p*-NPB), *p*-nitrophenylpalmitate (*p*-NPP), taurocholic acid, taurodeoxycholic acid, glycodeoxycholic acid, oleic acid, phosphatidylcholine, simvastatin, rutin trihydrate, cholestyramine, β-sitosterol, o-phthaldialdehyde (OPA), L-glutathione (GSH), pepsin, pancreatin, taurocholate, cholesterol, β-mercaptoethanol, sodium chloride (NaCl), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Dulbecco's modified eagle's medium (DMEM), antibiotics (penicillin, streptomycin, and amphotericin), fetal bovine serum, and phosphate buffered (PB) were all from Sigma-Aldrich (Saint Louis, MO, USA). Cholesterol oxidase kit was purchased from BioAssay Systems (Hayward, CA, USA). Total bile acid kit was from Bio-Quant (San Diego, CA, USA). Alcalase 2.4 L FG, produced by fermentation of a selected strain of *Bacillus licheniformis*, mainly composed by Subtilisin A, with catalytic activity on serine, and with an activity of 2.4 Anson units per gram, was kindly donated by Novozymes Spain S.A. (Madrid, Spain). All cell lines (HeLa, HT-29, and HK-2) were from the American Type Culture Collection ATCC (Rockwell, MD, USA). Raw olives

of ‘Manzanilla’ variety were kindly donated by the olive company FAROLIVA S.L. (Murcia, Spain).

2.2. Peptide production

Olive stones were crushed to release the seed and seeds were ground in a domestic mill. Olive seeds were defatted with hexane for 30 min (four times). Extraction of proteins from defatted olive seeds was performed following the method developed by Esteve *et al*. (2010). Briefly, 0.03 g of milled and defatted olive seeds were treated with 5 mL of an extracting buffer (100 mM Tris-HCl (pH 7.5), 0.5% (w/v) SDS, 0.5% (w/v) DTT) using a high intensity focused ultrasound (HIFU) probe (model VCX130, Sonic Vibra-Cell, Hartford, CT, USA) at 30% of amplitude for 5 min. After centrifugation (4000*g*, 10 min), proteins in the supernatant were precipitated with cold acetone (10 mL) in the fridge for 24 h, followed by centrifugation and drying at room temperature to obtain a protein isolate. The protein isolate was dissolved in a 5 mM borate buffer (pH 8.5) and hydrolyzed with Alcalase (0.15 UA/ g protein) by incubation in the Thermomixer Compact (Eppendorf, Hamburg, Germany) at 50 ºC during 4 h. The digestion was stopped by increasing the temperature to 100 ºC for 10 min and centrifuging for 10 min at 6000*g*. Finally, the supernatant, containing peptides, was stored at – 20 ºC.

2.3. Semipreparative RP-HPLC fractionation

The hydrolysate solution was firstly fractionated by ultrafiltration using molecular weight (Mw) cut-off filters Amicon® Ultra of 5 kDa and 3 kDa from Merck Millipore (Tullagreen, Ireland) to obtain three fractions: a fraction with peptides bigger than 5 kDa, a fraction with peptides with Mw between 5 and 3 kDa, and a fraction with peptides below 3 kDa.

Peptide separation by RP-HPLC was performed using a HPLC equipment from Agilent Technologies (Pittsburgh, PA, USA) model 1100, equipped with a vacuum degasser, a quaternary pump, an automatic injection system, a thermostatic column compartment, a diode array detector, and a fluorescence detector. Control of the equipment and data acquisition were performed with the HP ChemStation software. The separation was carried out in an Jupiter 4u Proteo (250 x 10 mm id) from Phenomenex (Torrance, CA, USA). Peptides were separated using an elution gradient from 25-54% B in 45 min, where mobile phase A was water with 0.1% (v/v) TFA and mobile phase B was ACN with 0.1% (v/v) TFA. Other conditions were: flow-rate, 1 mL/min; temperature, 25 ºC; injection volume, 600 µL; fluorescence detection at λexc = 280 nm and λem = 360 nm. Sample was injected two times and nine fractions (F1-F9) were collected at 5 min intervals in every injection. Every fraction was evaporated using a centrifugal evaporator (Eppendorf, Hamburg, Germany) and the remaining pellet was dissolved in 600 µL of digestion buffer (5 mM borate buffer, pH 8.5). Solutions corresponding to every fraction, injected two times, were pooled and used for future analysis.

2.4. Peptide content

The content of peptides was determined according to the method described by Wang *et al*. (2008) with some modifications. A 40 mg/mL solution of OPA reagent in MeOH was employed to prepare a fresh 5 mL mixture consisting of 2.5 mL of 100 mM disodium tetraborate, 1.0 mL of 5% (v/v) SDS, 1.39 mL of water, 10 µL of β-mercaptoethanol, and 100 µL of previous OPA solution. Next, 2.5 µL of sample was incubated with 100 µL of that mixture for 8 min at room temperature and the absorbance corresponding to the compounds formed by the reaction of OPA reagent with α-amino groups of peptides was measured at 340 nm using a spectrophotometer

Lambda 35 from Perkin-Elmer (Waltham, MA, USA). The peptide content was calculated by interpolation in a calibration curve obtained when using the tripeptide GSH (0–5 mg/ mL) as standard.

2.5. Evaluation of hypolipidemic activity of peptides

Hypolipidemic activity of hydrolysates and fractions was evaluated using four different *in vitro* assays to measure the ability of peptides to reduce absorption of dietary cholesterol (ability to reduce micellar cholesterol solubility, to inhibit the activity of pancreatic cholesterol esterase and to bind bile acids) and triglycerides (ability to inhibit the activity of pancreatic lipase). In every determination, three replicates were measured by triplicate.

2.5.1. Evaluation of the capacity to reduce the micellar cholesterol solubility

Cholesterol is solubilised in micelles for its transportation and absorption and the displacement of cholesterol in the micelle results in a decrease of cholesterol adsorption (Nagaoka, 2001). Artificial micelles were prepared according to the method of Zhang *et al.* (2012) with minor modifications. Briefly, a solution containing 0.5 mM cholesterol, 1 mM oleic acid, and 2.4 mM phosphatidylcholine in MeOH was prepared and dried before the addition of a 15 mM PB (pH 7.4) containing 6.6 mM taurocholate salt and 132 mM NaCl. This solution was sonicated for 1 min at 95% with the HIFU probe and incubated overnight at 37 ºC. After preparation of micelles, 150 µL of peptide or controls were added to 50 µL of the micelle solution, sonicated (1 min at 95%), and incubated in the Thermomixer for 2 h at 37 ºC. The mixture was then centrifuged for 10 min at 6000*g*. The supernatant was collected for the determination of the cholesterol remaining in micelles using a cholesterol kit. Determination of cholesterol consisted of the hydrolysis of cholesterol esters by cholesterol esterase enzyme, oxidation of the

resulting cholesterol to its ketone form and hydrogen peroxide by cholesterol oxidase enzyme, and detection of resulting hydrogen peroxide by its reaction with 10-acetyl-3,7-dihydroxyphenoxazine to obtain a product which absorbs at 570 nm. Cholestyramine was used as positive control. The reduction in the micellar solubility of cholesterol was calculated using the following equation:

$$\text{Cholesterol solubility reduction } (\%) = \frac{C_o - C_S}{C_o} \times 100$$

where C_o is the initial concentration of cholesterol in micelles (without peptides) and C_s is the concentration of cholesterol in micelles when adding peptides or positive control.

2.5.2. Evaluation of the capacity to inhibit pancreatic cholesterol esterase enzyme

The inhibition of the pancreatic cholesterol esterase enzyme, that hydrolyzes dietary cholesterol ester into free cholesterol, decreases the bioavailability of dietary cholesterol esters in animals (Krause, Sliskovic, Anderson, & Homan, 1998). This assay was carried out following the method described by Pietsch & Gütschow (2005). Peptides were incubated with 6 mM taurocholic acid and 20 mM *p*-NPB in 100 mM PB (pH 7.0) containing 100 mM NaCl at 25 ºC. The reaction was initiated by adding porcine pancreatic cholesterol esterase (25 µg/mL). After incubation for 5 min at room temperature, the absorbance corresponding to *p*-nitrophenol, obtained by the hydrolysis of *p*-NPB, was measured at 405 nm. Simvastatin was employed as positive control. The percentage of inhibition of the pancreatic cholesterol esterase enzyme was calculated using the following equation:

$$\text{Inhibition of pancreatic cholesterol esterase } (\%) = \frac{A_{Máx} - (A_S - A_{S_o})}{A_{Máx}} \times 100$$

where $A_{Máx}$ is the absorbance obtained under conditions of maximum enzyme activity (without peptides), A_S is the absorbance obtained in presence of peptides/positive control and A_{So} is the absorbance obtained when no enzyme was added.

2.5.3. Evaluation of the capacity to bind bile acids

Bile acids are synthesized in the liver by oxidation of endogenous cholesterol. Sequestering of bile acids results in the reduction of the bile acid pool which produces a greater conversion of endogenous cholesterol into bile acids and a decreased plasma cholesterol level (Insull, 2006). This assay was performed following the method described by Yoshie-Stark & Wäsche (2004). Three different biles acids were employed: taurocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid. Briefly, peptides were incubated in the Thermomixer with every bile acid (2 mM) in 0.1 M PB (pH 7.0) containing 0.1 M NaCl at 37 ºC for 90 min. The mixture was centrifuged (10 min, 6000*g*) and the supernatant was collected. The precipitate was washed with 1 mL of PB and centrifuged again and the supernatant was collected and combined with earlier supernatant. The pooled supernatant was frozen at 20 ºC until analysis. The amount of bile acids was determined using a total bile acid kit. The assay is based on an reaction driven by 3α-hydroxysteroid dehydrogenase enzyme in which bile acids are incubated in the presence of NADH and thio-NAD^+. Under these conditions, thio-NAD^+ is converted to its reduced form thio-NADH which is detected at 630 nm. β-sitosterol was used as positive control. The percentage of binding to bile acids was calculated using the following equation:

$$\text{Bile acid binding (\%)} = \frac{C_0 - C_S}{C_0} \times 100$$

where C_o is the initial concentration of bile acids in control (without peptides) and C_s is the concentration of bile acids when adding peptides or positive control.

2.5.4. Evaluation of the capacity to inhibit pancreatic lipase enzyme

Absorption of triglycerides can be ameliorated by the inhibition of pancreatic lipase enzyme that is responsible for their hydrolysis into monoglycerides and free fatty acids (Mattson & Beck, 1955). Based on a method previously published (Adisakwattana, 2010), peptides were incubated with 0.06 mg/ mL of porcine pancreatic lipase and 3.33 mM of *p*-NPP in 100 mM Tris-HCl buffer (pH 8.5) at 37 ºC for 25 min in the Thermomixer. The absorbance of released *p*-nitrophenol was measured at 405 nm. Rutin was used as positive control. Percentage of inhibition of pancreatic lipase was determined by the following equation:

$$\text{Inhibition of pancreatic lipase (\%)} = \frac{A_{Máx} - (A_S - A_{S_o})}{A_{Máx}} \times 100$$

where $A_{Máx}$ is the absorbance obtained when no peptides were added (maximum activity of the enzyme), A_S is the absorbance obtained when the enzyme is inhibited by added peptides or positive control, and A_{So} is the absorbance of samples without enzyme.

2.6. Resistance of peptides to simulated gastrointestinal digestion

In vitro gastrointestinal digestion (GID) was carried out according to the method of Garrett *et al.* (1999) with slight modifications. Briefly, the pH of hydrolysates was adjusted with 1 M HCl to pH 2.0 followed by digestion with pepsin enzyme (1:35 (w/w), enzyme: substrate ratio) at 37 °C with shaking for 1 h. Afterwards, the pH was adjusted to 5.0 with 0.1 M $NaHCO_3$ and to 7.0–8.0 with 0.1 M NaOH and pancreatin enzyme (mixture of pancreatic proteases) was added at a 1:25 (w/w) enzyme:substrate ratio. The mixture was incubated by shaking at 37 °C and after 3 h, the digestion was stopped by heating at 95 °C for 15 min.

2.7. Cell proliferation assay

The MTT assay was employed to measure the cytotoxicity of peptides. The assay is based on the protocol described for the first time by Mosmann (1983). The assay was optimized for the human cell lines used in the experiments: HeLa (human cervical cancer cells), HT-29 (human colorectal adenocarcinoma cells), and HK-2 (human renal proximal tubule cells). Culture medium was DMEN and it contained penicillin-streptomycin-amphotericin and 10% fetal bovine serum. Cell culture medium incorporated phenol red to control pH and was kept at 37 ºC in a humidified atmosphere with 5% CO_2 until use. Three days after seeding, cells were placed in 24-well plates (at a density of 10,000 cells/well) and incubated with 50 µL of peptide solution for 24 h (37 ºC/5% CO_2). A MTT stock solution of 5 mg/mL in PB was prepared and stored at 2–8 °C until its use. The MTT stock solution was added to every culture cell in a 1:10 (cell volume: MTT stock solution volume) ratio and incubated for 3 to 4 h. Afterwards, DMEM was removed and the formed formazan crystals were dissolved with 500 µL DMSO. Absorbance of formazan crystals was measured at a wavelength of 570 nm. Citotoxicity was expressed as the percentage of viability of cells treated with samples relative to the corresponding control.

2.8. Identification of peptides by RP-HPLC-MS/MS

MS/MS detection was carried out in an Electrospray Quadrupole-Time-of-Flight (ESI-Q-TOF) series 6530 mass spectrometer coupled to a liquid chromatograph (model 1100), both from Agilent Technologies. Peptide separation was carried out using an Ascentis Express Peptide ES-C18 (100 x 2.1 mm id, 2.7 µm particle size) column based on fused pore superficially porous particles using an Ascentis Express Peptide ES-C18 (5 x 2.1 mm id, 2.7 µm particle size) as guard column. Mobile phases A and B consisted of 0.3% (v/v) AA in water and 0.3% (v/v) AA in ACN, respectively. The flow-rate was

0.3 mL/min and the elution gradient was from 5 to 35% B in 35 min and from 35 to 95% B in 2 min followed by a reversed gradient from 95 to 5% B in 2 min to reach the initial chromatographic conditions. The column temperature was 55 ºC and the injection volume was 15 μL. Detection by ESI-Q-TOF was performed in the positive ion mode using a mass range from 50 to 3000 m/z. The ESI Jet Stream source conditions were: capillary voltage, 3500 V; fragmentator voltage, 200 V; drying gas, 10 L/min and 300 ºC; nebulizer pressure, 50 psig; sheath gas, 5.5 L/min and 250 ºC. MS/MS was carried out using *Auto* mode and collision induced dissociation was set at 4 V per each 100 Da Mw. Two Agilent compounds (HP0921 and purine), yielding ions at m/z 922.0098 and 121.0509, respectively, were simultaneously introduced and used as internal standards throughout the analysis. PEAKS Studio software from Bioinformatics Solutions Inc. (Waterloo, Canada) was used for the treatment of MS/MS data and *de novo* sequencing of peptide. Identified peptides showed an average local confidence (ALC, expected percentage of correct amino acids in the peptide sequence) ≥ 90% and a good precursor fragmentation pattern. Identifications were carried out using two individual samples injected by duplicate. Peptide identifications were accepted if they appeared in two independent samples. Since it is not possible to differentiate I from L by MS due to their equal molecular masses, both isoforms were presented in the results.

2.9. Statistical Analysis

Statistical analysis was performed using Statgraphics Software Plus 5.1 (Statpoint Technologies, Inc., Warranton, VA, USA). Data comparison was carried out by the analysis of variance (ANOVA) or the test-*t*. The level of significant was set at $P < 0.05$.

3. Results and discussion

The identification of lipid-lowering peptides has been scarcely performed or done using low selectivity and sensitivity techniques or conditions that have resulted in a scarce number of identified peptides. This work describes the optimization of a sensitive and selective method for the reliable identification of novel peptides with lipid-lowering capacity from a olive byproduct.

Figures 1A-D show the capacity of peptides from a hydrolysate of olive seeds proteins (obtained from a *Manzanilla* variety) to reduce exogenous lipid absorption by different mechanisms along with the peptide concentration (Figure 1E). The signals corresponding to the positive controls used in every assay (cholestyramine, β-sitosterol, simvastatin, and rutin) at concentrations identical to the peptide concentration in the hydrolysate (3.1 mg/mL, see Figure 1E) where included in Figures 1A-D. Capacity of peptides to reduce micellar cholesterol solubility was higher than the observed previously for peptides obtained from the *Picual* olive seed (García, González-García, Vásquez-Villanueva, & Marina, 2016). Main mechanism to reduce lipid absorption seemed to be the inhibition of the pancreatic lipase enzyme (70% of inhibition) while it did not show any capacity to sequester bile acids (glycodeoxycholic acid, taurocholic acid, and taurodeoxycholic acid). The capacity of peptides to inhibit cholesterol esterase and lipase enzymes ranged from 70-85% of the capacity of positive controls (simvastatin and rutin, respectively) while the capacity to reduce the micellar cholesterol solubility was higher than the observed for the positive control (cholestyramine). In comparison with bibliographic results (Rho, Park, Ahn, Shin, & Lee, 2007; Yust, Millán-Linares, Alcaide-Hidalgo, Milán, & Pedroche, 2012; Adisakwattana, Intrawangso, Hemrid, Chanathong, & Mäkynen, 2012; Jeon & Imm, 2014), *Manzanilla* olive seed peptides showed a high lipid-lowering capacity. In order

to isolate and identify those peptides responsible of this activity, fractionation of peptides was carried out.

3.1. Isolation of highly hypolipidemic peptides

Peptides were firstly separated in three fractions by ultrafiltration: fraction with peptides with Mw above 5 kDa, fraction with peptides with Mw between 5 and 3 kDa, and fraction with peptides with Mw below 3 kDa. Figures 1A-D show the hypolipidemic activity and peptide concentration of these fractions. Signals corresponding to the positive controls at concentrations identical to the peptide concentration in every fraction (fraction with peptides > 5 kDa, 0.4 mg/mL; fraction with peptides from 3-5 kDa, 0.3 mg/mL; fraction with peptides < 3 kDa, 2.4 mg/mL, see Figure 1E) were also graphed. Highest peptide concentration was observed in the fraction below 3 kDa. Fraction with peptides with Mw > 5 kDa showed a similar capacity to reduce the micellar cholesterol solubility to the observed for the whole hydrolysate but much higher than the obtained with the other two fractions ($P < 0.05$). Taurodeoxycholic bile acid binding capacity of fraction with peptides > 5 kDa was significantly higher than the obtained for the whole hydrolysate and for the other two fractions ($P < 0.05$). Moreover, this fraction showed a similar capacity to inhibit the cholesterol esterase enzyme to the observed for the whole hydrolysate and fractions ($P < 0.05$). Regarding the pancreatic lipase inhibition, the fraction with peptides > 5 kDa showed slightly lower capacity than the observed for the whole hydrolysate and the fraction with peptides < 3 kDa. Furthermore, the fraction with peptides > 5 kDa yielded a significant hypolipidemic activity in comparison with positive controls cholestyramine and simvastatin (at identical concentrations) and a similar hypolipidemic activity in comparison with positive controls β-sitosterol and rutin (at identical concentrations) ($P > 0.05$). Taking into account the hypolipidemic capacity of peptides in fraction > 5 kDa

and its peptide concentration, it was selected for its further fractionation by semipreparative RP-HPLC.

Different elution gradients were tried for the separation of peptides by semipreparative RP-HPLC and, finally, a gradient from 25 to 54% B in 45 min was selected. Figure 2A shows the chromatogram obtained. Nine fractions (F1-F9) were collected observing main signals in the fraction F3. Peptides in the nine fractions were characterized by measuring the same previous parameters and results are shown in Figures 2B-2E. No capacity to sequester bile acids was observed in any chromatographic fraction despite the fraction with peptides > 5 kDa did show certain ability to bind bile acids (see Figure 1B). The highest capacity to reduce the micellar cholesterol solubility was observed in fraction F3 (fraction showing main chromatographic peaks) while no capacity was observed for fractions F1, F2, F7, F8, and F9. Morever, the capacity observed for fraction F3 was statistically similar to the observed in the whole hydrolysate and in the fraction with peptides > 5 kDa ($P > 0.05$) (showed in Figure 1A) although peptide concentration was much lower. Pancreatic cholesterol esterase inhibition was significantly high in fraction F8 ($P > 0.05$) and below to 10 % in fractions F1, F2, F3, F4, F5, and F9. In fact, fraction F8 yielded a similar capacity to inhibit pancreatic cholesterol esterase to the observed for the fraction with peptides > 5 kDa and higher than the observed for the whole hydrolysate (shown in Figure 1C), although peptide concentration was much lower. Comparison of the activity of these fractions with that of positive control simvastatin (1.13 ± 0.81%), at the same concentration (0.1 mg/ mL), revealed that peptides in these fractions were highly active. Inhibition of pancreatic lipase activity was higher in fraction F5 followed by fractions F7 and F4. Activity in fraction F5 was slightly lower than in the whole hydrolysate and similar to the observed for the fraction with peptides > 5 kDa, but peptide concentration

in fraction F3 was also 60 and 10 times lower than in the whole hydrolysate and in the fraction with peptides > 5 kDa, respectively. The highest peptide contents were observed in fractions F1, F2, F3, and F4. Since there was no fraction yielding the greatest activity in all assays, it was not possible to select a single fraction but it was possible to select fractions F3, F4, F5, F6, F7, and F8 as most favorable.

Moreover, since lipid-lowering capacity could be altered during gastrointestinal digestion, this activity was next evaluated in the peptides obtained when submitting selected fractions (F3, F4, F5, F6, F7 and F8) to a simulated gastrointestinal digestion. Figures 3 A-C show the capacity to reduce the micellar cholesterol solubility and to inhibit the pancreatic cholesterol esterase and pancreatic lipase enzymes of the selected fractions. In comparison with results showed in Figure 2, the capacity to reduce micellar cholesterol solubility significantly decreased when peptides were submitted to gastrointestinal digestion (except in fractions F7 and F8). However, gastrointestinal digestion of peptides resulted in an increase in the capacity to inhibit the activity of the cholesterol esterase enzyme (in comparison with results showed in Figure 2) observing the highest inhibition in fractions F3 and F6. Fractions showed, in general, a decrease in the capacity to inhibit pancreatic lipase enzyme (comparing with Figure 2) observing the highest activity in fraction F7 followed by fraction F3 (in this case, there was no significant difference between the capacity observed after and before gastrointestinal digestion). Since fraction F3 showed the highest capacity to reduce the micellar cholesterol solubility and to inhibit pancreatic cholesterol esterase enzyme and the second highest capacity to inhibit pancreatic lipase enzyme, it was considered the most suitable fraction in terms of hypolipidemic capacity. Comparing fraction F3 (Figure 2) with the fraction with peptides > 5 kDa (Figure 1), it was observed that starting fraction showed a higher capacity to inhibit pancreatic lipase and cholesterol esterase than

fraction F3 and a similar capacity to reduce micellar cholesterol solubility, but fraction > 5 kDa also showed more than twice the peptide content than fraction F3. In comparison with the whole hydrolysate, fraction F3 showed a similar capacity to reduce micellar cholesterol solubility and a lower capacity to inhibit cholesterol esterase and pancreatic lipase enzymes but the whole hydrolysate was also 18 times more concentrated than fraction F3. Moreover, safety of peptides in fraction F3 was demonstrated by assessing cell viability of different human cell lines (one healthy cell line (HK-2) and two cancer cell lines (HeLa and HT-29)) treated with these peptides.

3.2. Optimization of a method for the identification of hypolipidemic peptides using high resolution RP-HPLC-MS/MS

Peptides in the fraction F3 were firstly analyzed by high resolution RP-HPLC-ESI-Q-TOF using a gradient from 5 to 95% B (ACN + 0.3% (v/v) AA) in 37 min. Although the number of identified peptides ranged from 9-15 peptides in four replicates, only five peptides were identified in all of them. In order to avoid potential signal suppression and to identify the highest number of peptides with reliability, the following parameters were next optimized: sample dilution, elution gradient, and collision energy. Rest of MS parameters (fragmentator voltage; nebuliser pressure; capillary voltage; gas temperature; gas flow; skimmer; sheath gas flow and temperature) were fixed based on knowledge of our reseach team and on previous results that demonstrated that these conditions were suitable to avoid source spontaneous fragmentation of peptides and to obatin a sensitive detection of peptides (Puchalska, García, & Marina, 2013). Sample was injected at three different concentrations (no dilution, dilution 1:3, and dilution 1:10) to find out whether sample concentration could affect the number of identified peptides. Dilution 1:3 enabled to increase the number of identified peptides from 5 to 9 while dilution 1:10 resulted in a sensible reduction in the number of identified peptides

(3 peptides). From this results, sample was diluted 1:3 times before its analysis. Next, the elution gradient was modified for the better separation of peptides and for avoiding potential signal supression. Figure 4 shows the separations obtained using three assayed elution gradients observing a better separation with the gradient from 5-35% B in 35 min. Collision energy was also optimized using 4, 5, 6, and 7 V/100 Da Mw. Increasing collision energy resulted in a significant reduction in the number of identified peptides and a collision energy of 4 V was selected.

3.3. Identification of peptides in fraction F3 by RP-HPLC-ESI-Q-TOF-MS/MS

Peptides in fraction F3 obtained by semipreparative RP-HPLC were sequenced by RP-HPLC-ESI-Q-TOF-MS/MS using optimized conditions. Figure 5 shows the TIC (Total Ion Chromatogram) corresponding to this fraction and the fragmentation spectra obtained for two peptides (EELVE and DYNDDQF) detected at 7.7 and 8.9 min, respectively. Despite many peptides could be detected, only those with ALC higher than 90% and appearing in two independent samples were selected. Table 1 shows the sequences of identified peptides together with the ALC, the retention time, the molecular mass, the mass accuracy (expressed in ppm), and isoelectric point. Peptides could not be traced to any protein since they had not been sequenced yet. Ten different peptides were identified, most of them showing Mw below 1 kDa and monocharged. Mw were much lower than expected but it could be explained by the poor selectivity of ultrafiltration filters, especially at low Mw (Puchalska, García, & Marina, 2014). Most peptides eluted in the first half of the chomatogram. Peptides presented between 4-9 amino acids, very acidic isoelectric points (Ip), and a good solubility in water (obtained using Innovagen's peptide property calculator). A high amount (52%) of acidic amino acids (aspartic acid (D) and glutamic acid (E)) and their amides (asparagine (N) and glutamine (Q)) within peptide sequences was the responsible of these low Ip.

Surprisingly, these amino acids were also present in other peptides yielding lipid-lowering capacity (Zhang, Yokoyama, & Zhang, 2012; Nagaoka, 2001) and their presence could be a common feature among hypolipidemic peptides. All identified peptides were checked against BIOPEP database but they had not been detected before. Specially interesting is peptide FDGEVEK since Nagaoka *et al.* (2001) identified a hypocholesterolemic peptide from a milk β-lactoglobulin tryptic hydrolysate with the same two C-terminal amino acids (IIAEK).

4. Conclusions

Ten different peptides with lipid-lowering capacity have been isolated by semipreparative RP-HPLC and identified by an optimized RP-HPLC-ESI-QTOF method from olive seed proteins. Peptides showed a multifunctional character since they could simultaneously fight hyperlipidemia by different pathways: reduction of micellar cholesterol solubility, inhibition of cholesterol esterase enzyme, and inhibition of lipase enzymes. Most active peptides remained in the fraction with Mw higher than 5 kDa. Semipreparative RP-HPLC enabled to obtain one fraction, fraction F3, that grouped peptides showing a high lipid-lowering capacity, even after gastrointestinal digestion. Peptides concentrated in this fraction presented a high amount of glutamic acid and aspartic acid and their amides, glutamine and asparagine, which could be a common feature among hypolipidemic peptides. This is the first reliable identification of peptides with multifunctional lipid-lowering capacity.

Acknowledgments

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Figure captions

Figure 1. Capacity to reduce micellar cholesterol solubility (A), to bind bile acids (B), and to inhibit pancreatic cholesterol esterase (C) and pancreatic lipase (D) enzymes of the whole olive seed hydrolysate, fractions obtained by ultrafiltration (> 5 kDa, 3-5 kDa, and < 3 kDa), and positive controls (cholestyramine, β-sitosterol, simvastatin, and rutin) used in every assay at concentrations identical to the peptide concentrations in whole hydrolysate and fractions (E). Values are expressed as mean ± standard deviation. Significant differences among whole hydrolysate and fractions in every assay, obtained by ANOVA, are indicated by a letter (a-d).

Figure 2. Separation by semipreparative RP-HPLC of peptides in fraction with peptides > 5 kDa (A) and capacity to reduce micellar cholesterol solubility (B), to inhibit pancreatic cholesterol esterase (C) and pancreatic lipase enzymes (D), and peptide content (E) of chromatographic fractions. Values are expressed as mean ± standard deviation. Significant differences among fractions in every assay, obtained by ANOVA, are indicated by a letter (a-f).

Figure 3. Capacity to reduce micellar cholesterol solubility (A) and to inhibit the activity of pancreatic cholesterol esterase (B) and pancreatic lipase (C) enzymes of fractions F3, F4, F5, F6, F7, and F8 obtained by semipreparative RP-HPLC after a simulated gastrointestinal digestion. Values are expressed as mean ± standard deviation. Significant differences among fractions in every assay, obtained by ANOVA, are indicated by a letter (a-d).

Figure 4. TIC corresponding to the separation of peptides in fraction F3 by RP-HPLC-ESI-Q-TOF using different elution gradients.

Figure 5. Total ion chromatogram (TIC) obtained by RP-HPLC-ESI-Q-TOF for the fraction F3 and fragmentation spectra corresponding to EELVE and DYNDDQF peptides.

Figure 3

Fig. 3

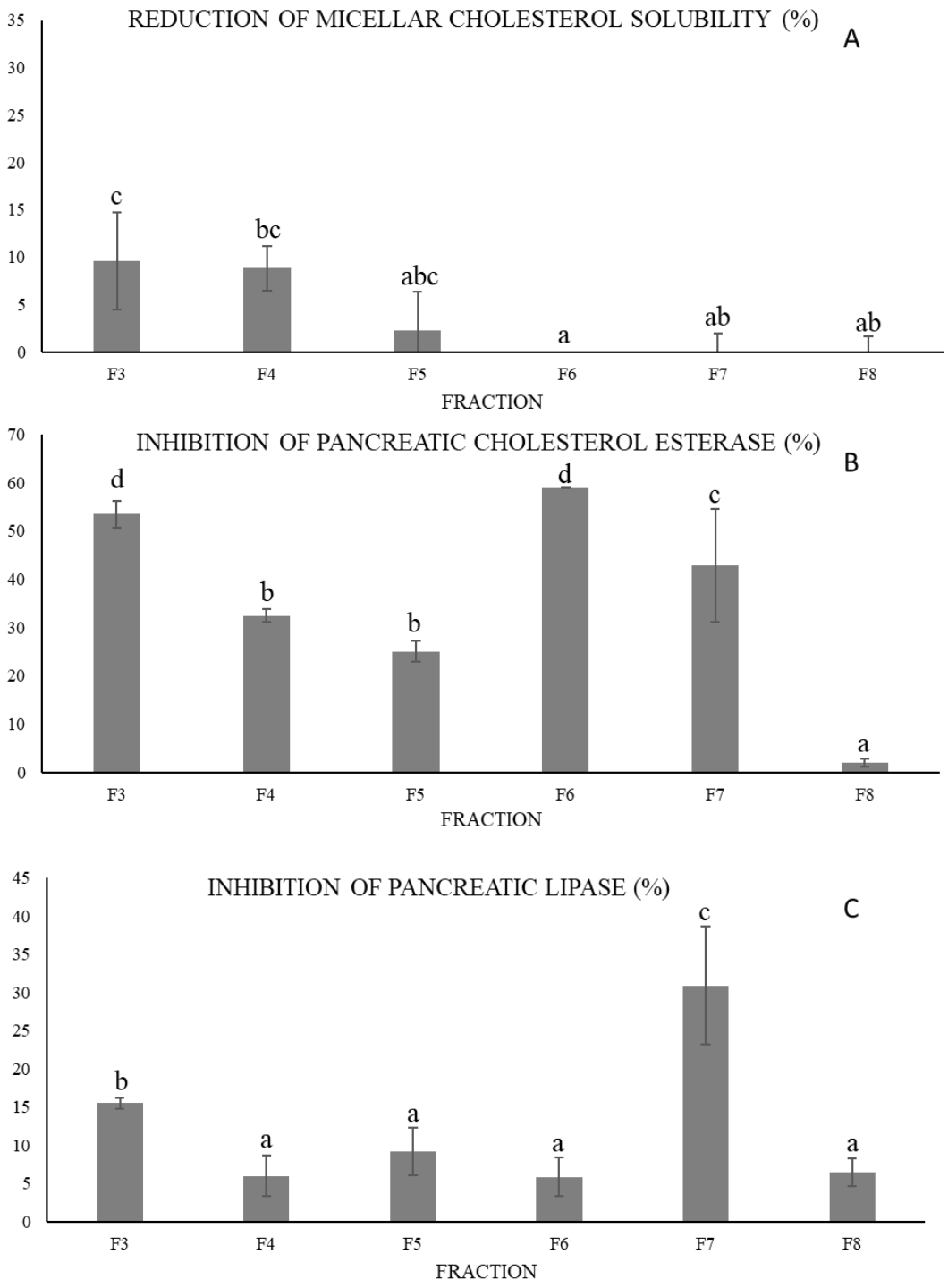


Figure 4

Fig. 4

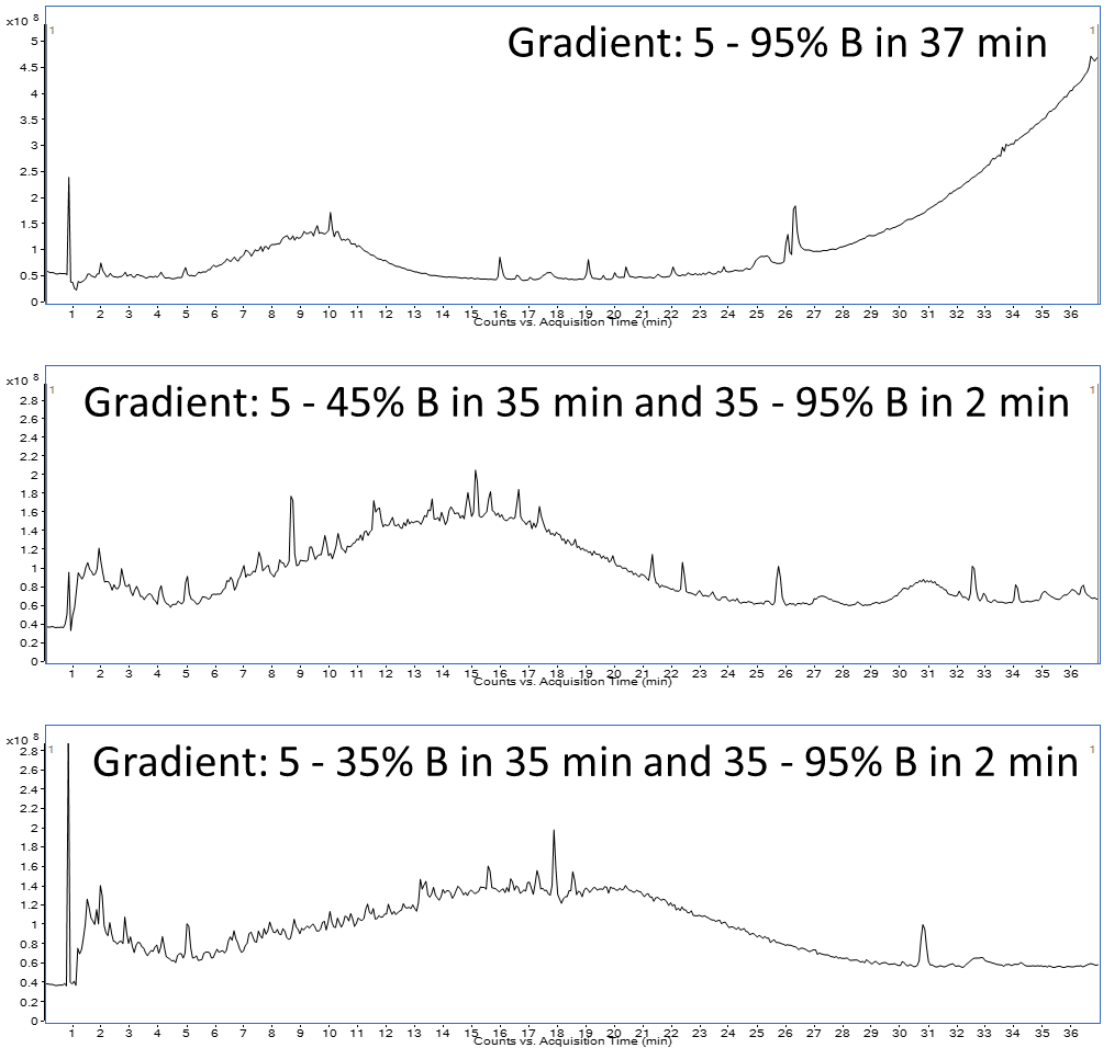
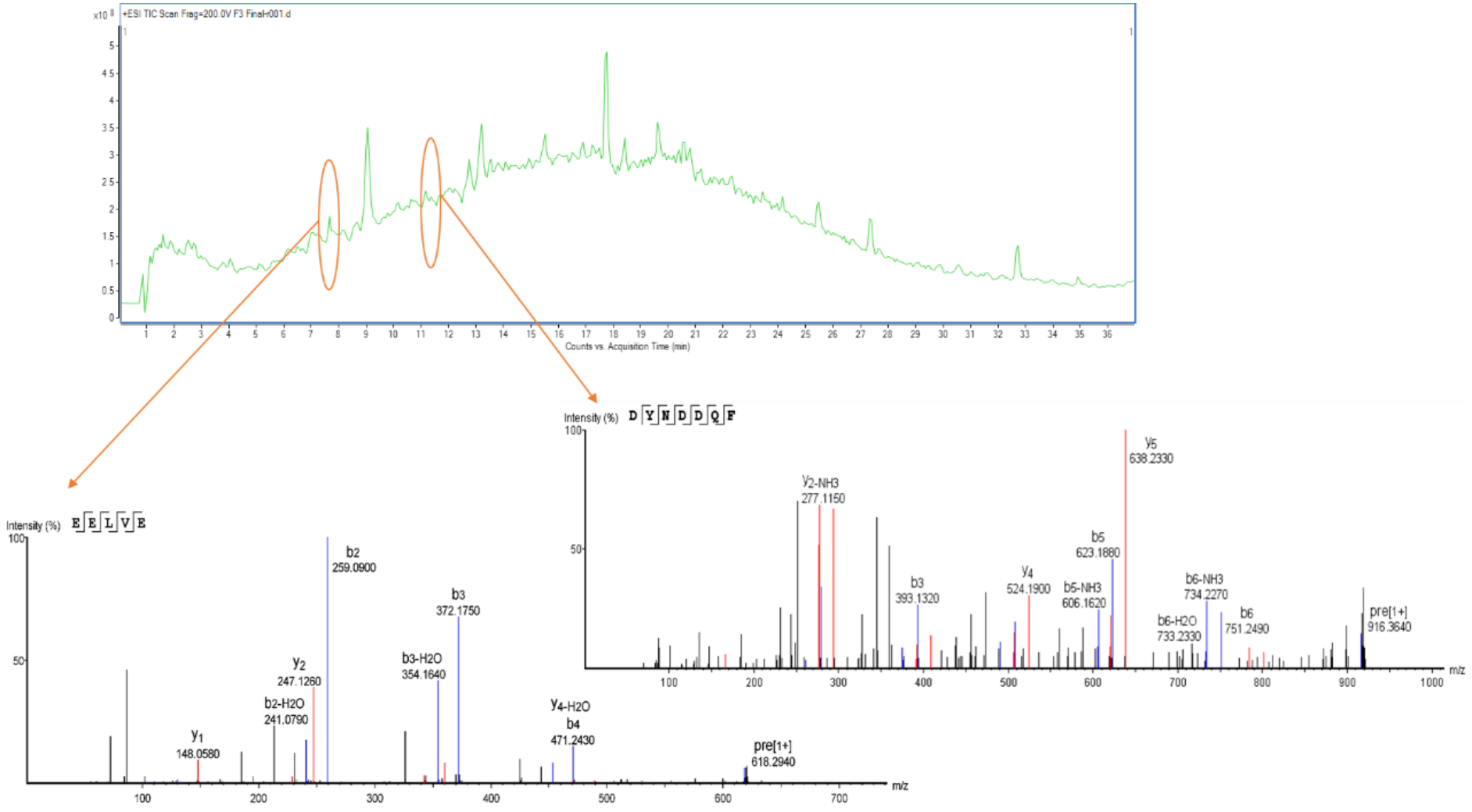


Fig. 5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b3-H2O 354.1640
y1 148.0580
y4-H2O b4 471.2430
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m/z
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y2-NH3 277.1150
b5 623.1880
b3 393.1320
y4 524.1900
b5-NH3 606.1620
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Figure 1

Fig. 1

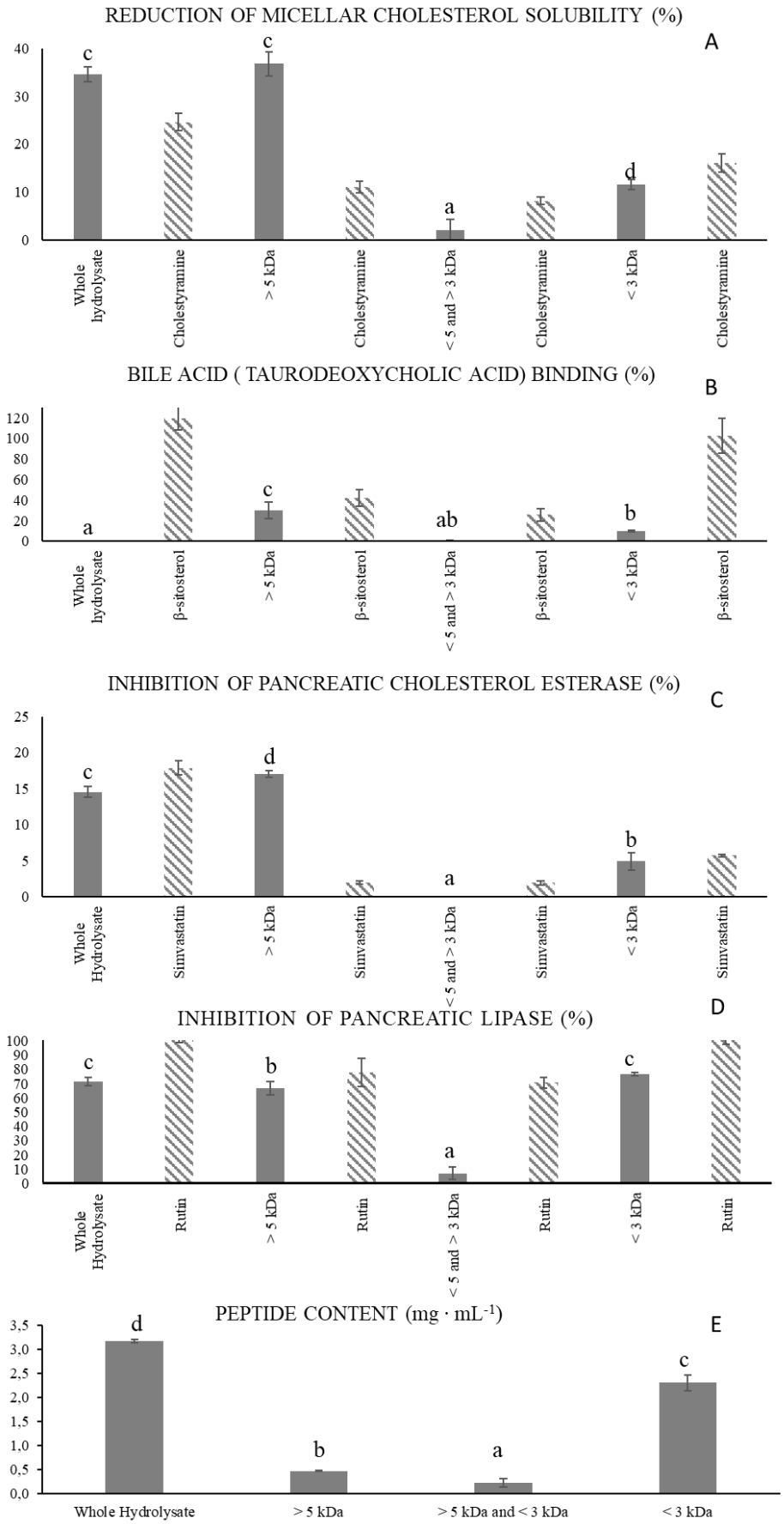
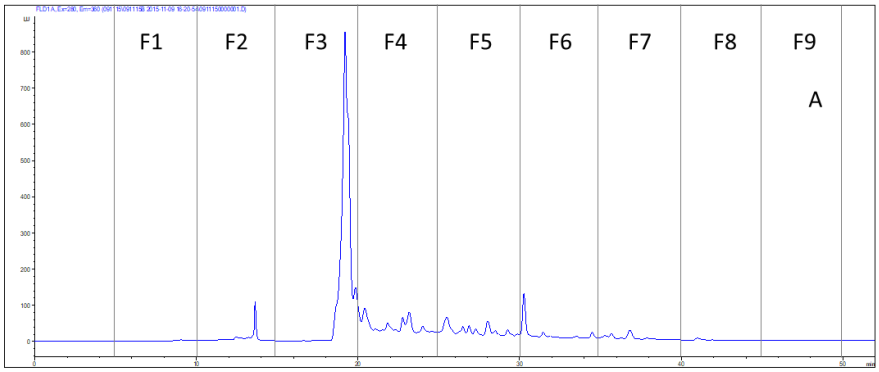
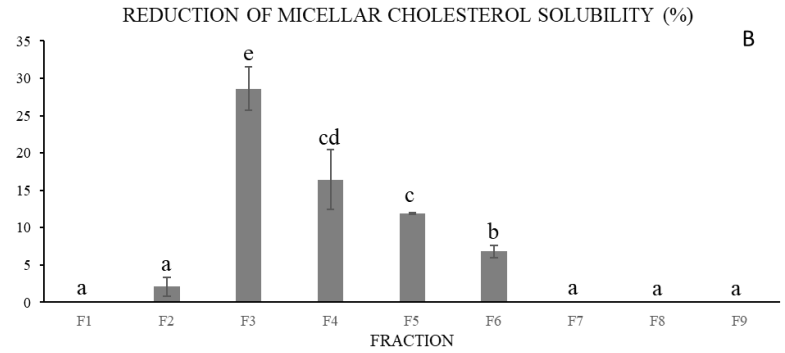
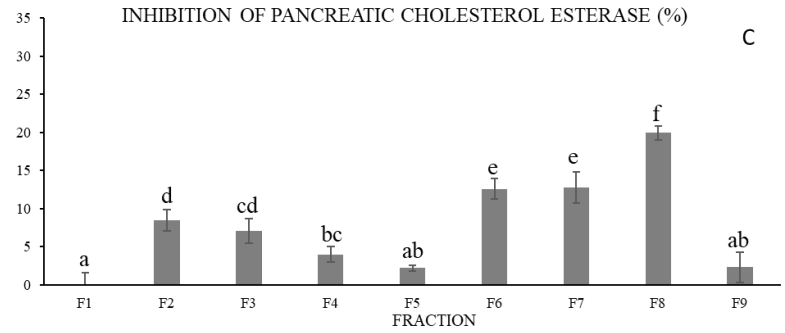


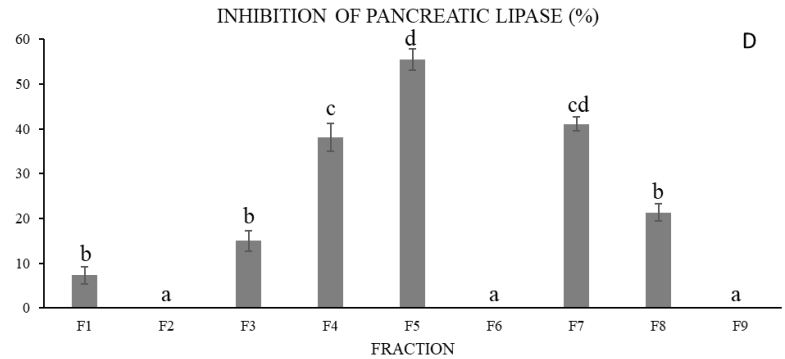
Figure 2

Fig. 2









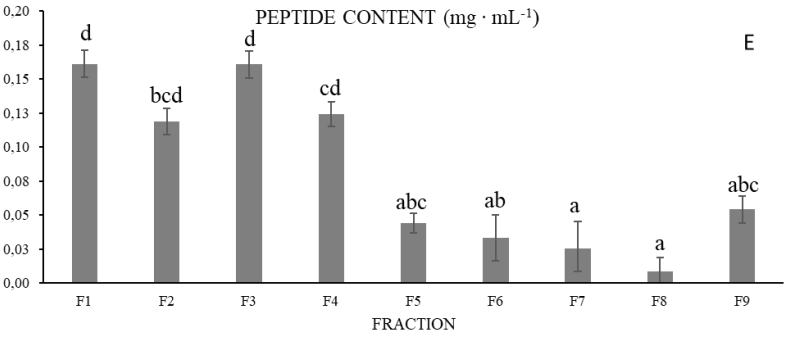


Table 1. Peptide sequence, average local confidence (ALC), retention time (RT), molecular mass, mass accuracy (ppm), and isoelectric point of peptides sequenced in fraction F3.

Peptide sequence	ALC (%)	RT (min)	Mass (Da)	ppm	Isoelectric point**
EELVE/EEIVE*	97	7.7	617.2908	7.7	0.76
DYNDDQF	96	11.8	915.3246	8.9	0.53
SAEDME	95	2.6	680.2323	5.6	0.66
YNDGFE	95	9.7	743.2762	7.7	0.71
AVFDDTLQE/AVFDDTIQE*	95	16.9	1036.4712	9.4	0.59
NVDLE/NVDIE	94	8.7	588.2755	6.8	0.71
ESGGVTE	94	2.2	677.2868	9.8	0.85
FDDTLEQ/FDDTIEQ*	93	13.2	866.3658	8.8	0.58
EMEE	93	1.8	536.1788	7.8	0.76
FDGEVEK	91	7.8	822.3759	6.8	3.69

* Since it is not possible to differentiate I from L due to their equal molecular masses, both sequences have been included in the table.
** Determined using Innovagen's peptide property calculator

*Graphical Abstract