

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA
Sección Departamental de Química Analítica



TESIS DOCTORAL

**Determinación indirecta de gluten mediante métodos basados en la
detección de ADN: PCR y genosensores electroquímicos**

**Indirect determination of gluten using DNA-based methods : PCR and
electrochemical genosensors**

**Determinação indireta de glúten usando métodos baseados em ADN :
PCR e genosensores eletroquímicos**

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Begoña Martín Fernández

Directores

Beatriz López-Ruiz
Noemí de los Santos López

Madrid, 2016



UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

SECCIÓN DEPARTAMENTAL DE QUÍMICA ANALÍTICA

**DETERMINACIÓN INDIRECTA DE GLUTEN MEDIANTE
MÉTODOS BASADOS EN LA DETECCIÓN DE ADN:
PCR Y GENOSENSORES ELECTROQUÍMICOS**

**INDIRECT DETERMINATION OF GLUTEN USING DNA-BASED METHODS:
PCR AND ELECTROCHEMICAL GENOSENSORS**

**DETERMINAÇÃO INDIRETA DE GLÚTEN USANDO MÉTODOS BASEADOS
EM ADN: PCR E GENOSENSORES ELETROQUÍMICOS**

2015

BEGOÑA MARTÍN FERNÁNDEZ

**DETERMINACIÓN INDIRECTA DE GLUTEN MEDIANTE
MÉTODOS BASADOS EN LA DETECCIÓN DE ADN:
PCR Y GENOSENSORES ELECTROQUÍMICOS**

*Memoria para optar al Grado de Doctor presentada por
Begoña Martín Fernández*

Bajo la dirección de las Doctoras
Beatriz López-Ruiz y Noemí de los Santos Álvarez

AGRADECIMIENTOS

A mi tutoras, las Dras. Beatriz López-Ruiz y Noemí de los Santos Álvarez, bajo cuya dirección se realizó este trabajo, por su inestimable dedicación y apoyo, sin el cual no hubiera sido posible la consecución de esta Tesis Doctoral. Por la confianza que pusieron en mí desde el primer momento, allá por 2007, cuando comencé la andadura en el campo de los biosensores enzimáticos electroquímicos, y más tarde, en 2009, cuando empezamos con los genosensores, por sus comentarios y consejos que durante todos estos años he recibido.

Al Grupo de Electroquímica de la Universidad de Oviedo, y en especial a los Dres. María Jesús Lobo Castañón y Arturo J. Miranda Ordieres por los conocimientos y los medios que han puesto a mi alcance en todo momento, por su acogida y dedicación todos y cada uno de los días que he pasado con ellos en Oviedo y a mi vuelta a Madrid. Al Dr. Juan Pedro Martín Clemente, por su disponibilidad, por facilitarnos el desarrollo de esta última y difícil parte del trabajo de investigación. Por ayudarnos poniendo a nuestra disposición todo el material y conocimientos a su alcance.

A Lorena por ser mi compañera de fatigas en todo momento de este trepidante viaje, por los buenísimos momentos que hemos compartido juntas, las risas, los llantos y por la amistad que nos une. Qué divertido es hacer investigación y reviews contigo, *menina!* Y sobre todo, por hacer esta etapa mucho más llevadera y agradable. *Volta já!*

A la Dra. Marta Sánchez-Paniagua López por estar siempre disponible durante todos estos años. A mis compañeros y amigos del laboratorio de Madrid: Adriany Amorim, Jana, Juan Pablo, Fernando, Alba, Pablo(s), Cristina, Felipe y Chesco. A los amigos de la Universidad de Oviedo: Daniel, Sonia, Susana, Laura, Emilio, Suely, Julien, Jairo, Eva y M^a José; gracias a todos por ayudarme tanto, por incentivar mi trabajo y hacerme pasar ratos inolvidables, ¡sois personas realmente increíbles! A la Dra. Rebeca Miranda-Castro, como iniciadora de mi incursión en el maravilloso, y a veces complejo, mundo de los genosensores.

À Doutora Isabel Mafra pela ajuda e novos conhecimentos que com ela adquiri, pela disponibilidade e por ter enriquecido o meu doutoramento durante o estágio na Universidade do Porto. E à Joana Costa, sem as quais parte deste trabalho não teria sido possível desenvolver. Agradeço à Doutora Beatriz Oliveira, à Doutora Cristina Delerue e à Doutora Fátima Barroso, por ter disponibilizado todos os recursos necessários para o decorrer do meu trabalho durante o estágio.

Aos meus amigos da Faculdade de Farmácia da Universidade do Porto: Lílíana, Caterina e Lucía pelos bons momentos que passamos juntas, e por escutar todas minhas locuras. Também não posso esquecer à Gori, Joana Amaral, Sónia e Filipa e aos milhões de bons amigos e as pessoas que me ajudaram e fizeram o meu estágio muito mais fácil e uma experiência inesquecível.

A todos los miembros de la Sección Departamental de Química Analítica de la Facultad de Farmacia que con cariño me han acogido desde los inicios de mi investigación. A los Dres. Ana Isabel Olives Barba, Sofía Ródenas de la Rocha, M^a Carmen Martín Gómez, Pedro Andrés Carvajales, Gregorio Carcedo Güemez, M^a Antonia Martín Carmona y Elena Rodríguez Rodríguez. A Rosa, Víctor y Teresa.

A mis padres y a mis hermanos por vuestro apoyo continuo y comprensión sin límites, a Carlos por estar siempre a mi lado; sin vuestro ánimo y ayuda constantes todo resultaría mucho más difícil.

A todos los que me acompañasteis en esta etapa, y que me apoyan en estas decisiones medio locas, a todos los que me ayudaron de una u otra forma a cumplir mis objetivos.

A la Universidad Complutense de Madrid por la concesión de una beca predoctoral para la consecución de mi trabajo de investigación. A los miembros de la Secretaría de la Facultad de Farmacia. A los miembros del Tribunal.

A todos, ¡GRACIAS!

OBRIGADA!

RESUMEN

La enfermedad celíaca es una enteropatía autoinmune muy frecuente (1% de la población) que se produce en individuos genéticamente predispuestos como resultado de una respuesta inmune exacerbada contra un factor ambiental bien definido, el gluten. De esta forma, es importante que los enfermos celíacos no consuman alimentos que contienen gluten. Además, parece que el consumo de cantidades relativamente pequeñas de gluten durante largos períodos de tiempo se correlaciona con un aumento del riesgo de desarrollo de complicaciones de tipo autoinmune.

Por lo tanto, la identificación de la presencia de gluten y su cuantificación en productos alimenticios son de primordial importancia, a la vez que un reto para los científicos, debido a la dificultad de su análisis. Hoy en día, la verificación analítica del cumplimiento de los requisitos de etiquetado y de detección de trigo en los productos alimenticios se realiza principalmente por métodos que detectan proteínas tipo inmunoensayo ligado a enzimas (ELISA). Sin embargo, a pesar de los enormes avances en la detección de proteínas de gluten, algunas de las limitaciones de los métodos existentes han fomentado el desarrollo de tecnologías alternativas. Así, los métodos basados en el ADN, como la reacción en cadena de la polimerasa (PCR), PCR en tiempo real o los sensores de ADN (también denominados genosensores), representan opciones atractivas. Todos ellos comparten una ventaja significativa: el analito en sí se puede amplificar y, por lo tanto, se pueden detectar cantidades extremadamente bajas del mismo. También la mayor estabilidad de las moléculas de ADN, en comparación con las proteínas, los hace métodos muy convenientes, especialmente para su aplicación al análisis de alimentos procesados. Sin embargo, la enorme complejidad del genoma del trigo (probablemente el más complejo de las especies vegetales domesticadas), junto con su gran variabilidad genética lo convierten en una tarea desafiante a la vez que de enorme interés. En general, los métodos basados en la detección de ADN se utilizan principalmente como herramientas de cribado para detectar la presencia de alimentos alergénicos, pero la PCR y los genosensores ofrecen una alternativa de alta sensibilidad y especificidad frente a los métodos ELISA, también con fines de cuantificación. Además, los métodos ELISA y PCR analizan diferentes tipos de analitos, de modo que un resultado positivo en ambos ensayos representa una evidencia casi absoluta de que el alérgeno está presente.

Debido a las ventajas de los métodos de ADN para la detección de trigo en alimentos, en la última década han surgido varios métodos de PCR y PCR en tiempo real, aunque la mayoría no alcanzan la sensibilidad necesaria. Una alternativa más sencilla y económica la constituyen los biosensores. Además de los métodos de PCR, los genosensores o sensores de ADN, en especial los

genosensores electroquímicos, son una alternativa en la detección de ADN por su relativa simplicidad, bajo coste de la instrumentación, la posibilidad del análisis en línea, o diseño de equipos miniaturizados y portátiles. Sin embargo su aplicación para la detección de trigo en alimentos está sin explotar.

La Tesis Doctoral se centra en la detección indirecta de gluten empleando secuencias de ADN específicas de cereales dañinos para enfermos celíacos, como alternativa a los métodos de detección de proteínas. Se realizó una búsqueda de las secuencias de ADN relacionadas con los cereales tóxicos para los enfermos celíacos adecuadas para emplearlas como analito. Se caracterizó ampliamente el comportamiento de tres secuencias de ADN por PCR en tiempo real y se evaluaron críticamente, obteniéndose con todas ellas una sensibilidad notablemente superior a la mencionada en la literatura hasta el momento (<20 ppm), cumpliendo con los requisitos impuestos por la legislación vigente. Se constató una fuerte influencia de la matriz en los resultados obtenidos, siendo las matrices de arroz y maíz las que ofrecieron las mejores prestaciones analíticas. Este hallazgo apunta a la necesidad de adecuar la matriz de los patrones a la de la muestra para evitar desviaciones negativas o positivas del valor verdadero. La secuencia de oligonucleótidos que codifica el péptido inmunodominante de la gliadina fue la que suministró los mejores resultados, motivo por el cual se diseñó un genosensor electroquímico para su detección, el primero específicamente diseñado para el análisis de gluten. Al contrario que para el caso de otros genosensores electroquímicos, la detección de fragmentos amplificados por PCR de muestras reales se pudo realizar sin ningún tipo de purificación previa de los amplicones y permitió cuantificar 15 pg de ADN de trigo o 0.001% de trigo en matriz de arroz, una detectabilidad que rivaliza directamente con los métodos más sensibles de PCR en tiempo real. El genosensor permitió la determinación de ADN de todos los cereales tóxicos incluida la avena mientras que no mostró reactividad cruzada con otras especies seguras como arroz y soja. Para disponer de un método específico de trigo, la selectividad del sensor fue modificada mediante el uso de sondas estructuradas que permitieron la detección selectiva de trigo frente a otros cereales filogenéticamente relacionados (cebada, centeno, avena) sin comprometer en gran medida las características analíticas del genosensor diseñado. Por último se exploró el método de análisis de curvas de disociación de alta resolución (HRM de sus siglas en inglés, *high resolution melting*) para su aplicación en la identificación de cereales tóxicos, pudiéndose discriminar el tipo de cereal del que proviene el gluten presente en una muestra.

En definitiva, los métodos propuestos en este trabajo, una vez caracterizados y optimizados, suponen un avance en la detección de gluten a través de secuencias de ADN específicas de trigo y constituyen sistemas pioneros en un campo que está aún por explorar, pero que tiene enormes posibilidades de desarrollo.

LISTA DE ABREVIATURAS

ADN: ácido desoxirribonucleico
ADNs: ADN de doble cadena
ADNs: ADN de hebra simple
ALP: fosfatasa alcalina
AOAC: Asociación Oficial de Químicos Analíticos
ARN: ácido ribonucleico
ARNr: ARN ribosómico
CP: sonda de captura
Ct: cycle threshold o ciclo umbral
CTAB: bromuro de hexadeciltrimetilamonio
dNTPs: desoxinucleótido trifosfato
DPV: voltamperometría diferencial de impulso
DR3, DR5/DR7 o DR4: genes de HLA
ELISA: inmunoensayo ligado a enzimas
ESPGHAN: Sociedad Europea de Gastroenterología, Hepatología y Nutrición
Pediátrica
FITC: isotiocianato de fluoresceína
HDT: hexanoditiol
HLA: antígeno humano leucocitario
HLA-DQ2, HLA-DQ8: genes de HLA
HMW: gluteninas de alto peso molecular
HPLC: cromatografía líquida de alta eficacia
HRM: curvas de disociación de alta resolución
HRP: peroxidasa de rábano silvestre
IFN- γ : interferón- γ
Ig: inmunoglobulina
IL-15: interleucina-15
LMW: bajo peso molecular
LOD o LD: límite de detección
MB: azul de metileno
MCH: 6-mercapto-1-hexanol
MIC-A: complejo mayor de histocompatibilidad con cadena A
NK: células natural-killer, un tipo de linfocito del sistema inmune
pb: pares de bases
PCR: reacción en cadena de la polimerasa
PDO: denominación de origen protegida
PGI: indicación geográfica protegida
ppb: partes por billón
ppm: partes por millón
RAPD: análisis amplificación aleatoria de ADN polimórfico
S/B. Relación señal/blanco
SDS. dodecil sulfato sódico
SP: sonda señalizadora o indicadora
TMB: tetrametilbenzidina
Tri a 18: proteína aglutinina isolectina
Tri a 25: proteína tiorredoxina h
TSG: especialidad tradicional garantizada
tTG: transglutaminasa tisular

ÍNDICE

1 INTRODUCCIÓN	16
1.1 Enfermedad celíaca	18
1.1.1 Patogénesis	19
<i>Gluten</i>	20
<i>Factores inmunes</i>	23
<i>Factores genéticos</i>	25
<i>Factores ambientales</i>	26
1.1.2 Manifestaciones clínicas	26
1.1.3 Diagnóstico	28
<i>Análisis serológicos</i>	29
<i>Análisis de alelos HLA</i>	29
<i>Biopsia e histología</i>	30
1.1.4 Tratamiento	30
1.1.5 Marco legislativo	32
1.2 Métodos de análisis de gluten en alimentos	33
1.2.1 Principios de la reacción en cadena de la polimerasa, PCR... 36	
1.2.2 Etapas en la detección de alérgenos y gluten mediante PCR 41	
1.2.3 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante PCR..... 43	
<i>Análisis para la detección de la presencia de cereales tóxicos</i>	44
<i>Análisis de trigo para prevenir el fraude alimentario</i>	48
1.2.4 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante genosensores..... 53	
<i>Métodos de inmovilización</i>	55
<i>Métodos de detección</i>	56
<i>Biosensores de ADN para la detección de alérgenos</i>	58
1.2.5 Puntos críticos en la detección de trigo y cereales mediante ADN	65
1.3 Bibliografía	67
2 OBJETIVOS	88

3	CRIBADO DE SECUENCIAS Y EFECTO DE MATRIZ.....	92
3.1	Cribado de secuencias.....	94
	Screening new gene markers for gluten detection in foods	94
3.2	Efecto de matriz.....	104
	Effect of matrix on the performance of quantitative real-time PCR of gluten-containing cereals.....	106
4	GENOSENSORES ELECTROQUÍMICOS	126
4.1	Genosensor para la detección de gliadina con sonda de captura lineal.....	128
	Strongly structured DNA sequences as targets for genosensing: Sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment	128
4.2	Acoplamiento del genosensor con PCR para el análisis de muestras alimentarias procesadas	142
	Challenging genosensors in food samples: the case of gluten determination.....	144
4.3	Genosensor para la detección de gliadina con sonda de captura estructurada	162
	Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a gliadin fragment	163
4.4	Genosensor para la detección de Tri a 18.....	172
5	ANÁLISIS DE CURVAS DE DISOCIACIÓN DE ALTA RESOLUCIÓN (HRM) 176	
	High resolution melting analysis as a new approach to discriminate gluten-containing cereals.....	180
6	CONCLUSIONES Y CONSIDERACIONES FINALES	196
7	SUMMARY.....	202
7.1	Introduction.....	203
7.2	Objetives	204
7.3	Conclusions and final considerations	205

8 RESUMO	208
8.1 Introdução	210
8.2 Objetivos	211
8.3 Conclusões e considerações finais	213
9 ANEXO I: PUBLICACIONES DERIVADAS DE LA TESIS DOCTORAL	216
Electrochemical Methods based on DNA in Food Safety Assessment	
220	

Introducción	Bloque 1 – Cribado de secuencias PCR	Bloque 2 – Genosensores electroquímicos	Bloque 3 – HRM analysis
--------------	--------------------------------------	---	-------------------------

Enfermedad celiaca	<ul style="list-style-type: none"> •Patofisiología e historia de la enfermedad celiaca •Métodos de ADN de análisis de gluten (y/o trigo) en alimentos: PCR y sensores de DNA •Tipo de secuencias empleadas 	<p>Screening secuencias: α2-gliadina, Tri a 18, Tri a 25</p> <p>Food control publicado</p> <ul style="list-style-type: none"> •α2-gliadina mejor sensibilidad •Método de PCR para analizar simultáneamente todos los cereales \rightarrow calibración da problemas porque no discrimina entre cereales (en cuantificación) No calibrado claro si son mezclas. 	<p>Genosensor α2-gliadina en muestras reales. <u>CP lineal</u></p> <p>B&B publicado</p> <ul style="list-style-type: none"> • Genosensor= selectividad adicional 	HRM para Tri a 18	<p>Food Chemistry enviado</p> <p>HRM como sistema específico para discriminar entre cereales, aunque no se puede cuantificar</p>
--------------------	---	--	---	-------------------	---

Efecto matriz	<p>Food and Chemical Toxicology enviado</p> <p>Efecto matriz en cuantificación por PCR de muestras reales (trigo en soja, arroz y maíz)</p>
---------------	--

Genosensor α 2-gliadina en sintético. <u>CP horquilla</u>	<p>ABC publicado</p> <ul style="list-style-type: none"> •CP horquilla aun mejora más la selectividad
Genosensor Tri a 18	<p>Hibridación entre CP y SP impide usarlo</p>

¿qué **secuencia** usamos?

Genosensores etapa de **selectividad** adicional

Método **alternativo** para identificación de especies de cereales toxicos

1 INTRODUCCIÓN

1.1 Enfermedad celíaca

- 1.1.1 Patogénesis
- 1.1.2 Manifestaciones clínicas
- 1.1.3 Diagnóstico
- 1.1.4 Tratamiento
- 1.1.5 Marco legislativo

1.2 Métodos de análisis de gluten en alimentos

- 1.2.1 Principios de la reacción en cadena de la polimerasa, PCR
- 1.2.2 Etapas en la detección de alérgenos y gluten mediante PCR
- 1.2.3 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante PCR
- 1.2.4 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante genosensores
- 1.2.5 Puntos críticos en la detección de trigo y cereales mediante ADN

INTRODUCCIÓN

1.1 Enfermedad celíaca

Actualmente alérgenos, organismos genéticamente modificados, patógenos y contaminantes químicos, constituyen los puntos principales de preocupación para los consumidores. De hecho, uno de los grandes problemas en materia alimentaria es la creciente incidencia de las alergias alimentarias e intolerancias, y entre ellas, la enfermedad celíaca. De entre los grandes alérgenos mundialmente reconocidos destaca el gluten, que desencadena la enfermedad celíaca. Desde el punto de vista más estricto, sin embargo, la enfermedad celíaca no debería ser clasificada como una alergia alimentaria sino como una enfermedad autoinmune, debido a que no intervienen anticuerpos de tipo inmunoglobulina E (IgE) (Janssen 2006; Tanabe 2008), pero frecuentemente se relaciona con este tipo de patologías y es difícil diferenciarlas entre sí (Fasano and Catassi 2012; Sicherer 2000).

La enfermedad celíaca es una enfermedad autoinmune que se desencadena debido tanto a componentes genéticos como ambientales (Denham and Hill 2013). Se trata de una enfermedad autoinmune única porque el precipitante ambiental es conocido, al contrario de lo que sucede con otras enfermedades de este tipo. Se produce en individuos genéticamente predispuestos, por la ingestión de gluten, proteína de almacenamiento mayoritaria en el trigo y en otros cereales similares, desencadenándose una respuesta del sistema inmune inadecuada en el intestino, la piel, el hígado, las articulaciones, el útero, y otros órganos (Nasr et al. 2015; Pividori et al. 2009). Al contrario que las alergias alimentarias, la enfermedad cursa con la activación inmunológica de linfocitos T, y los niveles de IgE específicos del gluten y totales en la mucosa no están elevados, por lo que la enfermedad celíaca no debe ser considerada una alergia (Tanabe 2008). Esta respuesta tiene un componente innato y otro adaptativo, mediado por linfocitos T CD₄⁺ específicos (van Heel and West 2006). La enfermedad celíaca se sabe que está producida por la gliadina y otras prolaminas, y está caracterizada por una combinación variable de presencia de gluten, anticuerpos específicos de gluten (contra transglutaminasa tisular, formas desamidadas de gliadina y anticuerpos antiendomiso), haplotipos HLA-DQ2 o HLA-DQ8, y enteropatía (Husby et al. 2012). Concretamente en el intestino, el daño causado da lugar a atrofia en las vellosidades de la mucosa, y con ello a la sintomatología típica de malabsorción y diarreas. De hecho, esta atrofia puede ser identificada en biopsias del intestino, y es considerada como el parámetro definitivo del diagnóstico de esta enfermedad.

En el pasado, la presentación clásica de la enfermedad celiaca se relacionó con la aparición de cuadros sintomáticos gastrointestinales, que incluían la diarrea y la pérdida de peso. Sin embargo, en los años 80 del siglo pasado, se hicieron cribados que demostraron que muchos celíacos eran asintomáticos. Además, la enfermedad celiaca es una de las enteropatías más frecuentes. Se estima que afecta hasta a un 1% de la población general, aunque este número puede estar subestimado debido a las dificultades en su diagnóstico (Denham and Hill 2013). Las manifestaciones clínicas de la enfermedad celíaca son altamente variables, incluyendo tanto síntomas gastrointestinales como no gastrointestinales, que pueden dar lugar a manifestaciones clínicas de tipo sistémico (Reilly et al. 2012). La edad de aparición de la enfermedad varía, pudiendo afectar tanto a niños como adultos. El primer pico de edad de presentación de la enfermedad se produce en la niñez, y se asocia generalmente con síntomas como malabsorción y retraso en el desarrollo, y existe otro pico en mujeres en edad fértil que aparece asociado a anemia. El tercer pico es la década de los 40 o 50 años de edad, en personas que presentan osteoporosis.

Debido a la complejidad de la enfermedad, el único tratamiento efectivo consiste en la eliminación de la dieta de cualquier alimento que pueda contener gluten, aunque para un 30% de los pacientes la mejoría clínica es pobre, y es frecuente la baja adherencia. Sin embargo, la absoluta certeza de que exista un mínimo grado de contaminación con gluten en el alimento es difícil de confirmar (Fasano and Catassi 2012). Aunque no se ha definido claramente un límite totalmente seguro de consumo de gluten, la literatura parece indicar que pequeñas cantidades de gluten, de 10-50 mg por día (un rebanada de pan de 25 g contiene aproximadamente 1,6 g de gluten) pueden ser suficientes para causar daño en la mucosa intestinal del paciente celiaco a lo largo del tiempo (Catassi et al. 2007). Además en estos pacientes, el consumo de gluten durante largos periodos de tiempo se ha relacionado con un aumento en la incidencia de complicaciones graves entre las que se encuentran adenocarcinoma, esprúe refractario y linfoma de células T asociado a enteropatía (Green and Cellier 2007). Incluso, la enfermedad celíaca no diagnosticada, y por tanto, el consumo de gluten de manera habitual se ha relacionado con un aumento de hasta cuatro veces del riesgo de muerte (Rubio-Tapia et al. 2009). En este sentido, el desarrollo de nuevos métodos de detección y diagnóstico precoz de la enfermedad celiaca y el análisis de la seguridad de los alimentos para el consumo por estos pacientes, es crucial.

1.1.1 Patogénesis

Las primeras descripciones de síntomas consistentes con la enfermedad celiaca fueron realizadas por el griego Aretaeus en el siglo II (Briani et al. 2008). En el siglo XIX Samuel Gee y otros médicos definieron los síntomas y

características de la enfermedad y propusieron varias ideas para su tratamiento. Sin embargo, Willem Karel Dicke, en los años 40, fue el primero en reconocer la importancia de eliminar los cereales de la dieta (De Re et al. 2013; van Berge-Henegouwen and Mulder 1993), mientras que John W. Paulley describió los cambios histológicos del intestino (Paulley 1954). El descubrimiento de la asociación de la enfermedad con marcadores de antígenos humanos leucocitarios (HLA) de clase II específicos y células T específicas para gluten y el descubrimiento de la transglutaminasa tisular (también conocida como transglutaminasa 2, tTG) y el auto-antígeno antiendomio se llevó a cabo en las décadas de 1980 y 1990 (Briani et al. 2008).

La patogénesis de la enfermedad, esto es, el mecanismo de desarrollo y evolución de la enfermedad es una interacción de cuatro factores fundamentales: gluten, factores genéticos, inmunes y ambientales, que se pueden desglosar por separado.

Gluten

El gluten es una sustancia de aspecto gomoso que permanece tras el lavado de la masa de pan hecha con harina de trigo, para eliminar el almidón (Wieser 2007). Se sabe que está constituido por un conjunto de proteínas de reserva que juegan un papel fundamental en la capacidad panificable de cereales como el trigo confiriéndole a la masa la propiedad de absorber agua, cohesividad, viscosidad y elasticidad (Goesaert et al. 2005). Además de en el trigo, el gluten puede encontrarse también en cereales filogenéticamente relacionados como la cebada y centeno (Figura 1). Generalmente las proteínas constituyentes del gluten son ricas en prolina y glutamina que se digieren poco en el tracto gastrointestinal de los seres humanos.

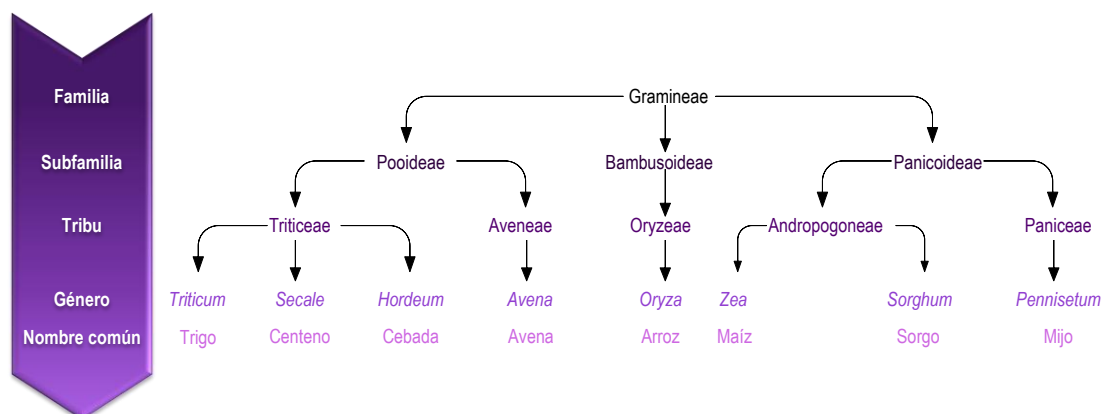


Figura 1. Árbol filogenético de algunos de los cereales más empleados en alimentación humana.

Como punto de partida, las proteínas del trigo se suelen clasificar en función de su solubilidad en diferentes disolventes o según su función. La clasificación más empleada es la propuesta por Osborne (1924) que distingue las diferentes fracciones de la proteína del vegetal en función de su solubilidad en: agua, disolución salina diluida, disolución de alcohol y disolución de ácidos o álcalis diluidos. De esta forma se pueden clasificar las proteínas del trigo en albúminas, globulinas, gliadinas y gluteninas respectivamente (Tabla 1) (Goesaert et al. 2005).

Tabla 1. Clasificación de Osborne de las proteínas del trigo.

Fracción de Osborne	Solubilidad en	Tipo de proteínas	Función biológica	Función en tecnología alimentaria
Albúminas	Agua	Monoméricas	Estructural y metabólica	Variable
Globulinas	Sales diluidas	Monoméricas	Estructural y metabólica	Variable
Gliadinas	Disoluciones de alcohol	Monoméricas y poliméricas de bajo peso molecular	Almacenamiento de la semilla	Viscosidad a la masa y extensibilidad
Gluteninas	Ácido acético diluido	Polímeros de alto peso molecular	Almacenamiento de la semilla	Elasticidad a la masa y tenacidad
Residuo	-	Polímeros	Almacenamiento de la semilla	Variable

Las proteínas estructurales representan un 20% del total del contenido proteico del trigo, y se encuentran en las capas externas del grano del trigo y en el endospermo, y constituyen las fracciones de Osborne de albuminas y globulinas. En su mayor parte son proteínas monoméricas, con funciones estructurales o de tipo enzimático. Se sabe que esta fracción está relacionada con las proteínas de almacenamiento de legumbres y en otros cereales, como la avena y el arroz (Shewry and Halford 2002).

Únicamente las proteínas de almacenamiento constituyen el gluten (gliadinas, gluteninas y residuo). Hasta un 80% del contenido proteico del trigo es gluten, representando la mayor parte de las proteínas de almacenamiento. Pertenecen a la clase de prolaminas y se encuentran principalmente en el endospermo del grano del trigo. Dentro de las prolaminas se pueden subclasificar en gliadinas (proteínas monoméricas) y gluteninas (poliméricas), que se encuentran aproximadamente en una proporción del 50% cada una en el gluten de trigo. Las gliadinas son un grupo relativamente extenso de proteínas monoméricas del gluten que abarca proteínas con pesos moleculares desde 30.000 a 80.000 Da, solubles en mezclas agua-alcohol. Según su estructura de residuos aminoacídicos las gliadinas se pueden dividir en α , γ y ω -gliadinas. Las gluteninas son proteínas relativamente grandes, insolubles en mezclas alcohol-agua presentando pesos moleculares desde 80.000 hasta varios millones de Da. Se pueden distinguir cuatro grupos de subunidades de gluteninas: las gluteninas de alto peso molecular (HMW) que oscilan entre 65 y

90 kDa; y las de bajo peso molecular (LMW) con pesos moleculares entre 30 y 60 kDa. El gluten es por tanto, una mezcla de polipéptidos de gliadina (monómeros) y glutenina (estructuras largas poliméricas). Existe una alta variación genética en las secuencias que codifican las proteínas del gluten, especialmente en las gliadinas. Como se ha dicho anteriormente, las proteínas del gluten son ricas en residuos de prolina y glutamina, mientras que algunos otros aminoácidos como el ácido glutámico y el aspártico son inusualmente poco frecuentes (Sollid 2002). Debido a este alto contenido en residuos aminoacídicos de este tipo, son proteínas altamente resistentes a la digestión proteolítica completa por las enzimas gástricas, pancreáticas y de las microvellosidades del intestino humano, principalmente porque estas enzimas son deficientes en actividad prolil endopeptidasa (Matysiak–Budnik et al. 2005; Stepniak et al. 2006). Este hecho hace que se acumulen grandes fragmentos de péptidos, de hasta 50 residuos aminoacídicos con alto contenido en prolina y glutamina en el intestino. Por lo tanto, la enfermedad celiaca es una enfermedad desencadenada por las características especiales de las proteínas que constituyen el gluten del trigo. La fracción de proteínas que constituye las gliadinas, ha sido la más estudiada (Altenbach et al. 2010; Anderson and Greene 1997; Anderson et al. 1997; Arentz-Hansen et al. 2000a; Arentz-Hansen et al. 2000b; De Re et al. 2013; Konic-Ristic et al. 2009; Nasr et al. 2015; Shan et al. 2002; Tollefsen et al. 2006; van de Wal et al. 1998; van Herpen et al. 2006; Wieser 2001), pero muchas o incluso todas las proteínas del gluten parece que están implicadas en la respuesta, así como proteínas similares en cebada (hordeínas) y centeno (secalinas) (Green and Jabri 2003).

En el año 2000 dos estudios independientes identificaron epítomos inmunodominantes peptídicos de la región 57-75 de la α -gliadina (Anderson et al. 2000; Arentz-Hansen et al. 2000b). Estos péptidos pasan la barrera epitelial del intestino, posiblemente durante infecciones intestinales o cuando aumenta la permeabilidad intestinal, e interaccionan con células presentadoras de antígeno en la lámina propia, siendo probablemente los desencadenantes de la respuesta inmune (Green and Cellier 2007). Además, se ha demostrado que algunos péptidos sin digerir de la gliadina, como el fragmento de la α 2-gliadina de 33 residuos aminoacídicos se mantienen en el lumen intestinal después de la ingestión del gluten, dado su resistencia a la degradación por enzimas proteasas gástricas, pancreáticas y de las microvellosidades intestinales (Shan et al. 2002). Algunos estudios también han indicado que las gluteninas pueden estar implicadas en el desencadenamiento de esta sucesión de reacciones (Dewar et al. 2006; Molberg et al. 2003; Vader et al. 2002; van de Wal et al. 1999).

Aunque se sabe que la exposición a estas proteínas no es suficiente para desarrollar la enfermedad celíaca por sí sola, parece que está estrechamente relacionado con la patología desarrollada. Sin embargo, no se han encontrado

diferencias muy significativas entre individuos sanos y enfermos en la capacidad de las enzimas para degradar los péptidos de gluten, y en la capacidad de éstos de atravesar la barrera epitelial del intestino. Probablemente el fracaso en la digestión completa de estas proteínas esté especialmente patente en el intestino de pacientes con celiaquía activa (Kagnoff 2007).

Factores inmunes

Las respuestas inmunes desencadenadas tras la entrada de estos péptidos procedentes del gluten ingerido se pueden clasificar según las localizaciones en las que generan una reacción (Alaedini and Green 2005). En la mucosa intestinal, las fracciones del gliadina en celíacos provocan una reacción inflamatoria, primeramente en el intestino delgado superior, que se caracteriza por la infiltración de la lámina propia y del epitelio con células de inflamación crónica y de atrofia de las vellosidades intestinales (Figura 2). El paso de péptidos derivados del gluten a través de la barrera epitelial intestinal posiblemente ocurra de forma paralela a infecciones intestinales o cuando la permeabilidad intestinal se encuentra aumentada. Esta interacción desencadena una reacción del sistema inmune innato y adaptativo (Gianfrani et al. 2005).

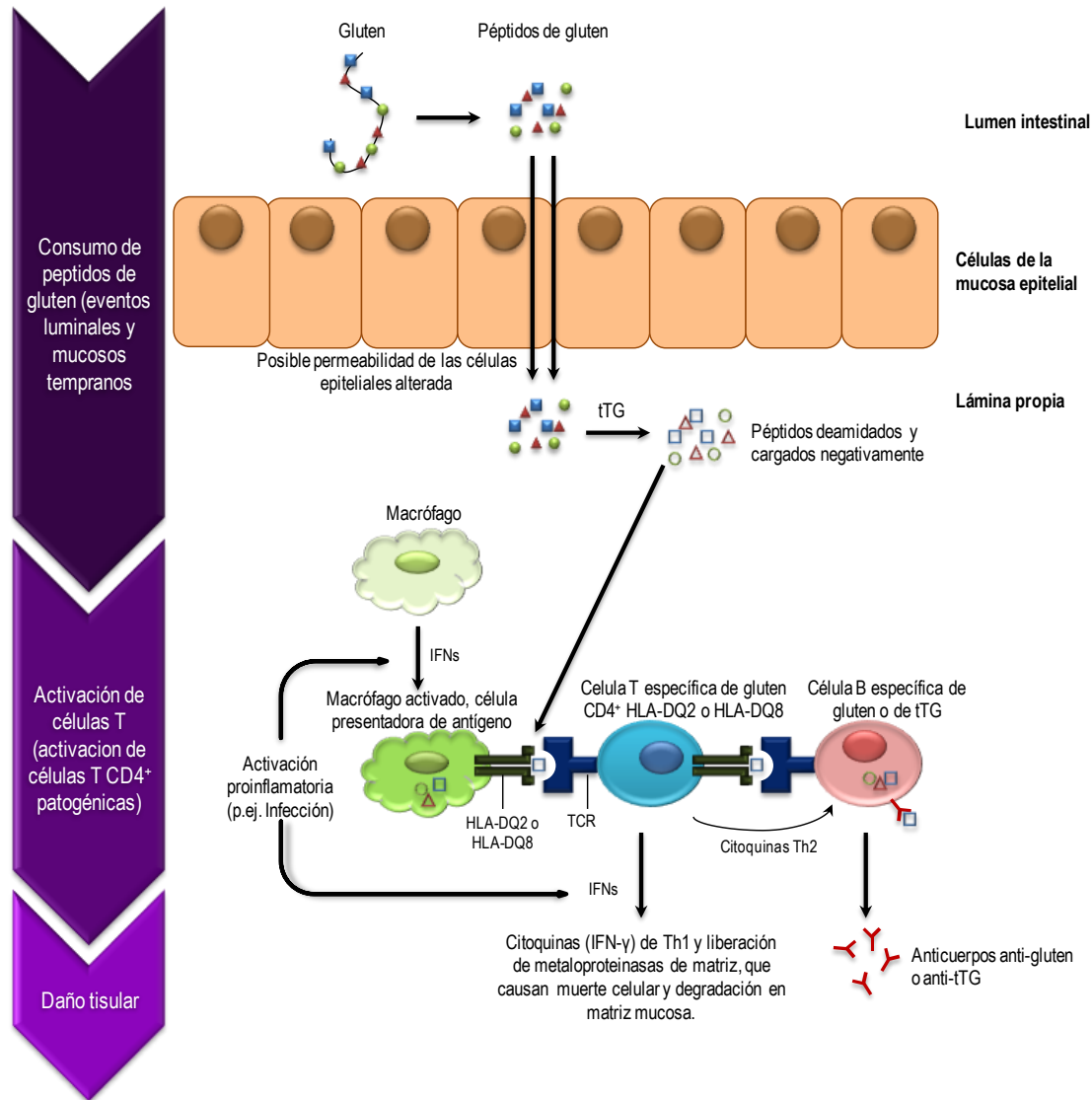


Figura 2. Mecanismo inmune de la enfermedad celíaca. Adaptado de Kagnoff (2007), Briani (2008) y Alaedini (2005).

La respuesta inmune adaptativa está mediada por las células T CD4⁺ reactivas frente a gliadina en la lámina propia. Los péptidos de gliadina son capaces de unirse a moléculas DQ2 o DQ8 del complejo mayor de histocompatibilidad de clase II (HLA, por sus siglas en inglés) en las células presentadoras de antígeno, y las células T producen citoquinas proinflamatorias, en concreto interferón-γ (IFN-γ) (Nilsen et al. 1998). De hecho, se han aislado células T CD4⁺ específicas frente a α-gliadina en pacientes celíacos, cuando no estaban siguiendo una dieta libre de gluten, pero no están presentes en un intestino normal ni en pacientes tratados (Nilsen et al. 1995).

La enzima transglutaminasa tisular (tTG) juega un papel fundamental en la cascada de reacciones nocivas de la enfermedad celíaca (Anderson et al. 2000; Arentz-Hansen et al. 2000b; McL Mowat 2003). Es una enzima del

intestino que desamida péptidos de gliadina, aumentando su inmunogenicidad, debido a que esta reacción enzimática genera aminoácidos cargados negativamente en la proteína que son esenciales para que la molécula HLA-DQ2 reconozca y se una a ella (Anderson et al. 2000; Arentz-Hansen et al. 2000b; Arentz-Hansen et al. 2002; Molberg et al. 1998; Vader et al. 2002). Una vez modificada y unida, la proteína es capaz de liberar una cascada de mediadores de daño tisular que inducen la hiperplasia de las criptas y el daño de las vellosidades de las células intestinales (Alaedini and Green 2005).

Además, los péptidos de gliadina activan también la respuesta inmune innata en el epitelio intestinal: aumentan la expresión de interleucina-15 (IL-15) por los enterocitos, lo que genera la expresión del receptor de activación NK-G2D por parte de los linfocitos intraepiteliales. Este receptor es un marcador de las células natural-killer, por lo que estas células activadas se vuelven citotóxicas y son capaces de provocar la muerte de los enterocitos cuya superficie expresa el complejo mayor de histocompatibilidad de tipo I relacionado con cadena A (MIC-A), un antígeno superficial inducido por estrés (como infección), lo que provoca que el daño en las células intestinales sea aún mayor. El mecanismo de interacción entre los procesos que ocurren en la lámina propia y en el epitelio no ha sido aún elucidado.

Factores genéticos

A pesar de todo lo expuesto anteriormente, la enfermedad celíaca no se desarrolla a menos que la persona tenga alelos que codifiquen determinadas proteínas HLA. Existe una elevada relación entre la enfermedad celiaca y determinados genes de HLA (DR3, DR5/DR7 o DR4) conocidos como HLA-DQ2 y HLA-DQ8, localizados en el cromosoma 6p21 (Catassi and Fasano 2008) que comprenden una cadena de DQA1*0501 con una cadena de DQB1*0201 o DQB1*0301, o DQA1*0301 con cadena de DRB1*0401 o DQB1*0302 (Sollid 2002; Sollid et al. 1989). Se asocia con HLA-DQ2 haplotipos DR-17 o DR5/7, y, en un menor grado con el haplotipo DR-4 de HLA-DQ8 (Tollefsen et al. 2006). Estos haplotipos se encuentran localizados en la membrana de las células presentadoras de antígeno presentes en la mucosa y se encargan de presentar antígenos peptídicos relativamente pequeños (epítomos) a linfocitos T CD4⁺. Estas moléculas son una de las piezas más importantes en la respuesta aberrante del sistema inmune al gluten en los pacientes celíacos (McL Mowat 2003). Aproximadamente el 95% de los pacientes celíacos presentan heterodímeros de HLA-DQ2, y el resto presentan HLA-DQ8 (Kim et al. 2004; Tollefsen et al. 2006). Tanto es así que la ausencia de estas moléculas tiene un valor predictivo negativo para enfermedad celiaca de casi el 100%, esto es, la probabilidad de no padecer enfermedad celiaca si no están presentes estas moléculas es del 100% (Kaukinen et al. 2002).

Aunque existen claras relaciones positivas entre la existencia de estas proteínas concretas y la enfermedad celíaca, se trata de una condición necesaria pero no es suficiente para el desarrollo de esta enfermedad. De hecho, se sabe que contribuyen en aproximadamente un 50% en su desarrollo. Si bien se han identificado algunos genes no HLA, no se conoce su influencia exacta (Romanos et al. 2009).

Factores ambientales

Dada la alta prevalencia de la enfermedad celíaca, se han hecho muchos estudios epidemiológicos al respecto. En las últimas décadas existe una enorme discusión respecto al momento idóneo de introducción del gluten en los lactantes, porque pudiera tener alguna relación con el desarrollo de la enfermedad celiaca (Norris et al. 2005). Se ha descrito la lactancia materna como un factor protector en el desarrollo de la enfermedad celíaca (D'Amico et al. 2005), así como la introducción de gluten después de 7 meses de edad y sin interrumpir el periodo de lactancia materna.

Recientemente se ha relacionado el padecimiento de algunas infecciones intestinales durante la infancia, como la de rotavirus, con el incremento en el riesgo de aparición de celiacía (Stene et al. 2006). En la actualidad numerosos estudios están tratando de caracterizar otros factores ambientales que pudieran estar afectando de forma tanto positiva como negativa al desarrollo de esta enfermedad.

1.1.2 Manifestaciones clínicas

El abanico que abarcan los síntomas de la enfermedad celíaca es muy amplio. Además, las manifestaciones clínicas de los enfermos varían mucho dependiendo del grupo de edad en el que se encuentren (Tack et al. 2010). En el caso de los niños se caracteriza por la presencia de diarrea, dolor y distensión abdominal, aunque también son frecuentes otros síntomas extra-intestinales como baja estatura, síntomas neurológicos y anemia (Fasano and Catassi 2012). En el caso de adultos, las mujeres lo padecen 2-3 veces más. Aunque no se conocen las causas, sucede de la misma forma con todas las enfermedades autoinmunes, que se presentan hasta en el doble de mujeres que de hombres. En pacientes en edad adulta, la enfermedad celiaca suele cursar con deficiencia de hierro y osteoporosis, diarrea, dolor abdominal, reflujo gastroesofágico y dermatitis herpetiforme (Dewar and Ciclitira 2005). También se ha observado una alta incidencia de infertilidad en personas con enfermedad celíaca (Meloni et al. 1999). Los pacientes celíacos tienen un riesgo incrementado de desarrollar enfermedades autoinmunes (Smedby et al. 2005).

Por lo tanto, la enfermedad celiaca es una enfermedad multisistémica, más que restringida al sistema gastrointestinal. La aparición de dermatitis herpetiforme, determinados problemas neurológicos, endocrinos y cánceres son síntomas patognomónicos de la enfermedad, esto es, si se encuentran presentes son claros definitorios de su existencia (Fasano and Catassi 2001). Aun así, hay variedades de la enfermedad celíaca, denominada atípica, que no presentan esta sintomatología clínica (Reilly and Green 2012; Reilly et al. 2012). También se han encontrado casos de enfermedad celiaca silente y latente, siendo ambas formas asintomáticas, aunque en la primera los individuos presentan test serológicos positivos y daños en la mucosa del intestino, y en el segundo, es positiva la serología pero no la histología, pudiendo en ocasiones desarrollar un cambio en la histología a lo largo del tiempo, e incluso, desarrollar sintomatología (Ferguson et al. 1993; Troncone et al. 1996). Esto hace que se haya denominado a la enfermedad celíaca como el *camaleón de la medicina*, debido a la variedad de formas y edades en las que se puede presentar, lo que provoca que existan multitud de pacientes sin diagnosticar (Fasano 2003). De hecho, un estudio internacional reciente a gran escala, investigó en una muestra de población en cuatro países diferentes de Europa, encontrando que, de media la prevalencia de la enfermedad celíaca era superior al 1%, y destacó que muchos de los casos no habrían podido ser detectados de no ser por el cribado serológico, debido a que no presentaban sintomatología ninguna (Mustalahti et al. 2010). También es interesante mencionar que actualmente se encuentra en controversia el riesgo/beneficio de hacer cribados poblacionales de enfermedad celíaca, pero los defensores argumentan que serían beneficiosos dado que existe un tratamiento conocido de la enfermedad y que los efectos a largo plazo del consumo de gluten pueden ser muy graves (Mearin et al. 2005; van Koppen et al. 2009).

La evolución natural de la enfermedad celíaca sigue el siguiente orden: en primer lugar aparecen anticuerpos característicos de enfermedad celíaca, que pueden aparecer incluso antes de que la biopsia sea anormal. Después se desarrollaría la enteropatía intestinal, y consecutivamente, los síntomas intestinales y extraintestinales característicos. Por último, si la enfermedad sigue su curso, harían aparición determinadas complicaciones (Fasano and Catassi 2012). Como se ha comentado anteriormente, a pesar de lo que se pensaba inicialmente, varios estudios han indicado que la seroconversión no tiene por qué darse en la infancia, sino que puede ocurrir en cualquier momento. También se ha observado que la pérdida de tolerancia al gluten puede ser reversible en algunos individuos (Simell et al. 2007).

1.1.3 Diagnóstico

El diagnóstico de la enfermedad celíaca suele ser bastante complejo. Existen guías que establecen la necesidad de encontrar dos factores fundamentales para establecer un diagnóstico claro de celiaquía:

1. Debe presentarse al menos una biopsia duodenal donde aparezca linfocitosis intraepitelial e hiperplasia de las criptas.
2. Debe existir una respuesta positiva a dieta libre de gluten.

Según la Sociedad Europea de Gastroenterología, Hepatología y Nutrición Pediátrica (ESPGHAN) solo es necesaria la mejora y remisión de la sintomatología con la dieta. Aunque algunos autores recomiendan para el diagnóstico la visualización de la mejora histológica de la biopsia tras dieta libre de gluten, sobre todo en adultos. Hasta un 10% de los casos son muy difíciles de diagnosticar por la falta de concordancia entre los hallazgos serológicos, clínicos e histológicos (Alaedini and Green 2005). Por este motivo, para facilitar el diagnóstico, algunos autores han desarrollado árboles de decisión que incluyen test serológicos y biopsias intestinales (Figura 3).

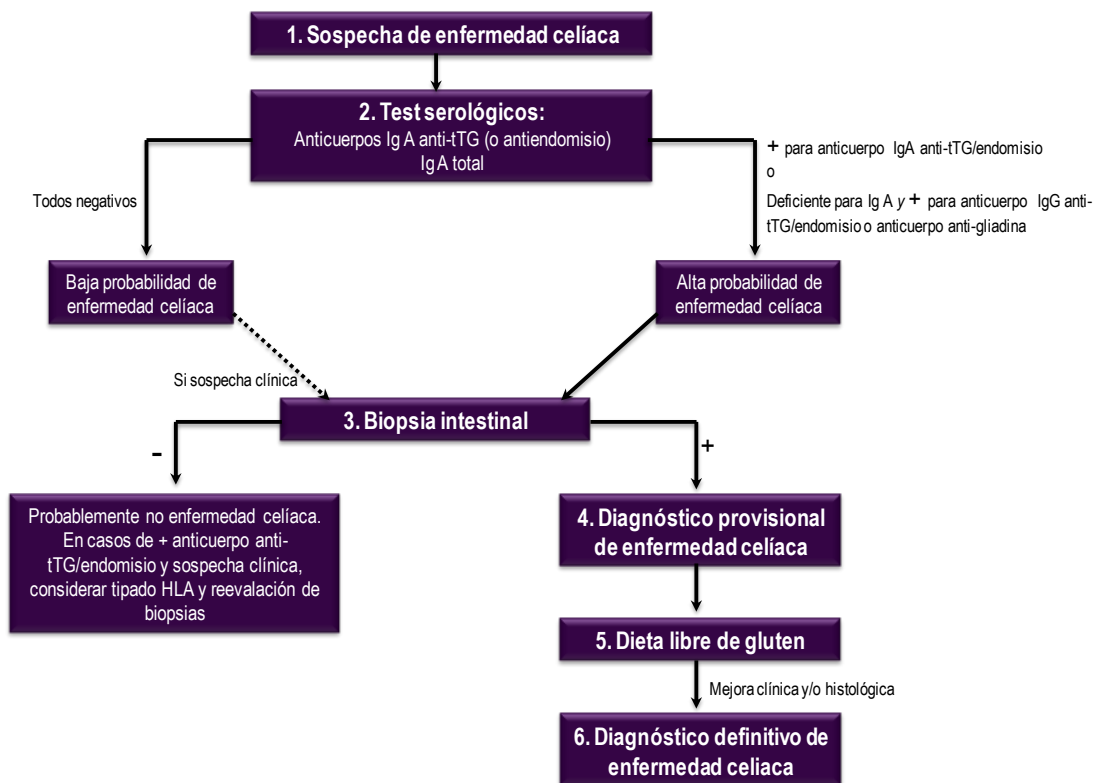


Figura 3. Algoritmo para la evaluación de pacientes sospechosos de presentar enfermedad celíaca. Adaptado de Alaedini (2005).

Análisis serológicos

Se recomienda realizar análisis serológicos cuando hay algún síntoma característico, como el síndrome de intestino irritable o ser familiar en primer grado de pacientes con enfermedad celíaca u otras enfermedades autoinmunes (Green and Jabri 2003). El anticuerpo más sensible es la inmunoglobulina A (IgA) (Hischenhuber et al. 2006). También existen anticuerpos antigliadina, anticuerpos para tejido conectivo (antirreticulina y antiendomiso), y anticuerpos contra tTG, la enzima responsable de la desamidación de la gliadina en la lámina propia (Green and Jabri 2006). La presencia de anticuerpos antigliadina se usó mucho en el pasado como método de diagnóstico serológico, pero no se consideran ya suficientemente específicos y sensibles (Rostami et al. 1999), excepto para niños de menos de 18 meses de edad. Los anticuerpos antirreticulina tampoco se suelen usar ya como marcadores de enfermedad celíaca (Alaedini and Green 2005).

El estándar diagnóstico basado en los análisis serológicos son los anticuerpos IgA de endomiso, que presentan casi 100% de precisión. Los anticuerpos anti tTG (Dieterich et al. 1997) permiten realizar test ELISA que son menos caros que los antiendomiso, y tienen un 90% de sensibilidad (Rostom et al. 2005). Las concentraciones de estos anticuerpos se relacionan con el grado de daño de la mucosa, por lo que la sensibilidad decrece fuertemente cuando los pacientes presentan una baja atrofia de las vellosidades intestinales.

Sin embargo, la deficiencia de IgA es frecuente en pacientes celíacos (1 de 40 en pacientes celíacos, frente a 1 de 400 en población general), por lo que se deben usar anticuerpos anti tTG, y comparar con los valores totales de IgA. Se recomienda detectar también IgM anti tTG (Alaedini and Green 2005; Cataldo et al. 1997).

Análisis de alelos HLA

El HLA-DQ2 se identifica en el 90-95% de los celíacos, y DQ8 en casi todos los demás pacientes (Kaukinen et al. 2002). Estos alelos aparecen en población general en 30-40% (HLA-DQ2 más frecuente que el HLA-DQ8), por lo que la ausencia de estos alelos es importante por su alto valor predictivo negativo. La ausencia de estos alelos define qué miembros de la familia deben ser analizados. Además, es un importante marcador de enfermedad celíaca para pacientes que ya siguen una dieta libre de gluten o para aquellos en los que el diagnóstico no está del todo claro.

Biopsia e histología

A pesar de los avances en el diagnóstico basados en análisis serológicos y de alelos, el estándar de diagnóstico de enfermedad celiaca continua siendo la biopsia y estudio histológico del duodeno (Valdimarsson et al. 1996). De esta forma, se recomienda realizar siempre que exista una sospecha clínica, independientemente de los test serológicos (Rostami et al. 1999). En general, suelen tomarse 4-6 especímenes de biopsias endoscópicas del duodeno. Se deben reconocer síntomas de atrofia de vellosidades durante la visualización microscópica de la muestra (Green and Jabri 2006). Sin embargo, es frecuente la aparición de falsos negativos debido a los cambios irregulares que puede sufrir la histología del intestino delgado en estos pacientes.

1.1.4 Tratamiento

El único tratamiento eficaz es la eliminación de trigo, cebada y centeno de por vida de la dieta (Tack et al. 2010). No se recomienda tratamiento farmacológico, salvo en el caso de que los pacientes celiacos presenten dermatitis herpetiforme, en los que se suele combinar la dieta libre de gluten con tratamiento farmacológico con dapsona.

Aunque existen opciones que permiten sustituir trigo, cebada y centeno por otros cereales como el arroz, maíz, quínoa, amaranto, sorgo y mijo, que no contienen gluten (el caso de la avena se discutirá más adelante), es difícil lograr una adherencia total al tratamiento. A pesar de la alta prevalencia de la enfermedad celiaca, los alimentos sin gluten son difíciles de encontrar o muy caros en países en desarrollo, mientras que en otros países el gobierno subvenciona estos productos.

La supresión del consumo de cereales en los enfermos celiacos, y consumo de harinas sustitutivas no fortificadas con vitaminas del grupo B, provoca la frecuente aparición de deficiencias de este grupo de vitaminas en pacientes que mantienen esta dieta restrictiva por un largo periodo (de más de 10 años) (Hallert et al. 2002). Por este motivo, las guías recomiendan suplementos vitamínicos para cubrir las carencias nutricionales que pudieran sufrir estos pacientes. Además, en pacientes diagnosticados, se deben controlar deficiencias en ácido fólico, B₁₂, vitaminas liposolubles, hierro y calcio, porque es frecuente la aparición de problemas como osteoporosis. En general, la eliminación de gluten de la dieta provoca una mejora clínica en un período corto de tiempo, días o semanas, mientras que la recuperación histológica suele requerir de un período de tiempo mayor, de meses o años especialmente en adultos (Green and Jabri 2003).

Sin embargo, hay casos en los que hay una respuesta pobre o nula al tratamiento (Abdallah et al. 2007; Daum et al. 2005). En estos casos es

necesario reevaluar el diagnóstico, debido a que la atrofia de las vellosidades intestinales no es exclusiva de la enfermedad celíaca, existiendo otras enfermedades que pueden cursar con la misma. En otros casos de baja remisión de los síntomas se debe sospechar también de pobre adherencia al tratamiento. En este sentido, la persistencia de anticuerpos antiendomiosio o antiTG después de un año de la supuesta instauración de una dieta libre de gluten, suelen indicar una mala adherencia al tratamiento (Dickey et al. 2000; Pietzak 2005). Es especialmente importante insistir en la instauración temprana del tratamiento y promover su seguimiento, debido a que, como se ha dicho anteriormente, el consumo de gluten puede provocar complicaciones graves a largo plazo como adenocarcinoma de intestino delgado, enteropatía asociada a linfoma de células T o esprúe refractario (Rubesin et al. 1989; Straker et al. 1989).

Dos temas importantes para los pacientes celíacos y que provocan controversia entre científicos y terapeutas de todo el mundo son el consumo de cereales malteados y de avena (Nasr et al. 2015). En el primer caso no existe evidencia de que el gluten tóxico se encuentre totalmente hidrolizado en los alimentos malteados aunque el cereal esté degradado. De hecho, se sabe que determinadas regiones de la gliadina son muy resistentes a la degradación enzimática (Arentz–Hansen et al. 2002; Shan et al. 2002) y además se han encontrado epítomos homólogos presentes en la gliadina en productos malteados y cervezas, procedentes de cebada (Ellis et al. 1994). Todo junto con el hecho de que algunos pacientes celíacos presentan sintomatología tras el consumo de grandes cantidades de productos malteados, parece apuntar a la necesidad de evitar el consumo de alimentos malteados por los enfermos celíacos.

Por otro lado, el potencial tóxico de la avena ha sido estudiado intensamente y existe una gran controversia al respecto (Thompson 2003). Se trata de un cereal filogenéticamente más alejado del trigo que la cebada y el centeno. Algunos autores señalan que la avena no es tóxica y es bien tolerada por los pacientes celíacos (Janatuinen et al. 2002), pero que los productos comercializados se encuentran generalmente contaminados con cereales que contienen gluten (Briani et al. 2008; Green and Cellier 2007). Otros autores opinan que existen grupos de pacientes celíacos que son intolerantes a la avena (Lundin et al. 2003), por lo que es difícil concluir si la avena es segura o no para el consumo por los enfermos celíacos, y en general, se recomienda evitarla.

Existen numerosos estudios encaminados a la búsqueda de tratamientos alternativos para la enfermedad celíaca. Entre ellos, se están desarrollando enzimas recombinantes que sean capaces de digerir las fracciones tóxicas de gliadina y otros componentes del gluten (Gass et al. 2007; Matysiak–Budnik et

al. 2005; McL Mowat 2003; Sollid and Khosla 2005; Stepniak et al. 2006). Otras líneas de investigación están orientadas al desarrollo de terapias que inhiban la respuesta inmune, como por ejemplo el bloqueo de la unión de la gliadina desamidada a HLA-DQ2 y DQ8 (Schuppan et al. 2009), o el bloqueo/modulación selectiva de la acción de la tTG (Reif and Lerner 2004), entre otras.

1.1.5 Marco legislativo

El consumo cada vez mayor de alimentos procesados ha obligado a los gobiernos a someter a la industria alimentaria a controles más estrictos de etiquetado de sus productos. Además las cadenas de suministro y los procesos de fabricación son cada vez más complejos, lo que genera que la presencia accidental de pequeñas cantidades de alérgenos y gluten sea inevitable en muchos productos (Crevel et al. 2008).

En el caso del gluten, al contrario de lo que ocurre para la mayoría de los alérgenos alimentarios, donde se debe etiquetar la presencia de alérgeno independientemente de la cantidad presente en el alimento, se han establecido umbrales límites bien definidos para el etiquetado de los alimentos con gluten. Sin embargo, aunque los niveles de seguridad universales para el consumo de gluten aún no se han definido, la literatura sugiere que pequeñas cantidades de gluten podrían ser suficientes para causar daño a la mucosa intestinal de los celíacos a largo plazo (Catassi et al. 2007) y un mayor riesgo de desarrollar trastornos autoinmunes (Ventura et al. 1999). En el caso de la enfermedad celíaca, el etiquetado es especialmente importante ya que ayuda al paciente a elegir el producto e identificar su contenido (Premanandh 2011). El Codex Alimentarius propone un límite de gluten de 0,01%, esto es, 100 ppm para alimentos libres de gluten y de 0,002 % (20 ppm) para alimentos naturalmente libres de gluten (Codex Alimentarius Commission 2008). En Europa se han adoptado una serie de normativas que establecen un contenido máximo de gluten de 20 mg/kg en el producto final, para etiquetar el alimento o producto alimenticio como "sin gluten", mientras que puede ser etiquetado como "con bajo contenido en gluten" si su contenido en gluten es inferior a 100 mg/kg (Regulation (EC) 41/2009 ; Regulation (EC) 609/2013 ; Regulation (EC) 828/2014 ; Regulation (EC) 1169/2011).

A pesar de existir un límite claro, para protegerse de sanciones y posibles perjuicios los fabricantes están comenzando a incrementar, y en ocasiones a abusar, del uso del etiquetado de precaución "puede contener" en los productos alimenticios, lo que conduce a una limitación innecesaria de elección de los consumidores a la devaluación de la información del etiquetado de todo tipo alérgenos, no sólo del gluten (van Hengel 2007).

Además de establecer claros límites para el etiquetado, la normativa vigente especifica que debería utilizarse el método basado en enzimoimmunoensayo (ELISA) R5 Méndez para el análisis de gluten. Si bien también establece que el análisis también podrá realizarse por otros métodos, siempre que el límite de detección sea igual o inferior a 10 mg/kg de gluten y que posea, como mínimo, la misma sensibilidad y especificidad que el ELISA antes mencionado (Codex Alimentarius Commission 2008). También se especifica que el análisis cualitativo de la presencia de gluten podrá realizarse por métodos ELISA o por métodos basados en el ADN.

1.2 Métodos de análisis de gluten en alimentos

Es importante contar con métodos analíticos capaces de detectar pequeñas cantidades de cereales y así poder identificar la presencia de trigo o cereales tóxicos en alimentos, incluso cuando se trate de alimentos muy procesados. La mayoría de los métodos empleados en la detección de gluten se basan en la detección de proteínas, en concreto suelen usarse ensayos ELISA (Diaz-Amigo and Popping 2013; Janssen 2006).

Skerrit et al. desarrollaron los primeros kits comerciales para la detección de gluten en alimentos (Skerritt and Hill 1990), que fueron adoptados como método oficial en 1991 por la Asociación Internacional Oficial de Químicos Analíticos (de sus siglas en inglés, AOAC). Se trata de un ensayo inmunoenzimático diseñado para detectar ω -gliadinas, que, tiene un importante inconveniente: su falta de sensibilidad contra prolaminas de cebada (Poms et al. 2004b). En los últimos años se han realizado grandes esfuerzos para desarrollar métodos para la detección de gluten, entre los que destaca el ELISA-R5 tipo sándwich desarrollado por Méndez et al. que es capaz de detectar gluten de trigo, cebada o centeno, en el intervalo de concentraciones de 5 a 200 ppm (Méndez et al. 2005). Este se clasificó como método de tipo I según el Codex sobre Métodos de Análisis y Toma de Muestras (Codex Alimentarius Commission 2006).

Sin embargo, en ocasiones pueden aparecer falsos negativos o reacciones cruzadas que podrían limitar la aplicación universal de estos métodos (Haraszi et al. 2011; Mujico et al. 2011). Algunos de los anticuerpos desarrollados tienen problemas para detectar gluten cuando se ha hidrolizado enzimáticamente o cuando se ha tratado térmicamente. Por ejemplo el ELISA de Skerritt no permitía la cuantificación de gluten modificado (Skerritt and Hill 1990). Por otro lado, los anticuerpos de Ellis (1998) se ven menos afectados por la hidrólisis porque reconocen epítomos lineales. Hay que tener en cuenta que la modificación de la toxicidad para el paciente celiaco cuando se altera química o enzimáticamente el gluten no se conoce con exactitud, y tampoco se

ha podido relacionar la disminución del efecto nocivo del gluten cuando este procede de alimentos altamente procesados. Además, la industria alimentaria realiza frecuentemente modificaciones sobre la proteína del gluten para mejorar su solubilidad o propiedades (Denery-Papini et al. 1999), que ponen en una encrucijada a los métodos que detectan proteínas, como los ELISA. Se han observado enormes diferencias cuando se analiza el contenido en prolaminas con diferentes kits de ELISA, que usan diferentes anticuerpos (van Eckert et al. 2006). Se sabe que la eficiencia de los test inmunoquímicos depende de varios factores: el método de producción del anticuerpo, el formato del test y el método de extracción del gluten. El tratamiento térmico que ha sufrido el alimento influye negativamente en la extractabilidad y detección de estas proteínas, y en general, da lugar a una subestimación del contenido en gluten. Las fracciones de prolaminas, generalmente detectadas por estos test, se suelen agregar y formar enlaces S-S intercatenarios, y en esta forma son insolubles en mezclas alcohol-agua, medio habitual de extracción. Por tanto, en muestras procesadas térmicamente, se hace necesario el uso de agentes reductores y caotrópicos adicionales como el mercaptoetanol o el cloruro de guanidina, capaces de romper los enlaces S-S intercatenarios de la proteína y solubilizar el gluten. El uso de estos reactivos tan diversos ha motivado que la disolución de extracción se denomine cocktail (Denery-Papini et al. 1999) siendo el más extendido el desarrollado por el grupo del Prof. Méndez et al. al que se debe el anticuerpo R5 y su método ELISA asociado (García et al. 2005). Sin embargo, algunos ensayos ELISA competitivos son incompatibles con este cocktail de extracción, porque sus componentes pueden desnaturizar el receptor proteico. En este sentido, se están estudiando condiciones de solubilización que sean compatibles con los métodos inmunoquímicos de cuantificación, y que mejoren la extracción tanto de proteína de gluten nativa como de desnaturizada (Gessendorfer et al. 2010; Kanerva et al. 2008; Mena et al. 2012).

Un tipo de receptor alternativo a los anticuerpos para el análisis de proteínas alergénicas la constituyen los aptámeros, receptores no proteicos formados por una hebra sencilla de ADN. Estos receptores se basan en la afinidad que tienen los ácidos nucleicos de cadena sencilla y estructura tridimensional por las proteínas, mediante interacciones débiles no covalentes de tipo electrostático, como enlace de hidrógeno o de van der Waals (Amaya-González et al. 2013). Una de las ventajas principales de los aptámeros es que se obtienen por síntesis química, por lo que se pueden obtener de forma muy reproducible y no requiere el uso de animales para su obtención (Liu and Zhang 2015) lo que permite utilizarlos como receptores biológicos en el diseño de sensores (aptasensores). Recientemente se han desarrollado aptasensores electroquímicos para la detección de fracciones de la proteína del gluten altamente tóxicas para los pacientes celíacos, como la gliadina (Pinto et al. 2014) y el fragmento 33-mer de la gliadina (Amaya-Gonzalez et al. 2014;

Amaya-González et al. 2015a; Amaya-González et al. 2015b). Pinto et al. (2014) utilizaron un ensayo aptaPCR, es decir, un ELISA competitivo y una amplificación por PCR en tiempo real cuantitativa. Sin embargo, su detectabilidad es insuficiente para el análisis de muestras reales, ya que es de 100 ppb sin tener en cuenta la extracción-dilución imprescindible de la muestra, que alcanza normalmente factores de 500-1000. Paralelamente Amaya-González et al. (2014; 2015a; 2015b) describieron varios aptámeros contra el péptido inmunodominante 33-mer que permitieron desarrollar un aptaensayo competitivo con detección electroquímica que responde selectivamente a cereales tóxicos y fue capaz cuantificar la concentración de gluten en muestras reales, tanto las sometidas a tratamientos de hidrólisis, como muestras no hidrolizadas, con un límite de detección de 0,5 ppm, esto es, incluso por debajo de los criterios exigibles por la normativa vigente, con una buena reproducibilidad. Los resultados obtenidos fueron validados por varios laboratorios usando ELISA, y se obtuvieron algunas diferencias en muestras complejas desde el punto analítico, como la cerveza, donde el aptasensor proporcionó un resultado positivo, mientras que el ELISA no fue capaz de detectar la presencia de gluten posiblemente debido a la hidrólisis del alimento, que provoca la degradación del fragmento proteico reconocido por el anticuerpo (Amaya-González et al. 2015a).

Sin embargo, a pesar de los enormes avances en el campo de la detección de las proteínas de gluten, algunas de las limitaciones de los métodos existentes han fomentado el desarrollo de tecnologías alternativas. Así, los métodos basados en el ADN, como la reacción de la amplificación en cadena de la polimerasa (PCR) o los genosensores, representan opciones atractivas. Poseen numerosas ventajas: la estabilidad térmica, la posibilidad de amplificación de analito, ADN, aumenta enormemente la sensibilidad que se puede alcanzar con estos métodos (Mafra et al. 2008a; Manzanares-Palenzuela et al. 2015; Martins-Lopes et al. 2013). La PCR se ha empleado con éxito en la identificación de organismos modificados genéticamente, de patógenos y en la identificación de especies de alimentos (Poms et al. 2004a). Los métodos de PCR han empezado a proliferar desde el año 2000, probablemente debido al establecimiento de normativas que exigen el etiquetado obligatorio de alérgenos. Sin embargo, en ocasiones estos métodos tropiezan con el problema de la transformación de los datos obtenidos, copias de ADN, a porcentaje de alérgeno en el alimento, dato que realmente tiene valor para los pacientes alérgicos y celíacos, y que es especialmente importante en el caso del gluten, donde la legislación establece una concentración límite a partir de la cual el alimento no es seguro para los enfermos celíacos. A pesar de esta limitación por el carácter indirecto de las cuantificaciones cuando se aplican estos métodos, en países como Alemania o Japón, se ha incluido la PCR como método analítico oficial para la detección de

alérgenos (Holzhauser and Röder 2015), además de haber sido empleada de forma rutinaria en análisis clínicos.

1.2.1 Principios de la reacción en cadena de la polimerasa, PCR

La PCR consiste en una amplificación exponencial específica de secuencias de ADN usando una enzima ADN polimerasa termoestable, que mimetiza la síntesis natural de estas moléculas.

En general se pueden distinguir las siguientes etapas (Figura 4): 1) desnaturalización, 2) unión de los cebadores a la hebra simple de ADN, 3) fase de extensión. 1) La primera etapa de desnaturalización consiste en la apertura de la hebra doble de ADN (ADNds) mediante la aplicación de una temperatura alta, de entre 93-96 °C, para romper los enlaces de hidrógeno que unen las dos hebras en el ADNds. La temperatura de disociación o T_m (*melting temperature*), es la temperatura a la que la mitad del ADN se encuentra como hebra simple. Esta temperatura varía según la naturaleza del disolvente, la fuerza iónica, pH y la relación entre las concentraciones de G/C y T/A. La segunda fase, unión de los cebadores, más conocida como *annealing*, tiene lugar a una temperatura próxima a la T_m de los cebadores (55-65 °C), que son secuencias cortas (15-25 nt), complementarias a los sitios de reconocimiento, que flanquean al target. Cuando la temperatura se reduce, las cadenas simples de ADN (ADNss) tienden a re-hibridizarse a ADNds. En esta fase, se produce un equilibrio entre la hebra simple, los cebadores por separado y el dúplex formado por la hebra unida a los cebadores. El cebador estabiliza la estructura ADNds naciente, y permite la unión de la polimerasa. 3) La fase de extensión consiste en la copia de la hebra de ADN que lleva a cabo una polimerasa, estable al calor, en presencia de dNTPs y $MgCl_2$, dando lugar a la duplicación del material de partida. La enzima tiene actividad 5'→3' polimerasa a partir del cebador usando una de las hebras de ADN como molde. Todas estas etapas constituyen un ciclo de amplificación de PCR y se llevan a cabo en un termociclador, que controla de forma precisa la temperatura y duración de cada etapa. La amplificación por PCR requiere, normalmente, entre 26 y 40 ciclos. Después de cada ciclo, cada una de las hebras de ADN sintetizadas actúa como molde en el siguiente ciclo, por lo que la cantidad de ADN se duplica en cada ciclo. Los productos del primer ciclo de amplificación tienen tamaños heterogéneos, que pueden exceder la distancia entre los sitios de unión de los dos cebadores. En el segundo ciclo, estas moléculas generan hebras de ADN de tamaños definidos que se acumulan siguiendo un modelo exponencial, a medida que transcurren los ciclos de amplificación en los siguientes ciclos de amplificación, y que serán los productos dominantes de la reacción. La cantidad de hebras obtenidas dependerá del número de ciclos y de la cantidad inicial y se puede calcular mediante la fórmula: $C_n = C_0 (1+E)^n$, siendo E la eficacia de la reacción de amplificación, y C_n y C_0 las cantidades final e inicial

de ADN, respectivamente (Rodríguez-Lazaro and Hernandez 2013). La especificidad y sensibilidad de la polimerización se puede modificar mediante el control de la temperatura de *annealing*.

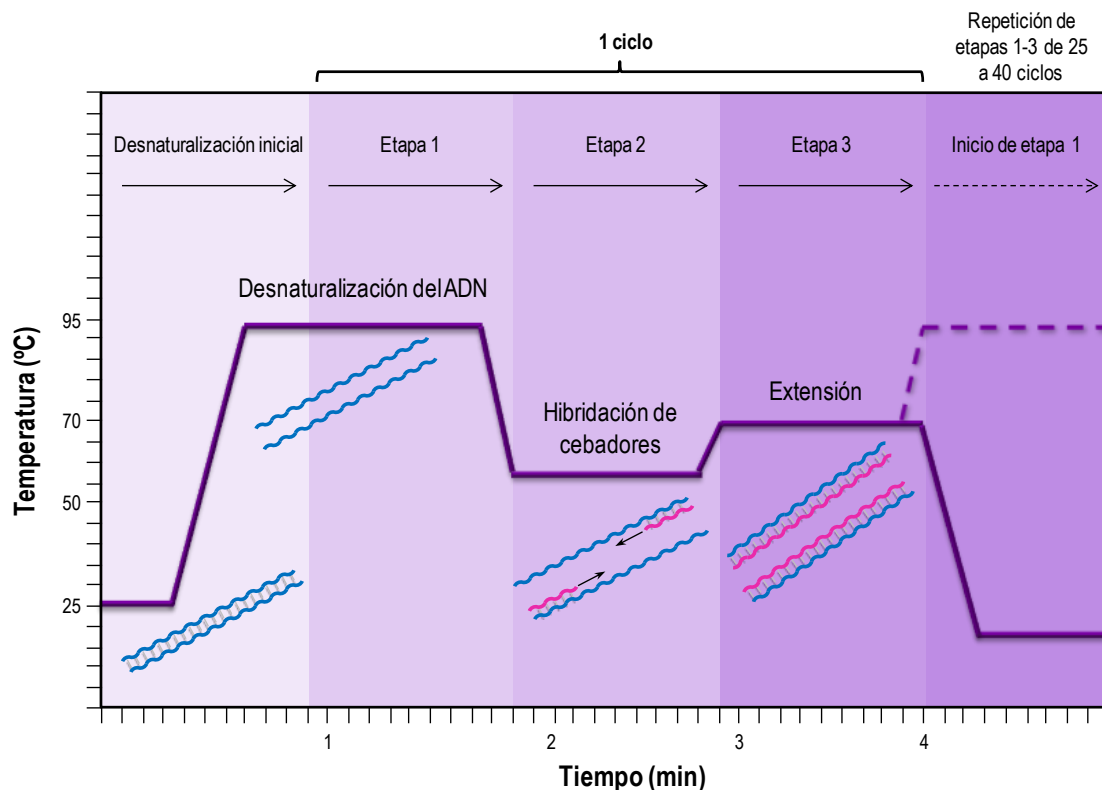


Figura 4. Etapas de la amplificación por PCR.

Históricamente la detección de los productos de PCR se llevaba a cabo mediante electroforesis en gel de agarosa que permite la separación de los fragmentos amplificados según su tamaño. Este método presenta importantes problemas que son: la aparición de contaminaciones por las manipulaciones del operador, los reactivos habitualmente empleados son carcinogénicos y que no permite el análisis cuantitativo de forma totalmente fiable.

Actualmente los métodos más empleados en la cuantificación de los amplicones son los de detección a tiempo real, PCR en tiempo real. El desarrollo de la PCR en tiempo real ha supuesto un gran avance para las técnicas de biología molecular, dado que permite la monitorización de la síntesis de nuevas moléculas de amplicón durante la PCR mediante medidas de fluorescencia, y no solo su detección final como en la PCR convencional. Pero además presenta dos ventajas destacables: la reacción y la detección se realizan en un único pocillo, lo que provoca una menor contaminación cruzada, y se trata de un método rápido con un intervalo lineal muy amplio (Rodríguez-Lazaro and Hernandez 2013).

Se pueden emplear dos tipos de sondas fluorescentes: las universales e inespecíficas, independiente de la secuencia de analito, y las específicas, esto es, dependientes de la secuencia a amplificar. En el primer caso se pueden usar como marcadores de amplificación moléculas fluorescentes que presentan diferente unión según el ADN se encuentre en su forma ADNss o ADNds, como por ejemplo, bromuro de etidio o SYBR Green. Sin embargo, usando este tipo de marcadores de fluorescencia la especificidad del método recae únicamente en los cebadores, por lo que se corre el riesgo de amplificar y detectar productos no específicos. Una forma de ganar especificidad en el análisis y cuantificación de ADN es el uso de sondas de ADN marcadas con compuestos fluorescentes, que se pueden clasificar en dos grupos: de hidrólisis y de hibridación, ambas capaces de hibridar con la región amplificada por los cebadores. La señal de fluorescencia obtenida se puede relacionar con la cantidad de producto amplificado en la PCR.

Las sondas de hidrólisis se rompen cuando se elongan por la actividad exonucleasa 5' → 3' de algunas ADN polimerasas durante la fase de elongación de los cebadores, dando lugar a una emisión de fluorescencia medible a tiempo real que es proporcional a la concentración de la secuencia de amplicón. Las sondas de hidrólisis más empleadas son las TaqMan (Figura 5A), desarrolladas por Applied Biosystems. Las sondas de hibridación no se hidrolizan durante la PCR. La fluorescencia en este caso se genera por un cambio en su estructura secundaria que pasa de ser en forma de horquilla a lineal al hibridarse con el amplicón, lo que resulta en un incremento de la distancia que separa la molécula fluorescente de la bloqueante. Entre los más usados se encuentran los *molecular beacons* (Figura 5B) y los cebadores *Scorpion*.

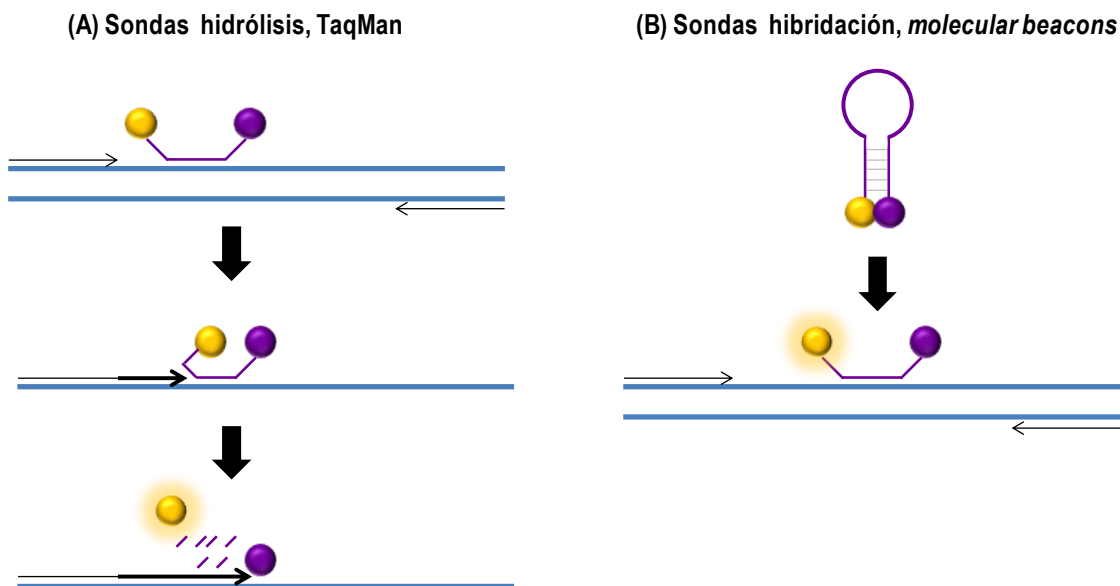


Figura 5. PCR en tiempo real, fundamentos de la detección según el tipo de sonda específica utilizada. Adaptado de www-bio-rad.com.

Sea cual sea el tipo de marcaje empleado, el análisis cuantitativo en PCR en tiempo real se basa en que la emisión de fluorescencia es proporcional al ADN sintetizado. Al representar la fluorescencia en función del número de ciclos de amplificación se obtienen curvas de amplificación (Figura 6). Éstas constan de 3 regiones: la primera correspondiente a la fase de iniciación, donde no se diferencia la fluorescencia de la línea base, una segunda región correspondiente a la fase exponencial, donde la fluorescencia aumenta exponencialmente, y la tercera región correspondiente a la fase de saturación, donde los reactivos empleados están agotándose y por tanto desaparece la relación entre la intensidad de fluorescencia y la cantidad de ADN amplificado. Por ello, es importante destacar que la cuantificación del contenido de ADN solo se puede llevar a cabo en la fase exponencial (Ginzinger 2002).

El ciclo umbral (*cycle threshold, Ct*) es el ciclo en el que la fluorescencia aumenta de forma estadísticamente significativa, y adquiere un valor límite establecido (Figura 6). Está inversamente relacionado con la cantidad de ácido nucleico en la muestra inicial, y por tanto, permite la cuantificación, y se encuentra siempre en la fase de amplificación exponencial, donde la amplificación es más eficiente, y de esta forma la cuantificación está menos afectada por las condiciones limitantes de la reacción.

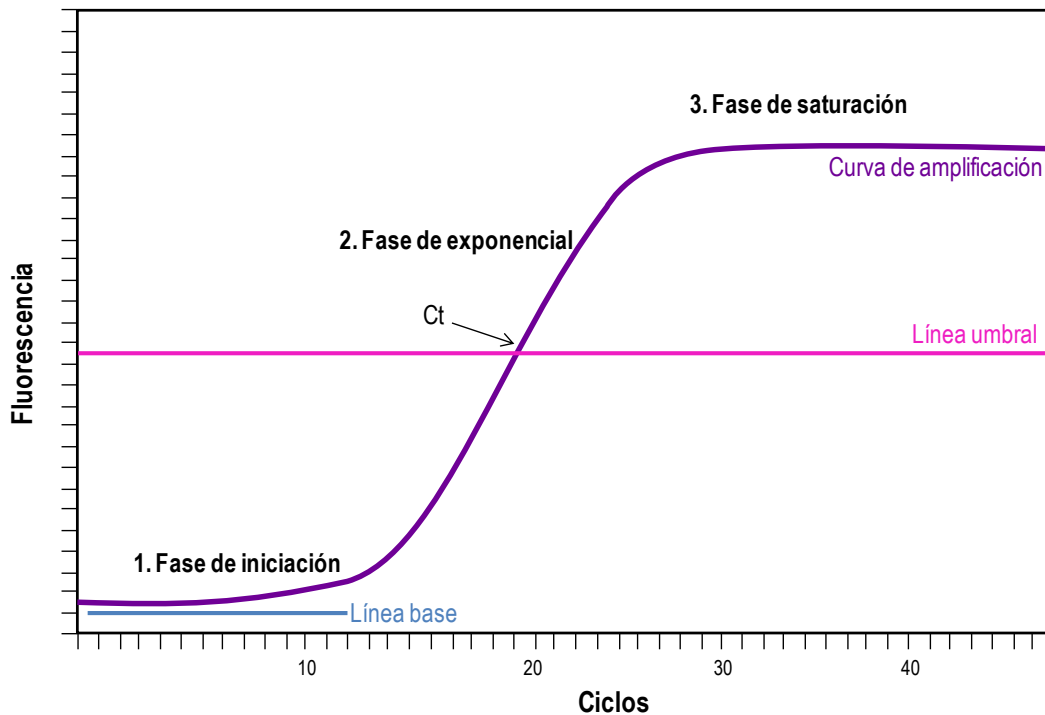


Figura 6. Curva de amplificación y fases que la componen.

Al no existir una normativa específica para la detección de ADN de alérgenos alimentarios, se usan los requisitos mínimos de la Red Europea de Laboratorios para la detección de organismos genéticamente modificados en

alimentos (ENGL 2008). Para poder cuantificar las cantidades iniciales de ADN molde con alta precisión se deben construir curvas de calibrado, donde lo que se representa es el ciclo umbral Ct hallado para diferentes concentraciones de ADN de partida, por ejemplo, de diluciones seriadas de estándares de concentraciones conocidas (Figura 7). De esta forma, la cantidad inicial de ADN de una muestra desconocida puede calcularse por interpolación del Ct en la curva de calibrado.

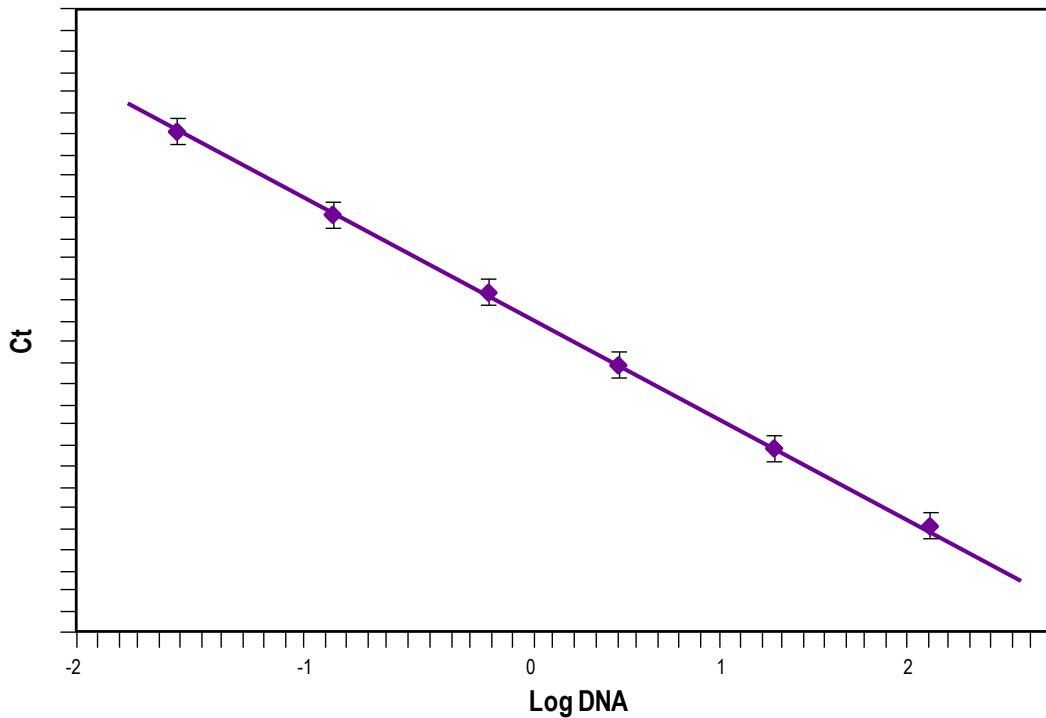


Figura 7. Curva de calibrado que representa la cantidad inicial conocida de ADN frente a los valores de Ct.

Según las guías anteriormente referidas, el límite de detección (LD) en los métodos de PCR, se define como la mínima cantidad de analito distinguible de cero detectado en al menos el 95% de los replicados, lo que asegura una cantidad de falsos negativos de menos del 5% (ENGL 2008). Muchos de los trabajos de detección indirecta de alérgenos y gluten han diferenciado entre el LD absoluto y relativo. El primero se obtiene con la última concentración a la que una dilución seriada de ADN extraído amplifica positivamente, y es distinguible del blanco. Para el LD relativo normalmente se realizan diluciones en peso del analito en una matriz inerte, expresándose así el LD como un valor relativo entre la cantidad de analito en una matriz inerte, para la concentración más pequeña que sea posible amplificar.

Además, a partir de la curva de calibrado se puede obtener la eficiencia de PCR, a partir de la fórmula: $\text{eficiencia} = (10^{(1/-s)}) - 1$, siendo s el valor de la pendiente del calibrado. La eficiencia de la PCR se ve muy afectada por las

concentraciones de $MgCl_2$, cebadores y sondas, por lo que deben ser controladas y optimizadas con cuidado. Para realizar ensayos cuantitativos se requiere que las eficiencias de PCR se encuentren preferiblemente alrededor o por encima del 90%. Una pendiente de -3,32 indica la reacción de PCR es 100% eficiente, es decir, la cantidad de ADN se duplica en cada ciclo (ENGL 2008; Rodriguez-Lazaro and Hernandez 2013).

Desde luego, la correcta selección de una secuencia de ADN como target es fundamental para obtener una detección sensible y selectiva, y en este sentido, la calidad y la cantidad del ADN extraído son muy importantes. El ADN es una molécula relativamente estable, pero el procesado del alimento puede causar su fragmentación, hidrólisis y oxidación. Por este motivo, debe evitarse utilizar amplicones de PCR de más 150-200 bp de longitud, debido a la probabilidad de que se encuentren fragmentados en el alimento procesado y no sea posible amplificarlos (Holzhauser and Röder 2015).

Además de lo anterior, para la correcta selección de la secuencia analito a amplificar debe evaluarse el número de copias en las que se encuentra en la especie a analizar. Las secuencias multicopia de orgánulos tipo mitocondria o cloroplastos, permiten una detección 10-100 veces más sensible (Holzhauser and Röder 2015). Pero, por el contrario, esta sensibilidad aumentada va ligada a la pérdida de especificidad de las secuencias multi-copia conservadas a lo largo de la evolución, aunque esta falta de selectividad se debe demostrar experimentalmente.

1.2.2 Etapas en la detección de alérgenos y gluten mediante PCR

Para la detección de alérgenos alimentarios por métodos basados en ADN, se deben llevar a cabo las siguientes etapas: extracción y purificación de ADN amplificable presente en la muestra problema, amplificación de la secuencia de ADN analito específica y por último, detección del producto generado por PCR, ya sea por la visualización de bandas de un determinado tamaño tras su electroforesis en gel (PCR convencional) o por la monitorización de la fluorescencia (PCR en tiempo real).

Es de gran importancia disponer de un método de extracción y purificación del ADN adecuado. Las impurezas de matriz tienen más repercusión en la detección de ADN que en la detección de proteínas (Holzhauser and Röder 2015; Sun 2010). Existen numerosos inhibidores de PCR tales como grasas, polisacáridos, minerales, enzimas o altas concentraciones de polifenoles que pueden dificultar tanto la extracción como la amplificación por PCR. Por estos y otros motivos, la sensibilidad del método de PCR es muy (o extraordinariamente) dependiente de la calidad y cantidad del ADN extraído y por tanto, del kit de extracción empleado, por lo que esta etapa debe optimizarse cuidadosamente. Las etapas de las que consta

generalmente la extracción de ADN son: la homogeneización de la muestra, la liberación del ADN nuclear y de orgánulos tras la rotura de las membranas por detergentes y proteasas, y la posterior purificación de ADN extraído, por precipitación del mismo con alcoholes o detergentes, o bien por unión selectiva a material de sílice en presencia de sales caotrópicas. Hoy en día, existen numerosos métodos de extracción de ADN para alimentos procesados basados en kits comerciales o protocolos desarrollados en el propio laboratorio, como el método basado en el reactivo bromuro de hexadeciltrimetilamonio (CTAB). Sin embargo, su elección depende de la matriz del alimento (Mafra et al. 2008b). En consecuencia, la obtención de ADN de los alimentos a veces puede ser todo un desafío. También es extremadamente importante adoptar una serie de precauciones, como la limpieza con disoluciones descontaminantes, el cambio frecuente de guantes, y la separación de áreas de trabajo para evitar la contaminación de arrastre o contaminación cruzada, especialmente importante debido a la extrema sensibilidad de los métodos de detección de ADN, lo que ayuda a asegurar que el amplicón final proviene de la muestra y no es una mera contaminación.

Las impurezas procedentes de la matriz afectan significativamente la detección eficiente de secuencias de ADN, y la alta calidad del extracto de ADN tiene un gran impacto en la sensibilidad del método de detección de PCR (Gryson 2010), PCR en tiempo real o genosensores, por lo que muchos de los autores que se reseñarán a continuación han optimizado más o menos exhaustivamente el método de extracción de ADN, y se han descrito numerosos y variados métodos con este propósito.

Una vez extraído el ADN se deben usar controles para verificar que el ADN extraído posee la suficiente pureza e integridad y se encuentra libre de inhibidores (Sun 2010). Para ello, se deben realizar medidas espectrofotométricas UV-vis a distintas longitudes de onda: la absorbancia a 260 nm proporciona la concentración aproximada de ADN extraído, a 280 nm la cantidad de proteínas, a 230 y a 320 nm la presencia de compuestos fenólicos y otros interferentes, respectivamente. La pureza del ADN se puede calcular mediante la relación entre las absorbancias a 260 y 280 nm. El ADN de la muestra es apto para su amplificación siempre que esta relación se encuentre entre 1,8 y 2. La integridad del ADN genómico se puede evaluar mediante la electroforesis en gel de los extractos, que debe dar lugar a bandas de tamaños superiores a 30 kb. Se han adoptado numerosas estrategias con el propósito de verificar la amplificabilidad del ADN extraído, entre las que destacan la amplificación de secuencias universales, el uso de calibradores y adición estándar. Entre los primeros, se usan genes que se encuentren en el ADN de todas las muestras extraídas, preferentemente que no exhiban variación alélica, por ejemplo el gen de referencia ITS ribosómico (Arlorio et al. 2003), una secuencia del gen *trnL* del cloroplasto (Terzi et al. 2004), un microsatélite

de ADN presente en el genoma A de trigo duro y común (Pasqualone et al. 2007) o gen 18S rRNA presente en organismos eucariotas (Debnath et al. 2009; Pegels et al. 2015; Tavoletti et al. 2009; Zeltner et al. 2009).

Más recientemente se ha incorporado el uso de calibradores competitivos internos que sirven a la par de calibrador y de control de inhibición de la amplificación (Dahinden et al. 2001; Taverniers et al. 2005; von Buren et al. 2001). Los calibradores internos son plásmidos recombinantes que contienen secuencias analito como material de referencia, y que se emplean como estándares internos para cuantificar ADN. En estos casos, estos calibradores se obtienen por clonación, posterior amplificaron por PCR, separación por electroforesis, y adición en cantidades conocidas a la muestra, y permiten establecer el grado de amplificabilidad de la muestra, dando lugar a una adecuada cuantificación, aunque debido a la complejidad en su preparación y aplicación puede ser empleado solo en contados casos.

1.2.3 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante PCR

El código genético se basa en codones de tres nucleótidos por un solo aminoácido y para la mayoría de aminoácidos existe más de un codón, por lo que existen tres veces más codones que aminoácidos transcritos. La degeneración del código genético dificulta la detección de ADN, pero amplía las opciones en cuanto a la selección de secuencias para su detección, en comparación con las proteínas. Hay que tener en cuenta que en el genoma existen secuencias no codificantes (intrones) que pueden ser empleadas para la detección, por lo que la detección de secuencias de ácidos nucleicos genera multitud de posibles analitos, en comparación con la proteína transcrita. Además, el desarrollo de métodos de PCR proporciona una especificidad aún mayor que los métodos basados en anticuerpos, porque la unión de los cebadores de PCR se puede regular modificando la temperatura de trabajo (temperatura de *annealing*). Se puede alcanzar una gran sensibilidad en la cuantificación de ADN, y dado que el analito en sí se amplifica, se pueden detectar muy pequeñas cantidades de ADN (Holzhauser et al. 2006).

Un inconveniente importante en los métodos basados en la detección de ADN está relacionado con que la relación entre el contenido en gluten y de ADN es variable en granos de diversas especies y cultivares, lo que dificulta la cuantificación conforme a la legislación vigente. Aun así, teniendo en cuenta que esta variabilidad no excede de un orden de magnitud, los métodos basados en PCR en tiempo real han sido considerados como excelentes alternativas a ELISA en el análisis de gluten (Goesaert et al. 2005; Leszczyńska et al. 2014; Piknova et al. 2008). Recientemente R-Biopharm ha desarrollado un kit para la detección de gluten basado en PCR en tiempo real,

SureFood® Allergen ID Gluten. Aun así, actualmente, el número de kits comerciales de PCR en tiempo real para la detección y cuantificación de alérgenos en los alimentos todavía es muy limitado debido principalmente a que su desarrollo apenas comenzó hace 15 años y las mejoras en este campo han ido incorporándose de forma gradual.

El objetivo del análisis de cereales mediante detección de secuencias específicas de ADN es doble: la búsqueda de cereales tóxicos para los pacientes celíacos y la prevención del fraude alimentario por sustitución de especies de cereales por otras de inferior valor en el alimento final. Ambos tipos de análisis están estrechamente relacionados porque, en definitiva, van encaminados a detectar trigo común en cantidades traza en diferentes matrices y pueden ser empleados para uno u otro fin. Muchos de los métodos desarrollados, además de trigo, también son capaces de detectar otros cereales tóxicos para los celíacos, como cebada, centeno, avena o diferentes variedades de trigo, cereales que los pacientes celíacos no deben consumir. La enorme complejidad del genoma del trigo, probablemente el más complejo de las especies de plantas domesticadas, junto con su alta variabilidad genética hace la selección y detección de secuencias específicas de ADN una tarea difícil, y a la vez que de gran interés.

El primer método para determinar trigo por PCR en productos alimenticios fue descrito por Allmann et al. en 1992 (Allmann et al. 1992). Después, se han propuesto multitud de métodos de PCR y PCR en tiempo real para la detección de cantidades traza de trigo en productos alimenticios (Tabla 2). Las secuencias a amplificar son muy variadas, desde las seleccionadas entre los genes que codifican proteínas alergénicas (gluteninas, gliadinas) hasta la amplificación de regiones específicas del genoma del trigo que no codifican proteínas.

Análisis para la detección de la presencia de cereales tóxicos

En las dos últimas décadas han aparecido numerosos artículos que describen métodos para la detección de gluten de forma indirecta, que se pueden clasificar según el tipo de método en: PCR convencional y PCR en tiempo real.

PCR convencional

El primer método de PCR para detectar gluten utilizó una secuencia de un gen que responde a giberelinas de 159 bp amplificado por PCR convencional (Allmann et al. 1992). Este método presentó falsos negativos observados en muestras procesadas, e insuficiente sensibilidad, por lo que posteriormente incluyeron un control para detectar falsos negativos, basado en la amplificación positiva de un fragmento muy conservado de ADN eucariota

(Allmann et al. 1993). En este caso Allmann y colaboradores evaluaron la contaminación con trigo de varios alimentos usando para ello la amplificación por PCR de una región intergénica ribosómica multicopia de 25S y 18S rRNA de 109 bp, encontrando que los resultados obtenidos concordaban con los obtenidos mediante un ensayo tipo ELISA. Koppel et al. (1998) usaron la misma secuencia de bases, obteniendo una detectabilidad para trigo en avena de entre 0.001% (10 mg/kg) y 0,01% (100 mg/kg) (Koppel et al. 1998).

Ko et al. usaron PCR y un análisis de la amplificación aleatoria de ADN polimórfico (RAPD, *Random Amplification of Polymorphic DNA*). Para la primera usaron cebadores que amplificaban una secuencia multicopia del 5S rRNA y para la segunda cebadores aleatorios de 10 bp (Ko et al. 1994). La técnica RAPD se basa en el análisis de fragmentos de ADN obtenidos por amplificación de regiones aleatorias del genoma, ya que los cebadores son secuencias arbitrarias de ADN sintético. Esta técnica se basa en la utilización de un único oligonucleótido de 10 bp que hibrida al azar con el ADN en estudio, por lo que no necesita conocimientos previos de la secuencia y permite distinguir entre muchos organismos de forma simultánea. Sin embargo, los autores concluyeron que la discriminación entre mezclas de diferentes cereales era complicada y que era especialmente difícil diferenciar los perfiles de amplificación para diferentes variedades de trigo debido al alto polimorfismo que presentaba.

Dahinden et al. (2001) utilizando el intrón del gen *trnL* del cloroplasto como segmento a amplificar por PCR, detectaron el trigo con un límite de detección (LD) de 0,02% (200 mg/kg). Compararon los resultados de la PCR con ELISA para diferentes alimentos obtenidos de comercios locales, y observaron diferencias que pueden atribuirse a la incapacidad del ELISA para reconocer proteínas que, al ser procesadas térmicamente, pierden drásticamente su estructura tridimensional (desnaturalización). Usando los mismos cebadores, Olexová et al. (2006) obtuvieron un límite de detección de 0,1% de harina de trigo en harina de soja, y el mismo límite de detección para bizcochos fabricados por ellos mismos de harina de trigo en harina sin gluten. También detectaron la contaminación de tres productos comerciales etiquetados sin gluten. Usando una amplificación por PCR convencional del gen *trnL* del cloroplasto, Masková et al. (2011) consiguieron el mismo límite de detección, y analizaron una veintena de productos comerciales catalogados como sin gluten, encontrando que varios de ellos presentaban un contenido más alto en cereales tóxicos del permitido.

El gen tricitin de *Triticum aestivum* fue escogido por Yamakawa et al. (2007) para desarrollar un ensayo cualitativo de PCR capaz de detectar 0,005% (50 mg/kg) de trigo en matriz de maíz.

Otros autores emplearon un gen de glutenina de bajo peso molecular para amplificarlo por PCR para detectar hasta 21,5 pg de ADN de trigo (Debnath et al. 2009). Sonnante et al. (2009) utilizaron el mismo fragmento con una amplificación en tiempo real con sondas TaqMan para detectar contaminaciones de trigo blando en trigo duro, consiguiendo detectar hasta un 1,25%.

Tavoletti et al. (2009) analizaron piensos para animales en los que detectaron la presencia de trigo, trigo duro y cebada obteniendo un LD del 0,9% de harina de cereal en harina inerte, mediante la amplificación de un gen que codifica una proteína de transferencia de lípidos.

La mayoría de los métodos descritos usaron PCR convencional y análisis por electroforesis en gel. Además de estos, también se ha empleado PCR convencional acoplada a electroforesis capilar para la detección de trigo-cebada-centeno-avena entre otros alérgenos, obteniendo un LD de hasta 10 copias de ADN de trigo o 0,01% de harina de trigo en harina soja (Mustorp et al. 2011).

PCR en tiempo real

Desde el desarrollo de la PCR en tiempo real, se han descrito numerosos métodos, que presentan, entre otras ventajas, la monitorización *in situ* de la síntesis de amplicón y su cuantificación con mayor rapidez. Se han empleado tanto marcajes de tipo inespecífico, como SYBR Green, como de tipo específico, con la adición de sondas TaqMan para la detección de cereales tóxicos.

Entre los primeros, la utilización de SYBR Green para la PCR en tiempo real permitió la detección de ADN de trigo entre 5-50 pg (Sandberg et al. 2003) y 5 pg (Mujico et al. 2011). Además compararon los resultados con los obtenidos con ELISA usando anticuerpo R5, y encontraron una correlación positiva entre el contenido en proteínas y ADN en la mayoría de las muestras alimentarias. Observaron que el contenido en ADN fue menor en las muestras tratadas térmicamente a altas temperaturas en comparación con alimentos que contienen almidón, que explicaron por el alto grado de degradación del ADN en las primeras muestras, y por la extracción selectiva de las proteínas del almidón, que justificaban estas observaciones (Mujico et al. 2011). Por este motivo, sólo en algunas muestras hidrolizadas se encontró una correlación positiva entre los resultados obtenidos por PCR en tiempo real y ELISA.

Entre los métodos que emplean la detección específica, usando sondas TaqMan, se pudo realizar el análisis de muestras alimentarias para cuatro de los cereales más empleados en alimentación, arroz, cebada, centeno, trigo común y trigo duro (Hernandez et al. 2005). Se observó que casi todas las

muestras analizadas coincidían con su etiquetado. También se informó de la insuficiente cantidad de ADN extraído en muestras de cerveza debido a su alto procesamiento.

Algunos sistemas basados en sondas TaqMan alcanzaron un LD absoluto de 26 pg de ADN de trigo, y un LD relativo de 200 mg/kg de trigo en matriz de trigo en una harina que no contenía gluten (Piknova et al. 2008) y 2,5 mg/kg de harina de trigo en harina de arroz (Zeltner et al. 2009).

Ronning et al. (2006) utilizaron una PCR en tiempo real dúplex para la detección de trigo y cebada en muestras alimentarias de diferente grado de procesamiento. Utilizaron solo un par de cebadores para amplificar un segmento monocopia de menos de 100 bp que se encuentra con mayor o menor homología en varios vegetales, y dos sondas TaqMan específicas para cebada y trigo con diferentes fluoróforos para la detección de varios productos comerciales y variedades de estos cereales.

Zeltner et al. (2009) amplificaron por PCR en tiempo real genes codificantes de gluteninas de alto peso molecular para detectar trigo, escanda, kamut y centeno; gen de hordeina para cebada y el gen que codifica una proteína de reserva 12S para avena. Utilizando gran diversidad de modelos binarios (harina de trigo en harina de maíz, y en salchicha de hígado; cebada en harina de arroz y maíz; y avena en harina de arroz), obtuvieron un LD de 2,5 mg/kg de trigo en matrices vegetales y 5 mg/kg para la matriz animal y de 10 mg/kg para avena y cebada en las matrices vegetales. Además compararon su método con un método comercial de PCR en tiempo real (Congen SureFood for gluten), encontrando que algunas muestras no podían ser detectadas por el kit (las de salchichas). Los autores atribuyeron estas diferencias a una inhibición parcial de la enzima por los componentes del alimento, que estarían afectando sólo a la polimerasa del kit y no a la polimerasa que utilizaron en el trabajo. Algunas muestras de arroz tampoco pudieron ser detectadas.

Pegels et al. (2015) propusieron recientemente la amplificación por PCR en tiempo real con sondas TaqMan de secuencias multicopia de regiones organizadas en tándem intergénicas ITS, que son secuencias conservadas entre plantas, para la detección de trigo, cebada y avena, usando diferentes cebadores y sondas. Para la detección de centeno la secuencia ITS no presentaba las características adecuadas, por lo que se recurrió a la amplificación de una secuencia que codifica una proteína importante en la síntesis del inositol, gen MIPS. Se obtuvo un LD absoluto de 1 pg, para trigo y cebada, y de 100 pg y 0,1 pg para centeno y avena, respectivamente; y un LD relativo de 1 mg/kg para trigo y cebada, 100 mg/kg y 0,1 mg/kg para centeno y avena, respectivamente, lo que les permitió analizar diferentes productos comerciales para consumo humano y piensos para perros y gatos de compañía. Todos los alimentos que declaraban en su etiquetado que contenían

cereales o gluten resultaron positivos, mientras que los que estaban etiquetados como “puede contener” (que es voluntario), resultaron casi todos negativos, lo que puso de manifiesto que, en muchos casos, el etiquetado preventivo es innecesario. Sin embargo, algunos productos, hasta el 25% de los que no decían nada, contenían gluten.

También la PCR en tiempo real se ha empleado acoplada a otros sistemas avanzados para el análisis de secuencias. Algunos autores han empleado el análisis de la curva de disociación con sondas de hidrólisis también para detectar cereales que contienen gluten (Mujico et al. 2011; Píknova et al. 2008; Sandberg et al. 2003; Terzi et al. 2004; Terzi et al. 2003; Zeltner et al. 2009).

Análisis de trigo para prevenir el fraude alimentario

El incorrecto etiquetado de los alimentos constituye en sí mismo un fraude alimentario. Por este motivo es muy importante verificar que las especies de alto valor comercial no han sido sustituidas en el alimento, de forma parcial o completa, por otras de menor valor (Mafra et al. 2008a). Además del fraude económico que esto supone, puede generar un potencial riesgo en la salud del consumidor, sobre todo cuando concierne especies alergénicas. Para evitar el fraude se han usado, además de métodos basados en la detección de proteínas, el análisis de secuencias de ADN. En general, el perfil de ADN sirve para identificar cereales en materiales crudos y procesados. A pesar del alto grado de conservación de genes, las fracciones de proteínas de almacenamiento entre las especies de cereales son diferentes, y esas diferencias se pueden traducir a nivel tecnológico. Por ejemplo, la avena posee un alto contenido en globulinas, lo que las hace especialmente idóneas para piensos. Por otro lado, debido a la composición del grano, la cebada posee la tan importante capacidad de malteado característica de las cervezas. Por su parte, el trigo, al ser rico en prolaminas proporciona las características de la red viscoelástica para la fabricación de masas (Terzi et al. 2005), pero no todas las variedades de trigo son iguales, ni presentan la mismas características para la preparación, por ejemplo, de pastas o de panes. De ahí la importancia de detectar posibles adiciones intencionales de cereales de variedades diferentes.

Si bien el fin es diferente, monitorizar la presencia de trigo como adulterante de inferior valor en matrices de mayor valor económico, la metodología seguida en los artículos aquí revisados es la misma que en los métodos descritos en el apartado anterior: detectar la presencia de trigo en cantidades traza en diferentes matrices.

Control de la adulteración del trigo duro

Triticum durum y *Triticum aestivum* son las especies más importantes de trigo que se pueden distinguir entre los *Triticales*. Tienen unas propiedades tecnológicas y reológicas peculiares derivadas principalmente de su fracción proteica, codificada por diferentes genomas. *T. durum* es una planta tetraploide (AABB), y reológicamente adecuada para producir pasta italiana. Por su parte, *T. aestivum* (trigo blando, panificable o común), es una planta hexaploide (AABBDD), que se utiliza en todo el mundo para producir pan, galletas y productos de panadería. La pasta hecha solo con trigo duro es de mejor calidad que la que se hace con una mezcla trigo común panificable y trigo duro. La adición de *T. aestivum* es la adulteración más común en las pastas de fabricación industrial. En España, Francia e Italia la pasta alimenticia, etiquetada como tal, debe ser fabricada exclusivamente con trigo duro, mientras que en los países del norte de Europa, están permitidas las pastas fabricadas con mezclas de trigo blando y duro. Sin embargo, debido a la posibilidad de contaminación accidental que puede ocurrir durante la recolección, almacenamiento y transporte de granos, la pasta alimenticia sólo se considera oficialmente como contaminada o impura cuando el nivel de trigo blando supera 3% (Regulation (EC) 1222/1994 ; Terzi et al. 2005).

El trigo común se diferencia por su genoma D, que codifica la fracción de albúmina especie-específica, y que se ha empleado para identificarlo en las pastas alimenticias de diferentes formas. De hecho, el método oficial para la detección de la adulteración de pastas alimenticias consiste en la detección de una proteína, la peroxidasa- α 7D, que es específica del genoma D del trigo (Kobrehel and Gautier 1974). También se han propuesto diversos métodos para distinguir entre estas especies, siendo el enfoque principal la determinación cualitativa y/o cuantitativa de algunas fracciones proteicas, en particular la fracción de albúmina. Estos métodos incluyen el uso de electroforesis en gel de poliacrilamida (SDS-PAGE, isoelectroenfoque) (Bietz and Wall 1972; Jones et al. 1959), HPLC (Cornec et al. 1994; Wieser et al. 1998), y ensayos inmunológicos (Ewart 1966; Nimmo and O'Sullivan 1967). Pero durante las últimas décadas el método de producción de pastas alimenticias ha evolucionado y ahora se hace un secado a alta temperatura (70-100°C) que limita el uso de estos métodos de detección de proteínas con fines cuantitativos. Como consecuencia, algunos ensayos de inmunodetección comerciales específicos para las muestras de pasta, tales como PastaScan (R-Biopharm Rhône Ltd), han incorporado una prueba adicional para determinar el grado de tratamiento térmico de la muestra, y así permitir la selección más adecuada del estándar a utilizar para poder realizar un análisis de tipo cuantitativo (Pasqualone 2011). Teniendo en cuenta estas limitaciones, dado que la molécula de ADN es más estable al tratamiento térmico que las proteínas, diferentes autores han aprovechado su estabilidad para diseñar

métodos de PCR para detectar la presencia de trigo blando en pastas alimenticias. Basándose en la amplificación de una secuencia multicopia presente en el cromosoma D del trigo hexaploide, pero ausente en el trigo duro, Dgas44, consiguieron obtener un LD de 1% de trigo blando en trigo duro en pastas y semolinas preparadas por ellos (Bryan et al. 1998).

El rendimiento en la extracción de ADN de trigo varía según avanza el procesado del cereal hasta formar el producto alimenticio final, de esta forma el ADN genómico extraído se encontraría menos fragmentado en los cereales con fibra, pero más en semillas y productos de molienda tipo harina. La amplificabilidad del ADN extraído también sufre cambios con el procesado del material, siendo de más a menos óptimo para la amplificación por PCR la harina, seguido de la masa y por último el pan. Para demostrarlo se tomaron muestras de cada una de las diferentes etapas de la preparación de productos de panadería y se amplificaron tres tipos de secuencias: una secuencia multicopia de 286 bp, Dgas44 específica de trigo común; una secuencia con bajo número de copias, tionina de 238 bp, también específica de trigo; y por último una secuencia que codifica una fosfatasa ácida de levadura, de bajo número de copias 201 bp para la detección de ADN extraño al trigo. Con todas estas secuencias se demostró que la amplificabilidad del ADN varía en cada una de las etapas de manufactura (Tilley 2004).

A falta de material de referencia válido, Alary et al. (2002) prepararon pastas alimenticias que contenían diferentes porcentajes de trigo blando en trigo duro y lo sometieron a secados a diferentes temperaturas. Al realizar la extracción de ADN observaron que la calidad de ADN extraído dependía fuertemente de la temperatura de secado de la pasta, del mezclado y la extrusión. Expresaron el contenido en trigo común de la pasta alimenticia como relación: trigo blando utilizando la amplificación por PCR a tiempo real del gen puroindolina b frente al contenido en trigo total calculado mediante la amplificación de un gen de proteína de transferencia de lípidos. Cada una de estas secuencias fue amplificada con sus cebadores específicos y detectada con una sonda TaqMan marcada con diferentes marcadores fluorescentes, FAM y VIC, respectivamente, para poder realizar una amplificación simultánea de ambos analitos. La detección del gen de puroindolina es un marcador de trigo común hexaploide frente a trigo duro tetraploide, porque son genes que se localizan en el cromosoma 5D del trigo hexaploide y están presentes en el trigo diploide, pero no en las especies tetraploides.

Usando uno de los cebadores de Alary (2002), y rediseñando el cebador directo, se obtuvo un amplicón de 95 bp que permitió la detección de trigo común, cebada y centeno mediante PCR en tiempo real con un LD de 200 mg/kg de harina de trigo en harina sin gluten (Piknova et al. 2008). Este es un ejemplo, en el que unos cebadores ideados para la detección de fraudes

alimentarios en los que el trigo común se presenta como adulterante, pueden usarse también para detectar trigo y cereales tóxicos en muestras, para monitorizar el contenido en gluten de las mismas. Se aplicó este diseño para analizar muestras comerciales y se obtuvieron resultados similares a los obtenidos por un método ELISA, donde se encontró que tres muestras contenían gluten cuando el etiquetado indicaba que eran seguras para pacientes celíacos.

Arlorio et al. (2003) utilizaron PCR convencional dúplex para detectar contaminaciones de trigo blando en pastas alimenticias, mediante la amplificación del gen de puroindolina b, específico de trigo hexaploide y diploide, junto con la amplificación del gen de referencia ITS ribosómico (región *Internal Transcriber Spacer*), de carácter universal, para comprobar la calidad del ADN procesado a altas temperaturas, obteniendo un LD de 0,2% de trigo blando en trigo duro, incluso para pastas secadas a alta temperatura.

El Reglamento 510/2006 del Consejo de la Unión Europea sobre “la protección de las indicaciones geográficas y de las denominaciones de origen de los productos agrícolas y alimenticios” establece que los productos que se consideran una PDO (de sus siglas en inglés de denominación de origen protegida) son un producto cuya producción, transformación y elaboración se realizan en una zona geográfica determinada, con reconocido “know-how”. En caso de una PGI (de sus siglas en inglés de indicación geográfica protegida) establece que el vínculo geográfico debe darse por lo menos una de las etapas de producción, transformación o elaboración. Por último, una TSG (de sus siglas en inglés de Especialidad Tradicional Garantizada) no es tanto el origen, sino el carácter tradicional, ya sea en la composición o medios de producción del producto alimenticio. Listados de las PDO registrada, PGI y TSG se pueden encontrar en Regulation (EC) 1151/2012.

Entre ellos, se encuentra registrado el pan PDO Altamura, que como característica principal debe estar fabricada con trigo duro, y no debe contener trigo común. Utilizando la detección de un microsatélite de ADN específico del genoma D del trigo común, y un microsatélite que se encuentra en trigo duro y común, se consiguió detectar la adición fraudulenta de trigo hasta en cantidades de 2,5 % de trigo común en los panes de trigo duro PDO Altamura (Pasqualone et al. 2007).

Control de la adulteración de escanda

La escanda [*Triticum aestivum spelta* (L.) Thell] es un cereal consumido tradicionalmente por los alamanes, tribu del centro de Europa, que solo en el siglo XX fue desplazado por los cultivares de alto rendimiento del trigo común panificable [*T. aestivum vulgare* (Vill.) Mackey]. También es una planta hexaploide (AABBDD), como el trigo común. La escanda se usa para

panificación, producción de pasta y como pienso para los animales. Aunque en ocasiones se le han atribuido ventajas medicinales a esta variedad de trigo, están lejos de ser justificadas. Además de eso, sus propiedades nutricionales no son superiores al trigo común y es menos tolerante a condiciones ambientales, como las regiones sub-alpinas. La escanda es más difícil de cosechar mecánicamente debido a que posee una paja larga. Además, los rendimientos de la cosecha son inferiores a los de trigo común porque las cáscaras representan pérdida de hasta el 30%, el proceso de molienda requiere una etapa adicional para separar estas cáscaras y la harina de escanda tiene por lo general una calidad de horneado menor. Sin embargo, el aumento de la demanda de productos orgánicos o naturales, ha generado un nicho de mercado para la escanda. Su harina solo se encuentra en tiendas especializadas y tiene un precio hasta un 50% mayor que la harina de trigo común. Los métodos de PCR pueden proporcionar el análisis requerido para diferenciar las dos especies y proteger a los consumidores de la posible adulteración. En este sentido se ha informado de métodos para realizar esta diferenciación (von Buren et al. 2001), usando una PCR cuantitativa para amplificar un fragmento del gen de γ -gliadina, que presenta diferentes alelos para el trigo común y para el trigo de escanda, y cuya calibración se realizó con ayuda de un estándar interno. También hicieron un estudio RFLP (*Restriction Fragment Length Polymorphism*), que consiste en el reconocimiento y la fragmentación mediante enzimas de restricción de secuencias específicas de nucleótidos en el ADN que presentan patrones de distancia, longitud y disposición diferentes, debido a que son secuencias polimórficas y presentan más de un alelo. Este análisis, junto con la PCR cuantitativa competitiva, permitió denunciar la frecuente contaminación de harinas comerciales de escanda con trigo común (sólo una muestra de siete no contenía contaminación por encima de 5%).

Otros autores amplificaron por PCR otra región de la misma gliadina reportada por Von Büren, utilizando cebadores diferentes que flanqueaban una región que presenta una inserción de 9 bases en el trigo común, que en la escanda no aparece, además de algunas bases diferentes entre estos dos tipos de trigo (Mayer et al. 2012). Sin embargo, con electroforesis en gel no fueron capaces de discriminar entre estas dos secuencias tan parecidas. Al usar una PCR acoplada a RFLP y detección mediante electroforesis capilar en gel, aparecieron dos bandas características de 101 y 177 bp en el caso de la escanda. Utilizando este procedimiento, se consiguió detectar contaminaciones de escanda con trigo común de tan solo 1%, lo que permitió analizar distintas muestras de panadería supuestamente fabricadas con escanda, encontrando que sólo una tercera parte contenían contaminaciones de trigo común por debajo del 1%, otra tercera parte por debajo del 10%, y el resto, contenían trigo común por encima del 70%.

Adulteración del trigo farro

Un cereal que aparece en los listados de PDO, PGI y TSG anteriormente referidos, es un tipo de pan italiano denominado Farro della Garfagnana, como PGI, que se fabrica con la escanda Garfagnana también conocido como farro (*T. dicoccum* L.), especie tetraploide (AABB) de trigo.

Utilizando la secuencia de Dgas44 se diseñó un microarray basado en sondas padlock con detección fluorescente para detectar contaminaciones de trigo blando en ese tipo especial de trigo duro con un LD de 2,5% (expresado en ADN). Las sondas padlock son dos secuencias de ADN de hebra simple cortas (~20 nucleótidos) que son complementarias al analito, que se encuentran conectados entre sí por 40 nucleótidos que actúan como espaciador. Cuando se unen con la diana complementaria las sondas padlock se circularizan, esto es, se unen para detectar ADN de secuencias conocidas (Prins et al. 2010).

Adulteración de castaña

Usando genes de puroindolina y homólogos para trigo común, cebada, centeno y gen de proteína de transferencia de lípidos, se consiguió detectar hasta el 1% de estos cereales en harina de castaña (Alary et al. 2007), y evitar así adulteraciones de esta harina tan cara con harina de cereales de menor valor.

1.2.4 Métodos descritos para la detección de alérgenos y gluten de forma indirecta, mediante genosensores

A pesar de su gran éxito para el análisis de ácidos nucleicos, los instrumentos de PCR en tiempo real que existen actualmente en el mercado se basan en componentes ópticos complejos y generalmente delicados, por lo que se trata de equipos más bien voluminosos, frágiles y costosos, lo que limita su uso a laboratorios de investigación o de grandes empresas. Con el objetivo de desarrollar una tecnología de análisis de ADN empleando instrumentos portátiles y más sencillos, se ha buscado recientemente reemplazar los sistemas de detección ópticos por sistemas no ópticos, que son más robustos, menos costosos y más fáciles de miniaturizar. Entre los pocos intentos encaminados a lograr este objetivo, los métodos de detección electroquímicos parecen los más prometedores, ya que requieren una instrumentación mínima, suministro de energía eléctrica bajo y se pueden integrar fácilmente con la microelectrónica en formatos tipo chip (Deféver et al. 2011).

Como sistemas alternativos a la detección de amplicones por métodos ópticos, surgen los genosensores electroquímicos. Los genosensores son biosensores de hibridación de ADN, y como tales, son dispositivos analíticos

capaces de transformar una respuesta biológica en una señal cuantificable. Los componentes característicos de los biosensores, de manera muy esquemática son un elemento receptor, en el caso de los genosensores, una sonda de ADN, que interaccionará de manera selectiva con el analito (Figura 8) y un transductor, en íntimo contacto con el elemento receptor y capaz de transformar la interacción biológica (hibridación de hebras de ADN) en una señal cuantificable y proporcional a la concentración de analito presente en el medio, que en el caso de los biosensores electroquímicos es una superficie electroquímica. La característica principal de los biosensores es que el elemento que interacciona con el analito o elemento sensor es de naturaleza biológica y es capaz de interaccionar de manera específica con el analito, lo que confiere al dispositivo analítico una alta selectividad (Lucarelli et al. 2008; Pividori and Alegret 2010; Pividori et al. 2000; Teles and Fonseca 2008).

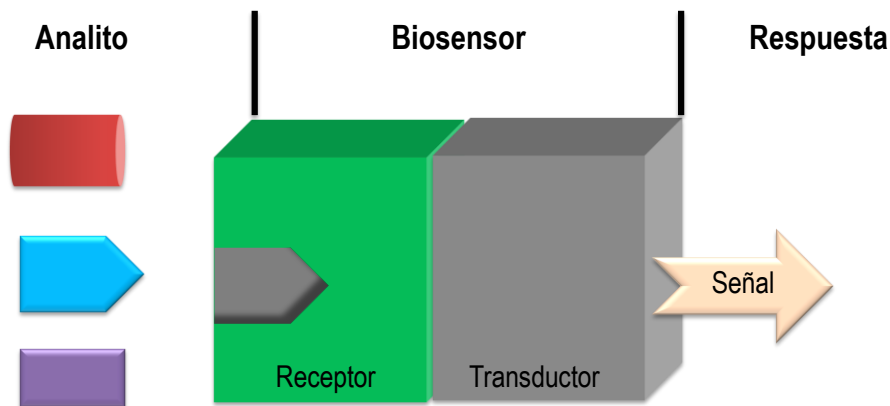


Figura 8. Componentes característicos de un biosensor.

Los biosensores de ADN o genosensores se fundamentan en la afinidad natural de la hebra simple, ADNss, por su hebra complementaria (Arora et al. 2011; Van Dorst et al. 2010) que mediante la reacción de hibridación dará lugar a un híbrido de doble hebra debido a la complementariedad de las bases. Esta afinidad natural permite la detección de genes específicos de una especie (Figura 9). La reacción de hibridación puede tener lugar aun cuando las dos hebras no sean absolutamente complementarias, pero las hélices dobles resultantes serán menos estables. La reacción de hibridación presenta dos características que justifican su enorme potencial analítico. Por un lado, la especificidad, ya que la unión viene determinada por el reconocimiento de la secuencia de bases, y por el otro la reversibilidad, proporcionada por las interacciones no covalentes entre ambas hebras. Los genosensores han experimentado un gran auge en las dos últimas décadas debido a los avances en la síntesis de ADN y el abanico de marcajes de sondas disponibles comercialmente.

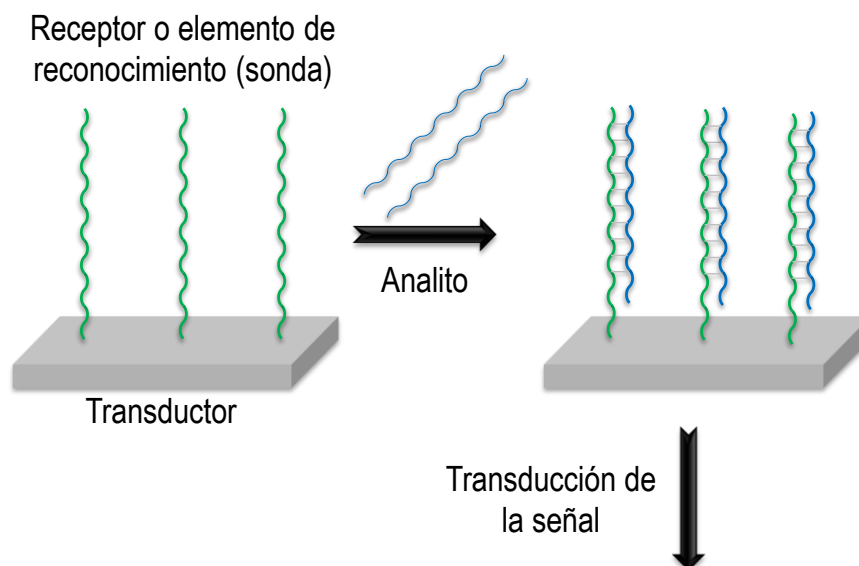


Figura 9. Genosensores o biosensores de ADN, reacción de hibridación del elemento de reconocimiento con el analito y transducción de la señal.

De entre los pasos para el diseño de genosensores electroquímicos destacan por su importancia la elección de los métodos de inmovilización y de detección más adecuados (Lucarelli et al. 2004).

Métodos de inmovilización

La inmovilización de las cadenas de ADN sobre la superficie eléctrica debe llevarse a cabo de forma que permita obtener un control preciso del empaquetado y orientación de los oligonucleótidos inmovilizados, con la mínima pérdida de capacidad de hibridación de las cadenas y de la forma más simple y estable que sea posible. Con estos objetivos se han explorado diferentes estrategias, como la adsorción asistida por la aplicación de potencial electroquímico o no (potencial abierto); la unión de afinidad entre moléculas pequeñas como digoxigenina o biotina, con sus receptores correspondientes, el anticuerpo antidigoxigenina y la avidina (o estreptavidina); la quimisorción sobre electrodos de oro de compuestos tiolados a través del átomo de azufre, formando monocapas autoensambladas (SAM); y el enlace covalente. Probablemente la estrategia más empleada en los últimos años para la inmovilización de ADN en la superficie eléctrica sea el uso de SAM, empleando oligonucleótidos funcionalizados con un grupo alcanotiol, dado que proporcionan una inmovilización simple y razonablemente estable, manteniendo la movilidad conformacional de la sonda, lo que facilita la hibridación con la cadena complementaria. Sin embargo, se ha observado que las monocapas de oligonucleótidos muy compactas son difíciles de obtener debido a su alta hidrofobicidad. Por eso, se ha descrito que, la adición de cationes monovalentes, o en mayor medida los divalentes, reducen la repulsión

electrostática entre cadenas de oligonucleótidos lo que mejora el grado de empaquetamiento. Sin embargo, un excesivo grado de empaquetamiento dificulta el acceso de la hebra complementaria, por lo que, en general, se debe encontrar un valor óptimo entre una excesiva cantidad de ADN sobre la superficie y una excesiva dilución de la misma, que impida obtener señales analíticas elevadas.

Métodos de detección

Para la detección de los ácidos nucleicos se han empleado dos tipos de formatos: directo o tipo sándwich. En ensayo directo consiste en aquel que emplea una sonda complementaria a la secuencia analito. El ensayo tipo sándwich se fundamenta en el uso de una tercera sonda, además de la sonda inmovilizada sobre el electrodo y el analito, que es la encargada de señalar el evento de la hibridación, y que, por ello, adquiere el nombre de sonda indicadora. El analito se encontraría parcialmente hibridado con ambas sondas la de captura (inmovilizada) y la indicadora, a modo de sándwich. Esta doble hibridación confiere una selectividad adicional a este tipo de formato de ensayo (Lucarelli et al. 2004).

La extensa investigación realizada sobre la detección electroquímica de ácidos nucleicos ha dificultado su clasificación, debido a la variedad de métodos que se han empleado. No obstante, la clasificación más clara es la propuesta por de-los-Santos-Álvarez et al. (2004), donde se diferencian entre los métodos directos de detección, esto es aquellos que se basan en la electroquímica de los ácidos nucleicos y los indirectos, que usan otro tipo de variaciones en las propiedades electroquímicas para la detección de la reacción de hibridación (Figura 10).

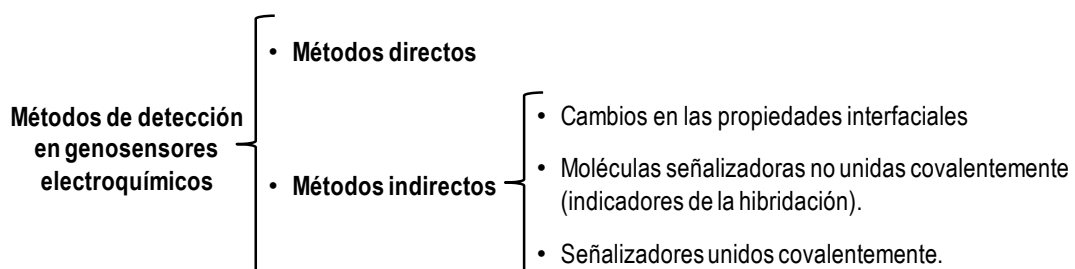


Figura 10. Clasificación de los métodos de detección empleados en genosensores electroquímicos para la detección de ácidos nucleicos. Adaptado de de-los-Santos-Álvarez (2004).

Detección electroquímica directa

La detección electroquímica directa se basa en que las nucleobases de la doble hélice se oxidan más difícilmente que aquellas que están presentes en

hebras sencillas, ADNss. El problema de usar este tipo de detección es que requiere aplicar potenciales altos y que suele generar altas corrientes de ruido de fondo. Además, aunque tienen la ventaja de no requerir elementos adicionales, la señal generada es totalmente inespecífica. Con este objetivo se han usado los complejos de rutenio (Steel et al. 1998; Zhang et al. 2007), que actúan como mediadores de la oxidación de la guanina, incrementando la transferencia electrónica entre los ácidos nucleicos y el electrodo, y por lo tanto, aumentan la señal analítica. Otra estrategia empleada para intentar reducir el ruido de fondo ha sido el empleo de sondas en las que se han sustituido las guaninas por inosinas (Kara et al. 2003; Kerman et al. 2002; Mascini et al. 2005).

Detección electroquímica indirecta

De entre los métodos indirectos, se han monitorizado los cambios en las propiedades interfaciales, o el uso de moléculas señalizadoras que se unen covalentemente o no (indicadores de hibridación) al ADN.

El primer caso se fundamenta en que es posible detectar que la reacción de hibridación ha tenido lugar porque las propiedades electrónicas de la interfase electrodo-disolución se alteran cuando ésta tiene lugar en la superficie del electrodo (Kara et al. 2003). Es posible monitorizar los cambios en las propiedades dieléctricas de la superficie (medidas de capacitancia) (Tichoniuk et al. 2010), o modificaciones en la transferencia electrónica de especies electroactivas en disolución, como el sistema $[\text{Fe}(\text{CN})_6]^{3-/4-}$, cuyo proceso redox se encuentra impedido en mayor grado cuando la reacción de hibridación ha tenido lugar, dado que provoca un incremento de las cargas negativas en la interfase (medidas de impedancia) (Bonanni et al. 2009; Bonanni and Pumera 2011; Ihalainen et al. 2014; Lucarelli et al. 2005; Ma et al. 2008; Pandey et al. 2011; Sun et al. 2008).

También se han usado estrategias que emplean moléculas señalizadoras (indicadores de hibridación) que no se unen covalentemente al ADN, sino que lo hacen a través de interacciones electrostáticas o mediante intercalación, y que poseen diferentes afinidades por el ADNss que por el ADNds (Miranda-Castro et al. 2009). Ejemplos de compuestos intercalantes frecuentemente utilizados son el azul de metileno (MB) (Ren et al. 2005; Tichoniuk et al. 2008; Xie et al. 2008; Xu et al. 2006; Zhang et al. 2008), daunomicina (Ligaj et al. 2003), y los complejos de rutenio y cobalto (Kerman et al. 2006; Lucarelli et al. 2004). Estas moléculas electroactivas pequeñas tienen la capacidad de intercalarse en el ADN de forma mucho más afín al híbrido de doble cadena, inmovilizado sobre la superficie del electrodo, que a una hebra monocatenaria de ADN. De esta forma, cuando se produce la hibridación, la concentración de intercalante aumenta en la superficie del electrodo, dando lugar a un incremento de la señal electroquímica. Sin embargo el azul de

metileno (MB), también muy empleado, tiene un mecanismo diferente: proporciona señales menores en el dúplex que en el ADNss, posiblemente debido a la interacción que posee este indicador por las bases de guanina, que en el caso del dúplex se encuentran impedidas.

También se han empleado señalizadores unidos covalentemente al ADN (conocidas como marcas), debido a la posibilidad de obtener oligonucleótidos funcionalizados de muy diversos tipos de forma comercial. Por ello, de entre todas las estrategias mencionadas, probablemente ésta haya sido la más ampliamente utilizada en genosensores electroquímicos para la amplificación del evento de hibridación. Así, se pueden diferenciar dos tipos: los que emplean marcadores electroactivos (como antraquinonas, ferroceno, MB o complejos de osmio o rutenio) (Duwensee et al. 2009; Farjami et al. 2012; Mix et al. 2012); y no electroactivos, como enzimas unidas directamente o no a la hebra de ADN. La marca enzimática puede incorporarse a través de enlaces específicos, en una etapa posterior. Esto es debido a que su enorme tamaño, en comparación a la sonda de ADN, podría dar lugar a impedimentos estéricos. Existen diferentes métodos de enlace de la enzima a las sondas, bien mediante enlaces de alta afinidad como biotina-avidina (Carpini et al. 2004; Del Giallo et al. 2005; Lucarelli et al. 2005; Martínez-Paredes et al. 2009) o mediante reconocimiento de un antígeno por su anticuerpo específico (fluoresceína, digoxigenina y los fragmentos Fab antifluoresceína, antidigoxigenina correspondientes) (Barroso et al. 2015; Gonzalez-Alvarez et al. 2013; Kuralay et al. 2012; Liao et al. 2013; Liu et al. 2013; Wu et al. 2010). Las enzimas más ampliamente empleadas para el diseño de genosensores electroquímicos han sido la fosfatasa alcalina, la glucosa oxidasa, la catalasa y la peroxidasa, debido a sus altos números de recambio.

Biosensores de ADN para la detección de alérgenos

Como se ha comentado anteriormente, dado que la PCR en tiempo real es una técnica laboriosa, y que requiere de personal cualificado y equipamiento e instalaciones adecuadas, actualmente, han surgido nuevas herramientas para la detección de ADN de alérgenos: los genosensores.

Wang et al. (2011) diseñaron un genosensor óptico para la detección de ocho alérgenos alimentarios, entre los que se encontraba la avena, que puede ser potencialmente tóxica para los enfermos celíacos. Para la detección de los alérgenos se procedió a una extracción del ADN de la muestra, seguido de dos amplificaciones de PCR tetraplex, esto es, usando cuatro pares de cebadores donde en dos reacciones diferentes se amplificaron los ocho alérgenos, cuatro en cada una, y posteriormente se detectaron en la superficie del biosensor por hibridación con las sondas inmovilizadas en la superficie. La reacción se reveló mediante una reacción enzimática que desarrolla color, lo que permitió la detección visual de las muestras, de forma cualitativa. A pesar de las ventajas

que presentan los biosensores electroquímicos, la determinación cuantitativa de la cantidad de amplicón generado mediante monitorización electroquímica se ha explorado mucho menos que en las técnicas ópticas (Deféver et al. 2011; Fang et al. 2009). De hecho, hasta el momento, no se han descrito biosensores electroquímicos para la detección de gluten, aunque sí para otros alérgenos, como el cacahuete y la avellana (Berti et al. 2009; Bettazzi et al. 2008; Sun et al. 2012; Sun et al. 2015; Sánchez-Paniagua López et al. 2014).

Tabla 2. Métodos de detección de trigo basados en ADN

Tipo de PCR	Tipo de analito	Analito	Método de extracción (cantidad muestra)	ng ADN muestra	Marcaje	Tipo de muestras analizadas	Características analíticas	Selectivo para	Ref.
PCR	1 copia	Gen que responde a giberelinas (159 bp trigo)	-	-	-	Harinas y pan.	0,1% de harina de trigo en harina de maíz 10 pg de ADN genómico de trigo.	Trigo, cebada, centeno, avena	(Allmann et al. 1992)
PCR	Multicopia	Región intergenica entre 25S y 18S rRNA (109 bp trigo)	Fenol-cloroformo (100 mg)	-	-	90 alimentos (procesadas, harinas, con y sin gluten). ADN de trigo en ADN de hongo.	LD absoluto = 1 pg ADN genómico trigo. LD relativo = 1 mg/kg de ADN de trigo mezclado con ADN de hongo. Comparan con ELISA competitivo sándwich anticuerpo monoclonal.	Trigo	(Allmann et al. 1993)
PCR (RAPD)	Multicopia	5S rRNA (230-700 bp) + cebadores arbitrarios de 10 bp para discriminar entre especies (RAPD)	CTAB (- mg)	-	-	Hojas, semillas	En mezclas de especies presentó problemas de discriminación	Trigo, cebada, centeno, avena, arroz, maíz, sorgo	(Ko et al. 1994)
PCR	Multicopia	Dgas44 (286 bp)	CTAB (5 g)	50 ng	-	% harina de trigo común en harina de trigo duro. Pasta de diferentes % secadas a diferentes temperaturas. Semillas: 66 cultivares trigo blando y 55 de trigo duro.	LD relativo = 1% (500 pg de trigo panificable en 50.000 pg de ADN de semillas y pasta). LD absoluto = 200 pg.	Trigo común	(Bryan et al. 1998)
PCR	Multicopia	Región intergenica entre 25S y 18S rRNA (109 bp) igual que Allmann et al. 1993	Wizard con pretratamiento (300 mg)	10 ng	-	% harina de trigo en harina de avena integral. Productos comerciales.	LD relativo = 0,001-0,01% (0,04-04 mg gliadin/100 g). Comparan con ELISA de Ridascreen de r-Biopharm (anticuerpo monoclonal contra w-gliadina).	Trigo	(Koppel et al. 1998)
PCR cuantitativa competitiva	Multicopia	Región no codificante de cloroplasto <i>trnL</i> (201 bp trigo) Y un estándar interno de ADN añadiendo 20 bp al producto de PCR original	Wizard (300 mg)	200 ng	- patrón interno	ADN de trigo en ADN de soja. Alimentos sin gluten.	Calibración entre 0,02% y 0,2% ADN trigo (=10 y 100 ppm gliadina). 0,02 % de harina de trigo en alimento (<5 copias de ADN = 40pg ADN trigo en 200.000 pg ADN total).	Trigos, cebada, centeno	(Dahinden et al. 2001)

Tipo de PCR	Tipo de analito	Analito	Método de extracción (cantidad muestra)	ng ADN muestra	Marcaje	Tipo de muestras analizadas	Características analíticas	Selectivo para	Ref.
PCR cuantitativa competitiva y RFLP	1 copia?	GAG56D (γ -gliadina) (236 bp)	Wizard (250-300 mg)	200 ng	- patron interno	Semillas trigo escanda y pan. % ADN trigo común en ADN trigo escanda.	LD relativo = 0,5% de ADN de trigo común en trigo escanda. LD = 0,01% (20 pg de ADN de trigo en 200.000 pg de ADN escanda).	Trigo	(von Buren et al. 2001)
PCR en tiempo real dúplex	1 -2 copias	Puroindolina b (63 bp, 1 copia) para trigo común. Gen que codifica una proteína de transferencia de lípidos para trigo total (61 bp, 2 copias).	Qiagen midi kit (1 g)	5 μ L of ADN diluido 160x (correspondiente a 0.31 μ L de ADN extraído de 1 g pasta)	TaqMan	% harina de trigo común en harinas de trigo duro (en muestras de pasta de diferentes secadas a muy alta, alta y baja temperatura).	LD relativo = 3% (220 pg de trigo panificable en 7.360 pg de ADN total extraído de pasta).	Trigo común	(Alary et al. 2002)
PCR dúplex	1 copia Multicopia	- Puroindolina b (551 bp) Especifico trigo igual que Alary et al. 2002 - Gen de referencia ITS ribosomal (310 bp; para comprobar calidad ADN)	Nucleon Phytopure (100 mg)	500 ng	-	% harina de trigo en harina de trigo duro. Semillas. Productos de panadería.	LD relativo = 0,2% de trigo panificable en duro (100 pg de trigo panificable en 500.000pg de ADN extraído de semillas, panadería y pasta seca).	Trigo común	(Arlorio et al. 2003)
PCR en tiempo real (+melting curve)	-	ω -gliadina (181 bp trigo y centeno, 164 bp cebada y 104 bp avena)	ADNeasy qiagen tissue (100 mg)	50 ng	SYBR green	ADN de cereal en ADN de soja.	LD = 50 pg ADN (= 0,1% en ADN soja). 0,1-0,01% (5-50 pg en 50.000 pg de ADN total).	Trigo, cebada, centeno, avena	(Sandberg et al. 2003)
PCR en tiempo real	-	Glutelina bajo peso molecular LMW (101 bp) γ -gliadina (101 bp)	CTAB (- mg)	100 ng	TaqMan y SYBR Green	% harina de trigo en harina de trigo duro. Semillas.	LD relativo = 1% de trigo panificable en duro (1.000 de ADN de trigo panificable en 100.000 pg de ADN total extraído de semillas o pasta)	Trigo, trigo duro	(Terzi et al. 2003)
PCR en tiempo real	-	O-methyltransferasa mRNA (101 bp)	CTAB para semillas (- mg) Wizard para alimentos (- mg)	100 ng	TaqMan y SYBR Green	% harina de centeno en harina de arroz. Semillas y alimentos con centeno. Dil seriada de ADN de centeno.	LD relativo = 1% (150 pg ADN de centeno)	Centeno Triticales	(Terzi et al. 2004)
PCR	Multicopia 1 copia Multicopia (bajo n°)	- Dgas44 (286 bp) igual que Bryan et al. 1998 - Tionina tipo I (238 bp) - Fosfatasa ácida de levadura (201 bp)	Wizard (100-500mg)	100 ng	-	Semillas. Harinas. Panes.	LD absoluto = 200 pg. LD relativo = 1% (100 pg de trigo panificable en 100.000 pg de ADN de pan de 5 días).	Trigo	(Tilley 2004)

Tipo de PCR	Tipo de analito	Analito	Método de extracción (cantidad muestra)	ng ADN muestra	Marcaje	Tipo de muestras analizadas	Características analíticas	Selectivo para	Ref.
PCR	Multicopia	Región no codificante de cloroplasto <i>trmL</i> (662 bp y 397 bp trigo)	ADNeasy plant Mini kit (25-100 mg)	50 ng	-	Semillas, hojas. Productos de panadería.	-	Trigo	(James and Schmidt 2004)
PCR en tiempo real	1 copia - bajo n° de copias	- γ -hordeína cebada - <i>gos9</i> arroz - heliantinina girasol - acetyl-CoA carboxylasa trigo - RALyasa trigo duro	CTAB (- mg)	100 ng	TaqMan	Semillas. Alimentos. Dilución de ADN en albúmina sérica bovina.	LD absoluto = 1 copia cebada, girasol y trigo; 3,3 arroz.	Cebada Arroz Girasol Trigo Trigo duro	(Hernandez et al. 2005)
PCR	Multicopia	Región no codificante de cloroplasto <i>trmL</i> (201 bp trigo) igual que Dahinden et al. 2001	Wizard GeneSpin (- mg)	-	-	% harina de trigo en harina de soja. Bizcochos % harina trigo en harina sin gluten (Promix). Semillas. Productos comerciales.	LD relativo = 0,1% trigo en soja y para los bizcochos. LD absoluto = 42 ± 12 pg = 10^0 copias de ADN.	Trigo Cebada Centeno	(Olexova et al. 2006)
PCR en tiempo real dúplex	1 copia	Gen proteína-kinasa serina-treonina PKABA1 amplifican con un par de cebadores, y dos sondas TaqMan diferentes para reconocer trigo y cebada (89 bp)	CTAB (- mg)	-	TaqMan	Semillas. Productos comerciales. Dilución seriada de ADN de centeno.	LD absoluto = 5 copias ADN para trigo y 16 para cebada.	Trigo Cebada	(Ronning et al. 2006)
PCR en tiempo real	Multicopia	- Microsatélite de ADN específico del genoma D del trigo común (GDM111, 210 bp) - Microsatélite de ADN del genoma A (GWM186, 240 bp) en trigo común y duro, verifica amplificabilidad.	Gene Elute plant kit (20-40 mg)	50 ng	SYBRgreen	% harina de trigo en harina de trigo duro. Panes de % harina de trigo en harina de trigo duro. Panes comerciales (PDO Altamura).	LD relativo = 2,5% de trigo común en trigo duro.	Trigo	(Pasqualone et al. 2007)
PCR	-	Gen precursor de triticina (141 bp)	DNesay Qiagen (200 mg)	50 ng	-	% harina de trigo en harina de maíz. Semillas. Productos comerciales.	LD relativo = 0,005% de trigo común en maíz.	Trigo Trigo duro	(Yamakawa et al. 2007)
PCR en tiempo real	1 copia - bajo n° de copias	Gen ALMT1 transportador de malato activado por aluminio (95 bp).	CTAB (200 mg)	40 ng	TaqMan	Semillas. Dilución seriada de ADN.	LD absoluto = 2 copias genoma haploide de trigo. LQ absoluto = 40 pg (=20 copias genoma haploide trigo).	Trigo	(Vautrin and Zhang 2007)

Tipo de PCR	Tipo de analito	Analito	Método de extracción (cantidad muestra)	ng ADN muestra	Marcaje	Tipo de muestras analizadas	Características analíticas	Selectivo para	Ref.
PCR duplex	1-2 copias	- Trigo común: gen puroindolina-a (381 bp) - Trigo duro: gen proteína transferencia de lípidos (262 bp) - Cebada: gen hordoindolina (262 bp) - Centeno: gen secaindolina-a (511 bp) - Avena: gen de tionina (265 bp)	DNeasy Plant Mini kit (100 mg)	60-100 ng	-	% harina de cereal en harina de castaña. Semillas. Productos comerciales.	LD relativo = 1% de cereal en castaña. LD absoluto = 10 copias.	Trigo Trigo duro Cebada Centeno Avena	(Alary et al. 2007)
PCR en tiempo real	1-2 copias	Puroindolina b (95 bp, 1 copia) para trigo común igual que Alary et al. 2002 uno de los cebadores, el otro rediseñado	NucleoSpin (- mg)	-	TaqMan	% harina de trigo en harina sin gluten (Promix). Dil seriada ADN de trigo. Productos comerciales.	LD relativo = 200 mg/kg. LD absoluto = 0,026 ng ADN trigo = 1,5 copias. Comparan con ELISA Ridascreen.	Trigo Cebada Centeno	(Piknova et al. 2008)
PCR en tiempo real	1 - bajo nº de copias	- Glutenina de alto peso molecular (HMW) (85 bp) para trigo, escanda, kamut y centeno. - Gen hordeina para cebada (73 bp) - Gen de proteína de reserva 12S para avena (64 bp)	CTAB (200 mg)	10 ng	TaqMan	% harina de trigo en harina de maíz, y en salchicha de hígado. % cebada molida en harina de arroz, y maíz. % avena molida en harina de arroz. Semillas.	LD relativo = 2,5 mg/kg de trigo en matriz vegetal y 5 mg/kg en carnes Para cebada 10 mg/kg Comparan con PCR en tiempo real Congen SureFood	Trigo Escanda Kamut Cebada Centeno Avena	(Zeltner et al. 2009)
PCR	1 - bajo nº de copias	Glutenina LMW (135 bp)	CTAB (- mg)	75 ng	-	Dilución seriada ADN de trigo. Semillas. Productos comerciales.	LD relativo = 0,1% en harina. LD absoluto = 21,5 pg (1-1,3 copias genoma), en alimentosaltamente procesados.	Trigo	(Debnath et al. 2009)
PCR en tiempo real	Multicopia	Microsatélite de ADN específico del genoma D del trigo común (GDM111, 210bp) igual que Pasqualone et al. 2007	CTAB (200 mg)	200 ng	TaqMan	% harina de trigo en harina de trigo duro. Dilución seriada ADN de trigo. Muestras de pasta secadas a alta temperatura	LD relativo = 1,25% de trigo común en trigo duro.	Trigo	(Sonnante et al. 2009)
PCR	-	- Trigo común y duro: gen proteína transferencia de lípidos igual que Alary et al. 2007 (262 bp) - Cebada: gen hordoindolina-A (145 bp)	Extract-N-Amp Seed Sigma (20-50 mg)	25 ng	-	% harina de trigo/trigo duro/cebada en matriz inerte Semillas Piensos	LD relativo = 0,9 % para trigo y cebada.	Trigo Trigo duro Cebada Centeno	(Tavoletti et al. 2009)

Tipo de PCR	Tipo de analito	Analito	Método de extracción (cantidad muestra)	ng ADN muestra	Marcaje	Tipo de muestras analizadas	Características analíticas	Selectivo para	Ref.
Padlock-microarray	Multicopia	- Dgas44 (45 bp) del genoma D del trigo igual que Bryan et al. 1998	CTAB (50 mg) + Enzimas de restricción	200ng para microarray	Sondas Padlock	Dilución de ADN de trigo común en ADN trigo dicoccum Farro della Garfagnana. Semillas.	LD relativo = 2,5% de ADN de trigo común en trigo duro.	Trigo	(Prins et al. 2010)
PCR	Multicopia	Región no codificante de cloroplasto <i>trmL</i> (201 bp trigo, 196bp cebada) igual que Dahinden et al. 2001	NucleoSpin, GeneSpin ₋ (mg)	-	-	% harina de trigo en harina de soja.	LD relativo = 0,1% (= 5 mg gliadina en 100 g alimento).	Trigo Cebada Centeno	(Maskova et al. 2011)
PCR en tiempo real	Multicopia	Región intergenica entre 25S y 18S rRNA (51 bp) Usada antes por Allmann et al. 1993; Koppel et al. 1998	Guanidina + SDS + Wizard modificado (20-40 mg)	1 ng	SYBR green	Dilución seriada ADN de trigo. Semillas Productos comerciales.	LD absoluto = 0,05 pg ADN (=5 pg/mg alimento). Comparan con ELISA R5 sándwich. Reproducibilidad 4%.	Trigo Trigo duro Triticale	(Mujico et al. 2011)
PCR multiplex (10-plex) + electrophoresis capilar en gel	Multicopia	Región no codificante de cloroplasto <i>trmL</i> (133 bp para trigo) igual que Dahinden et al. 2001	DNeasy plant mini kit (- mg)	50-300 ng	-	% harina de trigo en harina de soja. Semillas.	LD absoluto = 2.6 ng de ADN de trigo. LD relativo = 0,01% de trigo en soja.	Trigo Cebada Centeno Avena	(Mustorp et al. 2011)
Biosensor óptico + PCR tetraplex	-	- Avenina (84 pb) - Detectan también sésamo, lupino, mostaza, nuez, almendra, apio, avellana	CTAB (200 mg)	200 ng	Enzimático, cambio de color	Productos comerciales.	Cualitativo.	Avena	(Wang et al. 2011)
PCR + RFLP + lab-on-a-chip electrophoresis capilar en gel (indirecta cualitativ)	-	γ -gliadina cebadores igual que von Buren et al. 2001 (236 bp) y otros cebadores nuevos (278 bp escanda y 287 bp trigo común)	CTAB (200 mg) + EconoSpin	100 ng	-	% harina de trigo común en harina de trigo escanda. Semillas. Productos comerciales panadería.	LD relativo = 1 % de trigo común en trigo escanda. Comparan con PCR en tiempo real (TaqMan).	Trigo	(Mayer et al. 2012)
PCR en tiempo real	Multicopia	- Región intergenica ITS1 (par de cebadores y sonda específica para cada: 100 bp trigo, 81 bp cebada y 83 bp avena). - Y MIPs para centeno (107 bp)	Wizard (200 mg)	2 μ L (concentrac?)	TaqMan	% harina de trigo en harina de soja y/o maíz. Semillas. Dilución seriada ADN. Productos comerciales y piensos.	LD relativo: trigo y cebada = 0,0001% (1mg/kg); centeno = 0,01% (100mg/kg); avena = 0,00001% (0,1mg/kg) LD absoluto = 1pg trigo, cebada; 100 pg para centeno y 0,1 pg para avena. Discriminación del cereal de origen (cada 1, su sonda).	Trigo Cebada Centeno Avena	(Pegels et al. 2015)

1.2.5 Puntos críticos en la detección de trigo y cereales mediante ADN

En teoría, cualquier secuencia de ADN que relacione la especie y que permita ser discriminada de otros componentes presentes en el alimento, puede ser adecuada para la detección por métodos de PCR o genosensores. Sin embargo, se debe prestar especial atención en esta parte del diseño porque sistemas de detección específicos para especies de plantas como el trigo son especialmente difíciles de obtener por la presencia de secuencias de ADN homólogas en especies estrechamente relacionadas (cebada y centeno), y en otras especies de cereales más alejadas filogenéticamente. Por este motivo, se han realizado grandes esfuerzos en la búsqueda de secuencias para la detección de estos cereales, que se reflejan en la grandísima variedad de secuencias aquí revisadas.

Los métodos basados en PCR para la detección de alérgenos, presentan muchas ventajas frente a los métodos ELISA convencionales y se pueden aplicar en la mayoría de alérgenos que las diferentes legislaciones obligan a etiquetar. Sin embargo hay casos en que la detección de ADN para alérgenos no es aplicable (huevos, leche) (Holzhauser and Röder 2015). En otras ocasiones, la proteína puede estar ausente por el procesamiento que sufre el alimento, pero puede hallarse el ADN intacto, y a la inversa. Estos dos problemas pueden traducirse en falsos negativos al realizar, respectivamente el análisis ELISA o de PCR. Sin embargo, en los alimentos, en general la presencia de proteínas indica la presencia de ADN (Dahinden et al. 2001), aunque no es tan sencillo transformar los datos obtenidos a datos cuantitativos.

Sin embargo, la práctica ha demostrado la aplicabilidad de los métodos de PCR para la consecución de la detección de cereales de forma específica como se ha ido comentando a lo largo de este capítulo. Además de esta la especificidad que presentan este tipo de métodos, es importante hacer énfasis en la alta sensibilidad que se puede conseguir, habiéndose reportado LD relativos de tan solo 0,001% (Koppel et al. 1998), 0,005% (Yamakawa et al. 2007), 0,00025% (Zeltner et al. 2009), 0,0001% (Pegels et al. 2015) de trigo en diferentes matrices inertes; y LD absolutos de 5 copias (Ronning et al. 2006) , 2 copias (Piknova et al. 2008; Vautrin and Zhang 2007), ~1 copia (Debnath et al. 2009; Hernandez et al. 2005; Olexova et al. 2006). Para transformar esos LD para la detección de ADN de trigo a ppm de contenido en gluten se han empleado factores de transformación obtenidos empíricamente, que relacionan ambas magnitudes. Por ejemplo, Zeltner et al. (2009) asumieron que aproximadamente el 9% en peso de la harina es gluten para la transformación de sus datos a contenido de gluten.

La detección de secuencias multicopias, como las que proceden de orgánulos como mitocondrias y cloroplastos, como la región no codificante de

cloroplasto *trnL* usada por varios autores (Dahinden et al. 2001; James and Schmidt 2004; Mustorp et al. 2011; Olexova et al. 2006), pueden dar lugar a métodos de 10 a 100 veces más sensibles que aquellos que se fundamentan en la detección de secuencias que se encuentran sólo en una copia, como el gen de respuesta a giberelinas (Allmann et al. 1992), o los genes de puroindolina a y b (Alary et al. 2007; Alary et al. 2002; Arlorio et al. 2003) que permitieron un LD de en torno al 0,1% - 1%. Aunque las secuencias multicopia presentan mayor sensibilidad, y por este motivo han sido las más frecuentemente empleadas, suelen adolecer de una menor especificidad si la secuencia es altamente conservada a lo largo de la evolución, cosa que debe ser comprobada experimentalmente (Holzhauser and Röder 2015).

Comparando entre diferentes especies, para una misma cantidad de ADN de partida de PCR, el LD depende del tamaño del genoma de cada cereal (Hubner et al. 2001), cosa que se debe tener en cuenta cuando se establecen comparaciones entre diferentes tipos de alérgenos.

La detección de gluten a través del ADN no es sólo posible, sino también muy conveniente en muchos casos. Su aplicación como métodos analíticos en muestras reales permitió, por ejemplo, detectar que la contaminación de trigo común era muy habitual y en muy alta en los alimentos etiquetados como escanda (Mayer et al. 2012; von Buren et al. 2001); que hasta una cuarta parte de los piensos etiquetados como libres de gluten, no cumplían la normativa (Pegels et al. 2015); y que también los productos comerciales catalogados como sin gluten, presentan en ocasiones un contenido más alto en cereales tóxicos del permitido (Dahinden et al. 2001).

Por todo ello, la detección de cereales tóxicos a través de su ADN es altamente aconsejable, por un lado, para verificar la idoneidad de los alimentos para los pacientes celíacos, y por el otro, para evitar el fraude alimentario. Los problemas derivados de este tipo de metodologías de detección indirecta están siendo superados y las características analíticas incluso superan a las de los ELISA, llegando a cuantificar cantidades próximas a ppb. Por estos motivos, están comenzando a comercializarse e incorporarse como métodos analíticos recomendados por las diferentes legislaciones.

1.3 Bibliografía

Abdallah, H., Leffler, D., Dennis, M., Kelly, C., 2007. Refractory celiac disease. *Current Gastroenterology Reports* 9(5), 401-405.

Alaedini, A., Green, P.H.R., 2005. Narrative Review: Celiac Disease: Understanding a Complex Autoimmune Disorder. *Annals of Internal Medicine* 142(4), 289-298.

Alary, R., Buissonade, C., Joudrier, P., Gautier, M.-F., 2007. Detection and discrimination of cereal and leguminous species in chestnut flour by duplex PCR. *European Food Research and Technology* 225(3-4), 427-434.

Alary, R., Serin, A., Duviau, M., Joudrier, P., Gautier, M., 2002. Quantification of common wheat adulteration of durum wheat pasta using real-time quantitative polymerase chain reaction (PCR). *Cereal Chemistry* 79(4), 553-558.

Allmann, M., Candrian, U., Hofelein, C., Luthy, J., 1993. Polymerase chain-reaction (PCR) - a possible alternative to immunochemical methods assuring safety and quality of food - detection of wheat contamination in non-wheat food-products. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung* 196(3), 248-251.

Allmann, M., Candrian, U., Lüthy, J., 1992. Detection of wheat contamination in dietary non-wheat products by PCR. *The Lancet* 339(8788), 309.

Altenbach, S.B., Vensel, W.H., DuPont, F.M., 2010. Integration of transcriptomic and proteomic data from a single wheat cultivar provides new tools for understanding the roles of individual alpha gliadin proteins in flour quality and celiac disease. *Journal of Cereal Science* 52(2), 143-151.

Amaya-Gonzalez, S., de-los-Santos-Alvarez, N., Miranda-Ordieres, A.J., Lobo-Castanon, M.J., 2014. Aptamer Binding to Celiac Disease-Triggering Hydrophobic Proteins: A Sensitive Gluten Detection Approach. *Analytical Chemistry* 86(5), 2733-2739.

Amaya-González, S., de-los-Santos-Álvarez, N., Miranda-Ordieres, A., Lobo-Castañón, M., 2015a. Sensitive gluten determination in gluten-free foods by an electrochemical aptamer-based assay. *Analytical and Bioanalytical Chemistry*, 1-9.

Amaya-González, S., de-los-Santos-Álvarez, N., Miranda-Ordieres, A.J., Lobo-Castañón, M.J., 2013. Aptamer-Based Analysis: A Promising Alternative for Food Safety Control. *Sensors* 13(12), 16292-16311.

Amaya-González, S., López-López, L., Miranda-Castro, R., de-los-Santos-Álvarez, N., Miranda-Ordieres, A.J., Lobo-Castañón, M.J., 2015b. Affinity of aptamers binding 33-mer gliadin peptide and gluten proteins: Influence of immobilization and labeling tags. *Analytica Chimica Acta* 873(0), 63-70.

Anderson, O.D., Greene, F.C., 1997. The alpha-gliadin gene family. II DNA and protein sequence variation, subfamily structure and origins of pseudogenes. *Theor Appl Genet* 95, 59-65.

Anderson, O.D., Litts, J.C., Greene, F.C., 1997. The alpha-gliadin gene family. I. Characterization of ten new wheat alpha-gliadin genomic clones, evidence for limited sequence conservation of flanking DNA, and southern analysis of the gene family. *Theor Appl Genet* 95, 50-58.

Anderson, R.P., Degano, P., Godkin, A.J., Jewell, D.P., Hill, A.V.S., 2000. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med* 6(3), 337-342.

Arentz-Hansen, E.H., McAdam, S.N., Molberg, Ø., Kristiansen, C., Sollid, L.M., 2000a. Production of a panel of recombinant gliadins for the characterisation of T cell reactivity in coeliac disease. *Gut* 46(1), 46-51.

Arentz-Hansen, H., Körner, R., Molberg, Ø., Quarsten, H., Vader, W., Kooy, Y.M.C., Lundin, K.E.A., Koning, F., Roepstorff, P., Sollid, L.M., McAdam, S.N., 2000b. The Intestinal T Cell Response to α -Gliadin in Adult Celiac Disease Is Focused on a Single Deamidated Glutamine Targeted by Tissue Transglutaminase. *The Journal of Experimental Medicine* 191(4), 603-612.

Arentz-Hansen, H., McAdam, S.N., Molberg, Ø., Fleckenstein, B., Lundin, K.E.A., Jørgensen, T.J.D., Jung, G., Roepstorff, P., Sollid, L.M., 2002. Celiac lesion T cells recognize epitopes that cluster in regions of gliadins rich in proline residues. *Gastroenterology* 123(3), 803-809.

Arlorio, M., Coisson, J., Cereti, E., Travaglia, F., Capasso, M., Martelli, A., 2003. Polymerase chain reaction (PCR) of puroindoline b and ribosomal/puroindoline b multiplex PCR for the detection of common wheat (*Triticum aestivum*) in Italian pasta. *European Food Research and Technology* 216(3), 253-258.

Arora, P., Sindhu, A., Dilbaghi, N., Chaudhury, A., 2011. Biosensors as innovative tools for the detection of food borne pathogens. *Biosensors & Bioelectronics* 28(1), 1-12.

Barroso, M.F., Freitas, M., Oliveira, M.B.P.P., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Delerue-Matos, C., 2015. 3D-nanostructured Au electrodes for the event-specific detection of MON810 transgenic maize. *Talanta* 134, 158-164.

Bendich, A.J., McCarthy, B.J., 1970. DNA Comparisons among Barley, Oats, Rye, and Wheat. *Genetics* 65(4), 545-565.

Berti, F., Laschi, S., Palchetti, I., Rossier, J.S., Reymond, F., Mascini, M., Marrazza, G., 2009. Microfluidic-based electrochemical genosensor coupled to magnetic beads for hybridization detection. *Talanta* 77(3), 971-978.

Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G., Mascini, M., 2008. Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Analytica Chimica Acta* 614(1), 93-102.

Bietz, J.A., Wall, J.S., 1972. Wheat gluten subunits: Molecular weights determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Cereal Chemistry*.

Bonanni, A., Esplandiú, M., Del Valle, M., 2009. Impedimetric genosensors employing COOH-modified carbon nanotube screen-printed electrodes. *Biosensors and Bioelectronics* 24(9), 2885-2891.

Bonanni, A., Pumera, M., 2011. Graphene Platform for Hairpin-DNA-Based Impedimetric Genosensing. *ACS Nano* 5(3), 2356-2361.

Briani, C., Samaroo, D., Alaedini, A., 2008. Celiac disease: From gluten to autoimmunity. *Autoimmunity Reviews* 7(8), 644-650.

Bryan, G., Dixon, A., Gale, M., Wiseman, G., 1998. A PCR-based method for the detection of hexaploid bread wheat adulteration of durum wheat and pasta. *Journal of Cereal Science* 28(2), 135-145.

Cahoon, A.B., Sharpe, R.M., Mysayphonh, C., Thompson, E.J., Ward, A.D., Lin, A., 2010. The complete chloroplast genome of tall fescue (*Lolium arundinaceum*; Poaceae) and comparison of whole plastomes from the family Poaceae. *American Journal of Botany* 97(1), 49-58.

Campuzano, S., Kuralay, F., Lobo-Castañón, M.J., Bartošík, M., Vyavahare, K., Paleček, E., Haake, D.A., Wang, J., 2011. Ternary monolayers as DNA recognition interfaces for direct and sensitive electrochemical detection in untreated clinical samples. *Biosensors and Bioelectronics* 26(8), 3577-3583.

Carpini, G., Lucarelli, F., Marrazza, G., Mascini, M., 2004. Oligonucleotide-modified screen-printed gold electrodes for enzyme-amplified sensing of nucleic acids. *Biosensors & Bioelectronics* 20(2), 167-175.

Cataldo, F., Marino, V., Bottaro, G., Greco, P., Ventura, A., 1997. Celiac disease and selective immunoglobulin A deficiency. *The Journal of Pediatrics* 131(2), 306-308.

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., Fasano, A., 2007. A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *American Journal of Clinical Nutrition* 85(1), 160-166.

Catassi, C., Fasano, A., 2008. Celiac disease. *Current Opinion in Gastroenterology* 24(6).

Codex Alimentarius Commission, 2006. Report of the 27th session of the Codex Committee on Methods of Analysis and Sampling, Budapest, Hungary, 15–19 May 2006. ALINORM.

- Codex Alimentarius Commission, 2008. Codex Alimentarius Alinorm 08/31/26. Geneva, Switzerland.
- Cornec, M., Popineau, Y., Lefebvre, J., 1994. Characterisation of Gluten Subfractions by SE-HPLC and Dynamic Rheological Analysis in Shear. *Journal of Cereal Science* 19(2), 131-139.
- Costa, J., Mafra, I., Oliveira, M.B.P.P., 2012. High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry* 133(3), 1062-1069.
- Crevel, R.W.R., Ballmer-Weber, B.K., Holzhauser, T., Hourihane, J.O.B., Knulst, A.C., Mackie, A.R., Timmermans, F., Taylor, S.L., 2008. Thresholds for food allergens and their value to different stakeholders. *Allergy* 63(5), 597-609.
- Dahinden, I., von Buren, M., Luthy, J., 2001. A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients. *European Food Research and Technology* 212(2), 228-233.
- Daum, S., Cellier, C., Mulder, C.J.J., 2005. Refractory coeliac disease. *Best Practice & Research Clinical Gastroenterology* 19(3), 413-424.
- De Re, V., Caggiari, L., Tabuso, M., Cannizzaro, R., 2013. The versatile role of gliadin peptides in celiac disease. *Clinical Biochemistry* 46(6), 552-560.
- de-los-Santos-Álvarez, P., Lobo-Castañón, M.J., Miranda-Ordieres, A., Tuñón-Blanco, P., 2004. Current strategies for electrochemical detection of DNA with solid electrodes. *Analytical and Bioanalytical Chemistry* 378(1), 104-118.
- Debnath, J., Martin, A., Gowda, L., 2009. A polymerase chain reaction directed to detect wheat glutenin: Implications for gluten-free labelling. *Food Research International* 42(7), 782-787.
- Deféver, T., Druet, M., Evrard, D., Marchal, D., Limoges, B., 2011. Real-Time Electrochemical PCR with a DNA Intercalating Redox Probe. *Analytical Chemistry* 83(5), 1815-1821.
- Del Giallo, M., Lucarelli, F., Cosulich, E., Pistarino, E., Santamaria, B., Marrazza, G., Mascini, M., 2005. Steric factors controlling the surface hybridization of PCR amplified sequences. *Analytical Chemistry* 77(19), 6324-6330.
- Denery-Papini, S., Nicolas, Y., Popineau, Y., 1999. Efficiency and Limitations of Immunochemical Assays for the Testing of Gluten-free Foods. *Journal of Cereal Science* 30(2), 121-131.
- Denham, J., Hill, I., 2013. Celiac Disease and Autoimmunity: Review and Controversies. *Current Allergy and Asthma Reports* 13(4), 347-353.
- Dewar, D.H., Amato, M., Ellis, H.J., Pollock, E.L., Gonzalez-Cinca, N., Wieser, H., Ciclitira, P.J., 2006. The toxicity of high molecular weight glutenin subunits

of wheat to patients with coeliac disease. *European Journal of Gastroenterology & Hepatology* 18(5), 483-491.

Dewar, D.H., Ciclitira, P.J., 2005. Clinical features and diagnosis of celiac disease. *Gastroenterology* 128(4, Supplement 1), S19-S24.

Diaz-Amigo, C., Popping, B., 2013. Accuracy of ELISA Detection Methods for Gluten and Reference Materials: A Realistic Assessment. *Journal of Agricultural and Food Chemistry* 61(24), 5681-5688.

Dickey, W., Hughes, D.F., McMillan, S.A., 2000. Disappearance of endomysial antibodies in treated celiac disease does not indicate histological recovery. *Am J Gastroenterol* 95(3), 712-714.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., Schuppan, D., 1997. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature medicine* 3(7), 797-801.

Duwensee, H., Mix, M., Broer, I., Flechsig, G., 2009. Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine. *Electrochemistry Communications* 11(7), 1487-1491.

D'Amico, M.A., Holmes, J., Stavropoulos, S.N., Frederick, M., Levy, J., DeFelice, A.R., Kazlow, P.G., Green, P.H.R., 2005. Presentation of Pediatric Celiac Disease in the United States: Prominent Effect of Breastfeeding. *Clinical Pediatrics* 44(3), 249-258.

Ellis, H.J., Doyle, A.P., Day, P., Wieser, H., Ciclitira, P.J., 1994. Demonstration of the Presence of Coeliac-Activating Gliadin-Like Epitopes in Malted Barley. *International Archives of Allergy and Immunology* 104(3), 308-310.

Ellis, H.J., Rosen-Bronson, S., O'Reilly, N., Ciclitira, P.J., 1998. Measurement of gluten using a monoclonal antibody to a coeliac toxic peptide of A gliadin. *Gut* 43(2), 190-195.

ENGL, E.N.o.G.L., 2008. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing. European Commission, Brussels, Belgium.

Ewart, J.A.D., 1966. Cereal proteins: Immunological studies. *Journal of the Science of Food and Agriculture* 17(6), 279-284.

Fang, T.H., Ramalingam, N., Xian-Dui, D., Ngin, T.S., Xianting, Z., Lai Kuan, A.T., Peng Huat, E.Y., Hai-Qing, G., 2009. Real-time PCR microfluidic devices with concurrent electrochemical detection. *Biosensors and Bioelectronics* 24(7), 2131-2136.

Farjami, E., Campos, R., Ferapontova, E., 2012. Effect of the DNA End of Tethering to Electrodes on Electron Transfer in Methylene Blue-Labeled DNA Duplexes. *Langmuir* 28(46), 16218-16226.

Fasano, A., 2003. Celiac disease: how to handle a clinical chameleon. *N Engl J Med* 348(25), 2568-2570.

Fasano, A., Catassi, C., 2001. Current approaches to diagnosis and treatment of celiac disease: An evolving spectrum. *Gastroenterology* 120(3), 636-651.

Fasano, A., Catassi, C., 2012. Celiac Disease. *New England Journal of Medicine* 367(25), 2419-2426.

Ferguson, A., Arranz, E., O'Mahony, S., 1993. Clinical and pathological spectrum of coeliac disease--active, silent, latent, potential. *Gut* 34(2), 150-151.

García, E., Llorente, M., Hernando, A., Kieffer, R., Wieser, H., Méndez, E., 2005. Development of a general procedure for complete extraction of gliadins for heat processed and unheated foods. *European Journal of Gastroenterology & Hepatology* 17(5).

Gass, J., Bethune, M.T., Siegel, M., Spencer, A., Khosla, C., 2007. Combination Enzyme Therapy for Gastric Digestion of Dietary Gluten in Patients With Celiac Sprue. *Gastroenterology* 133(2), 472-480.

Gessendorfer, B., Wieser, H., Koehler, P., 2010. Optimisation of a solvent for the complete extraction of prolamins from heated foods. *Journal of Cereal Science* 52(2), 331-332.

Gianfrani, C., Auricchio, S., Troncone, R., 2005. Adaptive and innate immune responses in celiac disease. *Immunology Letters* 99(2), 141-145.

Ginzinger, D.G., 2002. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology* 30(6), 503-512.

Goesaert, H., Brijs, K., Veraverbeke, W.S., Courtin, C.M., Gebruers, K., Delcour, J.A., 2005. Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology* 16(1-3), 12-30.

Gonzalez-Alvarez, M., Perez-Ruiz, E., Miranda-Castro, R., de-los-Santos-Alvarez, N., Miranda-Ordieres, A., Lobo-Castanon, M., 2013. Effect of Tags and Labels on the Performance of Enzyme-Amplified Electrochemical Genomagnetic Assays. *Electroanalysis* 25(1), 147-153.

Green, P., Cellier, C., 2007. Medical progress: Celiac disease. *New England Journal of Medicine* 357(17), 1731-1743.

Green, P., Jabri, B., 2006. Celiac disease. *Annual Review of Medicine* 57, 207-221.

Green, P.H.R., Jabri, B., 2003. Coeliac disease. *The Lancet* 362(9381), 383-391.

Gryson, N., 2010. Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review. *Analytical and Bioanalytical Chemistry* 396(6), 2003-2022.

Hallert, C., Grant, C., Grehn, S., Grännö, C., Hultén, S., Midhagen, G., Ström, M., Svensson, H., Valdimarsson, T., 2002. Evidence of poor vitamin status in coeliac patients on a gluten-free diet for 10 years. *Alimentary Pharmacology & Therapeutics* 16(7), 1333-1339.

Haraszi, R., Chassaing, H., Maquet, A., Ulberth, F., 2011. Analytical methods for detection of gluten in food--method developments in support of food labeling legislation. *Journal of AOAC International* 94(4), 1006-1025.

Hernandez, M., Esteve, T., Pla, M., 2005. Real-time polymerase chain reaction based assays for quantitative detection of barley, rice, sunflower, and wheat. *Journal of Agricultural and Food Chemistry* 53(18), 7003-7009.

Hischenhuber, C., Crevel, R., Jarry, B., MÄKi, M., Moneret-Vautrin, D.A., Romano, A., Troncone, R., Ward, R., 2006. Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease. *Alimentary Pharmacology & Therapeutics* 23(5), 559-575.

Holzhauser, T., Röder, M., 2015. 13 - Polymerase chain reaction (PCR) methods for detecting allergens in foods. In: Flanagan, S. (Ed.), *Handbook of Food Allergen Detection and Control*, pp. 245-263. Woodhead Publishing.

Holzhauser, T., Stephan, O., Vieths, S., 2006. 7 - Polymerase chain reaction (PCR) methods for the detection of allergenic foods. In: Koppelman, S.J., Hefle, S.L. (Eds.), *Detecting Allergens in Food*, pp. 125-143. Woodhead Publishing.

Hubner, P., Waiblinger, H., Pietsch, K., Brodmann, P., 2001. Validation of PCR methods for quantitation of genetically modified plants in food. *Journal of Aoac International* 84(6), 1855-1864.

Husby, S., Koletzko, S., Korponay-Szabó, I.R., Mearin, M.L., Phillips, A., Shamir, R., Troncone, R., Giersiepen, K., Branski, D., Catassi, C., Lelgeman, M., Mäki, M., Ribes-Koninckx, C., Ventura, A., Zimmer, K.P., for the Espghan Working Group on Coeliac Disease Diagnosis, o.b.o.t.E.G.C., 2012. European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Guidelines for the Diagnosis of Coeliac Disease. *Journal of Pediatric Gastroenterology and Nutrition* 54(1).

Ihalainen, P., Pettersson, F., Pesonen, M., Viitala, T., Määttänen, A., Österbacka, R., Peltonen, J., 2014. An impedimetric study of DNA hybridization on paper-supported inkjet-printed gold electrodes. *Nanotechnology* 25(9), 094009.

James, D., Schmidt, A.-m., 2004. Use of an intron region of a chloroplast tRNA gene (trnL) as a target for PCR identification of specific food crops including sources of potential allergens. *Food Research International* 37(4), 395-402.

Janatuinen, E., Kempainen, T., Julkunen, R., Kosma, V., Maki, M., Heikkinen, M., Uusitupa, M., 2002. No harm from five year ingestion of oats in coeliac disease. *Gut* 50(3), 332-335.

Janssen, F., 2006. Detecting wheat gluten in food. In: Koppelman, S.J., Hefle, S.L. (Eds.), *Detecting Allergens in Food*, pp. 244-272. Woodhead Publishing, Boca Raton.

Jones, R.W., Taylor, N.W., Senti, F.R., 1959. Electrophoresis and fractionation of wheat gluten. *Archives of Biochemistry and Biophysics* 84(2), 363-376.

Kagnoff, M., 2007. Celiac disease: pathogenesis of a model immunogenetic disease. *Journal of Clinical Investigation* 117(1), 41-49.

Kanerva, P., Sontag-Strohm, T., Brinck, O., 2008. Improved extraction of prolamins for gluten detection in processed foods. *Agricultural and Food Science* 20(3), 206-216.

Kara, P., Ozkan, D., Erdem, A., Kerman, K., Pehlivan, S., Ozkinay, F., Unuvar, D., Itirli, G., Ozsoz, M., 2003. Detection of achondroplasia G380R mutation from PCR amplicons by using inosine modified carbon electrodes based on electrochemical DNA chip technology. *Clinica Chimica Acta* 336(1-2), 57-64.

Kaukinen, K., Partanen, J., Maki, M., Collin, P., 2002. HLA-DQ typing in the diagnosis of celiac disease. *Am J Gastroenterol* 97(3), 695-699.

Kerman, K., Ozkan, D., Kara, P., Meric, B., Gooding, J., Ozsoz, M., 2002. Voltammetric determination of DNA hybridization using methylene blue and self-assembled alkanethiol monolayer on gold electrodes. *Analytica Chimica Acta* 462(1), 39-47.

Kerman, K., Vestergaard, M., Nagatani, N., Takamura, Y., Tamiya, E., 2006. Electrochemical genosensor based on peptide nucleic acid-mediated PCR and asymmetric PCR techniques: Electrostatic interactions with a metal cation. *Analytical Chemistry* 78(7), 2182-2189.

Kim, C.-Y., Quarsten, H., Bergseng, E., Khosla, C., Sollid, L.M., 2004. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proceedings of the National Academy of Sciences* 101(12), 4175-4179.

Ko, H.L., Henry, R.J., Graham, G.C., Fox, G.P., Chadbone, D.A., Haak, I.C., 1994. Identification of Cereals using the Polymerase Chain Reaction. *Journal of Cereal Science* 19(2), 101-106.

Kobrehel, K., Gautier, M.-F., 1974. Variability in peroxidase isozymes in wheat and related species. *Canadian Journal of Botany* 52(4), 755-759.

Konic-Ristic, A., Dodig, D., Krstic, R., Jelic, S., Stankovic, I., Ninkovic, A., Radic, J., Besu, I., Bonaci-Nikolic, B., Jojic, N., Djordjevic, M., Popovic, D., Juranic, Z., 2009. Different levels of humoral immunoreactivity to different wheat cultivars gliadin are present in patients with celiac disease and in patients with multiple myeloma. *Bmc Immunology* 10.

Koppel, E., Stadler, M., Luthy, J., Hubner, P., 1998. Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked

immunosorbent assay (ELISA). *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology* 206(6), 399-403.

Kuralay, F., Campuzano, S., Wang, J., 2012. Greatly extended storage stability of electrochemical DNA biosensors using ternary thiolated self-assembled monolayers. *Talanta* 99, 155-160.

Leszczyńska, J., Majak, I., Bartos, A., 2014. Gluten testing in foods, pharmaceuticals and cosmetics. *Food Allergen Testing*, pp. 151-184. John Wiley & Sons, Ltd.

Liao, W., Chuang, M., Ho, J., 2013. Electrochemical sensor for multiplex screening of genetically modified DNA: Identification of biotech crops by logic-based biomolecular analysis. *Biosensors & Bioelectronics* 50, 414-420.

Ligaj, M., Oczkowski, T., Jasnowska, J., Musiał, W., Filipiak, M., 2003. Electrochemical genosensors for detection of *L. monocytogenes* and genetically-modified components in food and genetically-modified components in food. *Polish journal of food and nutrition sciences* 12(Suppl. 2), 61-63.

Liu, X., Zhang, X., 2015. Aptamer-Based Technology for Food Analysis. *Applied Biochemistry and Biotechnology* 175(1), 603-624.

Liu, Y., Li, H., Chen, W., Liu, A., Lin, X., Chen, Y., 2013. Bovine Serum Albumin-Based Probe Carrier Platform for Electrochemical DNA Biosensing. *Analytical Chemistry* 85(1), 273-277.

Lucarelli, F., Marrazza, G., Mascini, M., 2005. Enzyme-based impedimetric detection of PCR products using oligonucleotide-modified screen-printed gold electrodes. *Biosensors & Bioelectronics* 20(10), 2001-2009.

Lucarelli, F., Marrazza, G., Turner, A., Mascini, M., 2004. Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors. *Biosensors & Bioelectronics* 19(6), 515-530.

Lucarelli, F., Tombelli, S., Minunni, M., Marrazza, G., Mascini, M., 2008. Electrochemical and piezoelectric DNA biosensors for hybridisation detection. *Analytica Chimica Acta* 609(2), 139-159.

Lundin, K.E.A., Nilsen, E.M., Scott, H.G., Løberg, E.M., Gjøen, A., Bratlie, J., Skar, V., Mendez, E., Løvik, A., Kett, K., 2003. Oats induced villous atrophy in coeliac disease. *Gut* 52(11), 1649-1652.

Ma, Y., Jiao, K., Yang, T., Sun, D., 2008. Sensitive PAT gene sequence detection by nano-SiO₂/p-aminothiophenol self-assembled films DNA electrochemical biosensor based on impedance measurement. *Sensors and Actuators B-Chemical* 131(2), 565-571.

Mafra, I., Ferreira, I.M.P.L.V.O., Oliveira, M.B.P.P., 2008a. Food authentication by PCR-based methods. *European Food Research and Technology* 227(3), 649-665.

Mafra, I., Silva, S., Moreira, E., da Silva, C., Beatriz, M., Oliveira, P., 2008b. Comparative study of DNA extraction methods for soybean derived food products. *Food Control* 19(12), 1183-1190.

Manzanares-Palenzuela, C.L., Martín-Fernández, B., Sánchez-Paniagua López, M., López-Ruiz, B., 2015. Electrochemical genosensors as innovative tools for detection of genetically modified organisms. *TrAC Trends in Analytical Chemistry* 66, 19-31.

Martins-Lopes, P., Gomes, S., Pereira, L., Guedes-Pinto, H., 2013. Molecular Markers for Food Traceability. *Food Technology and Biotechnology* 51(2), 198-207.

Martínez-Paredes, G., González-García, M.B., Costa-García, A., 2009. Genosensor for SARS Virus Detection Based on Gold Nanostructured Screen-Printed Carbon Electrodes. *Electroanalysis* 21(3-5), 379-385.

Mascini, M., Del Carlo, M., Minunni, M., Chen, B., Compagnone, D., 2005. Identification of mammalian species using genosensors. *Bioelectrochemistry* 67(2), 163-169.

Maskova, E., Paulickova, I., Rysova, J., Gabrovska, D., 2011. Evidence for Wheat, Rye, and Barley Presence in Gluten Free Foods by PCR Method - Comparison with Elisa Method. *Czech Journal of Food Sciences* 29(1), 45-50.

Matysiak-Budnik, T., Candalh, C., Cellier, C., Dugave, C., Namane, A., Vidal-Martinez, T., Cerf-Bensussan, N., Heyman, M., 2005. Limited Efficiency of Prolyl-Endopeptidase in the Detoxification of Gliadin Peptides in Celiac Disease. *Gastroenterology* 129(3), 786-796.

Mayer, F., Haase, I., Graubner, A., Heising, F., Paschke-Kratzin, A., Fischer, M., 2012. Use of Polymorphisms in the γ -Gliadin Gene of Spelt and Wheat as a Tool for Authenticity Control. *Journal of Agricultural and Food Chemistry* 60(6), 1350-1357.

McL Mowat, A., 2003. Coeliac disease—a meeting point for genetics, immunology, and protein chemistry. *The Lancet* 361(9365), 1290-1292.

Mearin, M.L., Ivarsson, A., Dickey, W., 2005. Coeliac disease: is it time for mass screening? *Best Practice & Research Clinical Gastroenterology* 19(3), 441-452.

Meloni, G.F., Dessole, S., Vargiu, N., Tomasi, P.A., Musumeci, S., 1999. The prevalence of coeliac disease in infertility. *Human Reproduction* 14(11), 2759-2761.

Mena, M.C., Lombardía, M., Hernando, A., Méndez, E., Albar, J.P., 2012. Comprehensive analysis of gluten in processed foods using a new extraction method and a competitive ELISA based on the R5 antibody. *Talanta* 91(0), 33-40.

Miranda-Castro, R., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2009. Structured nucleic acid probes for electrochemical devices. *Electroanalysis* 21(19), 2077-2090.

Mix, M., Rüger, J., Krüger, S., Broer, I., Flechsig, G.-U., 2012. Electrochemical detection of 0.6% genetically modified maize MON810 in real flour samples. *Electrochemistry Communications* 22, 137-140.

Molberg, O., McAdam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E.A., Sjostrom, H., Sollid, L.M., 1998. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* 4(6), 713-717.

Molberg, Ø., Solheim flÆete, N., Jensen, T., Lundin, K.E.A., Arentz-Hansen, H., Anderson, O.D., Kjersti Uhlen, A., Sollid, L.M., 2003. Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. *Gastroenterology* 125(2), 337-344.

Mujico, J., Lombardia, M., Mena, M., Mendez, E., Albar, J., 2011. A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chemistry* 128(3), 795-801.

Mustalahti, K., Catassi, C., Reunanen, A., Fabiani, E., Heier, M., McMillan, S., Murray, L., Metzger, M.-H., Gasparin, M., Bravi, E., Mäki, M., 2010. The prevalence of celiac disease in Europe: Results of a centralized, international mass screening project. *Annals of Medicine* 42(8), 587-595.

Mustorp, S., Dromtorp, S., Holck, A., 2011. Multiplex, Quantitative, Ligation-Dependent Probe Amplification for Determination of Allergens in Food. *Journal of Agricultural and Food Chemistry* 59(10), 5231-5239.

Méndez, E., Vela, C., Immer, U., Janssen, F.W., 2005. Report of a collaborative trial to investigate the performance of the R5 enzyme linked immunoassay to determine gliadin in gluten-free food. *European Journal of Gastroenterology & Hepatology* 17(10).

Nasr, I., Messing, J., Nasr, I.H., Ciclitira, P.J., 2015. 19 - Detection and control of gluten as a food allergen. In: Flanagan, S. (Ed.), *Handbook of Food Allergen Detection and Control*, pp. 367-377. Woodhead Publishing.

Nilsen, E.M., Jahnsen, F.L., Lundin, K.E.A., Johansen, F.E., Fausa, O., Sollid, L.M., Jahnsen, J., Scott, H., Brandtzaeg, P., 1998. Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* 115(3), 551-563.

Nilsen, E.M., Lundin, K.E., Krajci, P., Scott, H., Sollid, L.M., Brandtzaeg, P., 1995. Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. *Gut* 37(6), 766-776.

Nimmo, C.C., O'Sullivan, M.T., 1967. Immunochemical comparisons of antigenic proteins of durum and hard red spring wheats. *Cereal Chem* 44, 584-591.

Norris, J.M., Barriga, K., Hoffenberg, E.J., et al., 2005. Risk of celiac disease autoimmunity and timing of gluten introduction in the diet of infants at increased risk of disease. *JAMA* 293(19), 2343-2351.

Olexova, L., Dovicovicova, L., Svec, M., Siekel, P., Kuchta, T., 2006. Detection of gluten-containing cereals in flours and "gluten-free" bakery products by polymerase chain reaction. *Food Control* 17(3), 234-237.

Osborne, T.B., 1924. The vegetable proteins. *Journal of the Society of Chemical Industry* 43(17), 440-440.

Pandey, C., Singh, R., Sumana, G., Pandey, M., Malhotra, B., 2011. Electrochemical genosensor based on modified octadecanethiol self-assembled monolayer for *Escherichia coli* detection. *Sensors and Actuators B-Chemical* 151(2), 333-340.

Pasqualone, A., 2011. Authentication of durum wheat-based foods: Classical vs. innovative methods. In: Oliveira, M.B.P.P., Mafra, I., Amaral, J.S. (Eds.), *Current topics in food authenticity*, pp. 23-39. Transworld Research Network, Kerala, India.

Pasqualone, A., Montemurro, C., Grinn-Gofron, A., Sonnante, G., Blanco, A., 2007. Detection of Soft Wheat in Semolina and Durum Wheat Bread by Analysis of DNA Microsatellites. *Journal of Agricultural and Food Chemistry* 55(9), 3312-3318.

Paulley, J.W., 1954. Observations on the Aetiology of Idiopathic Steatorrhoea. *BMJ* 2(4900), 1318-1321.

Pegels, N., González, I., García, T., Martín, R., 2015. Authenticity testing of wheat, barley, rye and oats in food and feed market samples by real-time PCR assays. *LWT - Food Science and Technology* 60(2, Part 1), 867-875.

Pietzak, M.M., 2005. Follow-up of patients with celiac disease: Achieving compliance with treatment. *Gastroenterology* 128(4, Supplement 1), S135-S141.

Piknova, L., Brezna, B., Kuchta, T., 2008. Detection of gluten-containing cereals in food by 5'-nuclease real-time polymerase chain reaction. *Journal of Food and Nutrition Research* 47(3), 114-119.

Pinto, A., Polo, P., Henry, O., Redondo, M., Svobodova, M., O'Sullivan, C., 2014. Label-free detection of gliadin food allergen mediated by real-time apta-PCR. *Analytical and Bioanalytical Chemistry* 406(2), 515-524.

Pividori, M., Lermo, A., Bonanni, A., Alegret, S., del Valle, M., 2009. Electrochemical immunosensor for the diagnosis of celiac disease. *Analytical Biochemistry* 388(2), 229-234.

Pividori, M.I., Alegret, S., 2010. Electrochemical biosensors for food safety. pp. 173-191. Contributions to Science.

Pividori, M.I., Merkoci, A., Alegret, S., 2000. Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods. Biosensors and Bioelectronics 15(5), 291-303.

Poms, R.E., Anklam, E., Kuhn, M., 2004a. Polymerase Chain Reaction Techniques for Food Allergen Detection. Journal of AOAC International 87(6), 1391-1397.

Poms, R.E., Klein, C.L., Anklam, E., 2004b. Methods for allergen analysis in food: a review. Food Additives and Contaminants 21(1), 1-31.

Premanandh, J., 2011. Global consensus—Need of the hour for genetically modified organisms (GMO) labeling. Journal of Commercial Biotechnology 17(1), 37-44.

Prins, T.W., van Dijk, J.P., Angeline Van Hoef, A.M., Voorhuijzen, M.M., Broeders, S., Trapmann, S., Seyfarth, R., Pardigol, A., Schoen, C.D., Aarts, H.J.M., Kok, E.J., 2010. Towards a multiplex cereal traceability tool using padlock probe ligation on genomic DNA. Food Chemistry 118(4), 966-973.

Regulation (EC) 41/2009, of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. Official Journal of European Union, pp. 3-5.

Regulation (EC) 609/2013, of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. Official Journal of European Union, pp. 35-56.

Regulation (EC) 828/2014, of 30 July 2014 on the requirements for the provision of information to consumers on the absence or reduced presence of gluten in food. Official Journal of European Union, pp. 5-8.

Regulation (EC) 1151/2012, of 21 November 2012 on quality schemes for agricultural products and foodstuffs. Official Journal of European Union, pp. 1-29.

Regulation (EC) 1169/2011, of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, . Official Journal of European Union, pp. 18-63.

Regulation (EC) 1222/1994, of 30 May 1994 laying down common detailed rules for the application of the system of granting export refunds on certain agricultural products exported in the form of goods not covered by Annex II to the Treaty, and the criteria for fixing the amount of such refunds. Official Journal of European Union, pp. 5-32.

Reif, S., Lerner, A., 2004. Tissue transglutaminase—the key player in celiac disease: a review. *Autoimmunity Reviews* 3(1), 40-45.

Reilly, N., Green, P.R., 2012. Epidemiology and clinical presentations of celiac disease. *Seminars in Immunopathology* 34(4), 473-478.

Reilly, N.R., Fasano, A., Green, P.H.R., 2012. Presentation of Celiac Disease. *Gastrointestinal Endoscopy Clinics of North America* 22(4), 613-621.

Ren, Y., Jiao, K., Xu, G., Sun, W., Gao, H., 2005. An electrochemical DNA sensor based on electrodepositing aluminum ion films on stearic acid-modified carbon paste electrode and its application for the detection of specific sequences related to bar gene and CP4 Epsps gene. *Electroanalysis* 17(23), 2182-2189.

Rodriguez-Lazaro, D., Hernandez, M., 2013. Real-time PCR in Food Science: Introduction. *Current Issues in Molecular Biology* 15(2), 25-37.

Romanos, J., van Diemen, C.C., Nolte, I.M., Trynka, G., Zhernakova, A., Fu, J., Bardella, M.T., Barisani, D., McManus, R., van Heel, D.A., Wijmenga, C., 2009. Analysis of HLA and Non-HLA Alleles Can Identify Individuals at High Risk for Celiac Disease. *Gastroenterology* 137(3), 834-840.e833.

Ronning, S., Berdal, K., Andersen, C., Holst-Jensen, A., 2006. Novel reference gene, PKABA1, used in a duplex real-time polymerase chain reaction for detection and quantitation of wheat- and barley-derived DNA. *Journal of Agricultural and Food Chemistry* 54(3), 682-687.

Rostami, K., Kerckhaert, J., Tiemessen, R., von Blomberg, B.M.E., Meijer, J.W.R., Mulder, C.J.J., 1999. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. *Am J Gastroenterol* 94(4), 888-894.

Rostom, A., Dubé, C., Cranney, A., Saloojee, N., Sy, R., Garritty, C., Sampson, M., Zhang, L., Yazdi, F., Mamaladze, V., Pan, I., MacNeil, J., Mack, D., Patel, D., Moher, D., 2005. The diagnostic accuracy of serologic tests for celiac disease: A systematic review. *Gastroenterology* 128(4, Supplement 1), S38-S46.

Rubesin, S.E., Herlinger, H., Saul, S.H., Grumbach, K., Laufer, I., Levine, M.S., 1989. Adult celiac disease and its complications. *RadioGraphics* 9(6), 1045-1066.

Rubio-Tapia, A., Kyle, R.A., Kaplan, E.L., Johnson, D.R., Page, W., Erdtmann, F., Brantner, T.L., Kim, W.R., Phelps, T.K., Lahr, B.D., Zinsmeister, A.R.,

Melton Iii, L.J., Murray, J.A., 2009. Increased Prevalence and Mortality in Undiagnosed Celiac Disease. *Gastroenterology* 137(1), 88-93.

Sandberg, M., Lundberg, L., Ferm, M., Yman, I., 2003. Real Time PCR for the detection and discrimination of cereal contamination in gluten free foods. *European Food Research and Technology* 217(4), 344-349.

Schuppan, D., Junker, Y., Barisani, D., 2009. Celiac Disease: From Pathogenesis to Novel Therapies. *Gastroenterology* 137(6), 1912-1933.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G., Sollid, L., Khosla, C., 2002. Structural basis for gluten intolerance in Celiac sprue. *Science* 297(5590), 2275-2279.

Shewry, P.R., Halford, N.G., 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany* 53(370), 947-958.

Sicherer, S.H., 2000. Determinants of systemic manifestations of food allergy. *Journal of Allergy and Clinical Immunology* 106(5, Supplement), S251-S257.

Simell, S., Hoppu, S., Hekkala, A., Simell, T., Ståhlberg, M.-R., Viander, M., Yrjänäinen, H., Grönlund, J., Markula, P., Simell, V., 2007. Fate of five celiac disease-associated antibodies during normal diet in genetically at-risk children observed from birth in a natural history study. *The American journal of gastroenterology* 102(9), 2026-2035.

Skerritt, J.H., Hill, A.S., 1990. Monoclonal antibody sandwich enzyme immunoassays for determination of gluten in foods. *Journal of Agricultural and Food Chemistry* 38(8), 1771-1778.

Smedby, K.E., Åkerman, M., Hildebrand, H., Glimelius, B., Ekbom, A., Askling, J., 2005. Malignant lymphomas in coeliac disease: evidence of increased risks for lymphoma types other than enteropathy-type T cell lymphoma. *Gut* 54(1), 54-59.

Sollid, L., 2002. Coeliac disease: Dissecting a complex inflammatory disorder. *Nature Reviews Immunology* 2(9), 647-655.

Sollid, L.M., Khosla, C., 2005. Future therapeutic options for celiac disease. *Nat Clin Pract Gastroenterol Hepatol* 2(3), 140-147.

Sollid, L.M., Markussen, G., Ek, J., Gjerde, H., Vartdal, F., Thorsby, E., 1989. Evidence for a primary association of celiac disease to a particular HLA-DQ alpha/beta heterodimer. *The Journal of Experimental Medicine* 169(1), 345-350.

Sonnante, G., Montemurro, C., Morgese, A., Sabetta, W., Blanco, A., Pasqualone, A., 2009. DNA Microsatellite Region for a Reliable Quantification of Soft Wheat Adulteration in Durum Wheat-Based Foodstuffs by Real-Time PCR. *Journal of Agricultural and Food Chemistry* 57(21), 10199-10204.

Steel, A., Herne, T., Tarlov, M., 1998. Electrochemical quantitation of DNA immobilized on gold. *Analytical Chemistry* 70(22), 4670-4677.

Stene, L.C., Honeyman, M.C., Hoffenberg, E.J., Haas, J.E., Sokol, R.J., Emery, L., Taki, I., Norris, J.M., Erlich, H.A., Eisenbarth, G.S., Rewers, M., 2006. Rotavirus Infection Frequency and Risk of Celiac Disease Autoimmunity in Early Childhood: A Longitudinal Study. *Am J Gastroenterol* 101(10), 2333-2340.

Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., Koning, F., 2006. Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease. *American Journal of Physiology - Gastrointestinal and Liver Physiology* 291(4), G621-G629.

Straker, R.J., Gunasekaran, S., Brady, P.G., 1989. Adenocarcinoma of the Jejunum in Association with Celiac Sprue. *Journal of Clinical Gastroenterology* 11(3).

Sun, W., 2010. Nucleic Extraction and amplification. *Molecular Diagnostics: Techniques and Applications for Clinical Laboratory*. Academic Press Inc. .

Sun, W., Zhong, J., Qin, P., Jiao, K., 2008. Electrochemical biosensor for the detection of cauliflower mosaic virus 35 S gene sequences using lead sulfide nanoparticles as oligonucleotide labels. *Analytical Biochemistry* 377(2), 115-119.

Sun, X., Guan, L., Shan, X., Zhang, Y., Li, Z., 2012. Electrochemical Detection of Peanut Allergen Ara h 1 Using a Sensitive DNA Biosensor Based on Stem-Loop Probe. *Journal of Agricultural and Food Chemistry* 60(44), 10979-10984.

Sun, X., Jia, M., Ji, J., Guan, L., Zhang, Y., Tang, L., Li, Z., 2015. Enzymatic amplification detection of peanut allergen Ara h1 using a stem-loop DNA biosensor modified with a chitosan-mutiwalled carbon nanotube nanocomposite and spongy gold film. *Talanta* 131, 521-527.

Sánchez-Paniagua López, M., Frutos Cabanillas, G., Lobo Castañón, M.J., López-Ruiz, B., 2014. Development of a genosensor for peanut allergen ARA h 2 detection and its optimization by surface response methodology. *Biosensors and Bioelectronics* 62, 350-356.

Tack, G.J., Verbeek, W.H.M., Schreurs, M.W.J., Mulder, C.J.J., 2010. The spectrum of celiac disease: epidemiology, clinical aspects and treatment. *Nat Rev Gastroenterol Hepatol* 7(4), 204-213.

Tanabe, S., 2008. Analysis of food allergen structures and development of foods for allergic patients. *Bioscience Biotechnology and Biochemistry* 72(3), 649-659.

Taverniers, I., Windels, P., Vařilingom, M., Milcamps, A., Van Bockstaele, E., Van den Eede, G., De Loose, M., 2005. Event-Specific Plasmid Standards and Real-Time PCR Methods for Transgenic Bt11, Bt176, and GA21 Maize and

Transgenic GT73 Canola. *Journal of Agricultural and Food Chemistry* 53(8), 3041-3052.

Tavoletti, S., Iommarini, L., Pasquini, M., 2009. A DNA method for qualitative identification of plant raw materials in feedstuff. *European Food Research and Technology* 229(3), 475-484.

Teles, F.R.R., Fonseca, L.P., 2008. Trends in DNA biosensors. *Talanta* 77(2), 606-623.

Terzi, V., Infascelli, F., Tudisco, R., Russo, G., Stanca, A., Faccioli, P., 2004. Quantitative detection of *Secale cereale* by real-time PCR amplification. *Lebensmittel-Wissenschaft Und-Technologie-Food Science and Technology* 37(2), 239-246.

Terzi, V., Malnati, M., Barbanera, M., Stanca, A., Faccioli, P., 2003. Development of analytical systems based on real-time PCR for *Triticum* species-specific detection and quantitation of bread wheat contamination in semolina and pasta. *Journal of Cereal Science* 38(1), 87-94.

Terzi, V., Morcia, C., Gorrini, A., Stanca, A.M., Shewry, P.R., Faccioli, P., 2005. DNA-based methods for identification and quantification of small grain cereal mixtures and fingerprinting of varieties. *Journal of Cereal Science* 41(3), 213-220.

Thompson, T., 2003. Oats and the gluten-free diet. *Journal of the American Dietetic Association* 103(3), 376-379.

Tichoniuk, M., Gwiazdowska, D., Ligaj, M., Filipiak, M., 2010. Electrochemical detection of foodborne pathogen *Aeromonas hydrophila* by DNA hybridization biosensor. *Biosensors & Bioelectronics* 26(4), 1618-1623.

Tichoniuk, M., Ligaj, M., Filipiak, M., 2008. Application of DNA hybridization biosensor as a screening method for the detection of genetically modified food components. *Sensors* 8(4), 2118-2135.

Tilley, M., 2004. PCR amplification of wheat sequences from DNA extracted during milling and baking. *Cereal Chemistry* 81(1), 44-47.

Tollefsen, S., Arentz-Hansen, H., Fleckenstein, B., Molberg, xD, yvind, xE, ki, M., Kwok, W.W., Jung, G., xFc, nther, Lundin, K.E.A., Sollid, L.M., 2006. HLA-DQ2 and -DQ8 signatures of gluten T cell epitopes in celiac disease. *The Journal of Clinical Investigation* 116(8), 2226-2236.

Troncone, R., Greco, L., Mayer, M., Paparo, F., Caputo, N., Micillo, M., Mugione, P., Auricchio, S., 1996. Latent and potential coeliac disease. *Acta Pædiatrica* 85, 10-14.

Vader, W., Kooy, Y., van Veelen, P., de Ru, A., Harris, D., Benckhuijsen, W., Peñaş, S., Mearinş, L., Drijfhout, J.W., Koning, F., 2002. The gluten response in children with celiac disease is directed toward multiple gliadin and glutenin peptides. *Gastroenterology* 122(7), 1729-1737.

Valdimarsson, T., Franzen, L., Grodzinsky, E., Skogh, T., Ström, M., 1996. Is small bowel biopsy necessary in adults with suspected celiac disease and IgA anti-endomysium antibodies? *Digestive Diseases and Sciences* 41(1), 83-87.

van Berge-Henegouwen, G.P., Mulder, C.J., 1993. Pioneer in the gluten free diet: Willem-Karel Dicke 1905-1962, over 50 years of gluten free diet. *Gut* 34(11), 1473-1475.

van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Molberg, O., Lundin, L., Mutis, T., Benckhuijsen, W., Drijfhout, J.W., Koning, F., 1998. Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc Natl Acad Sci U S A* 95, 10050-10054.

van de Wal, Y., Kooy, Y.M.C., van Veelen, P., Vader, W., August, S.A., Drijfhout, J.W., Peña, S.A., Koning, F., 1999. Glutenin is involved in the gluten-driven mucosal T cell response. *European Journal of Immunology* 29(10), 3133-3139.

Van Dorst, B., Mehta, J., Bekaert, K., Rouah-Martin, E., De Coen, W., Dubruel, P., Blust, R., Robbens, J., 2010. Recent advances in recognition elements of food and environmental biosensors: A review. *Biosensors & Bioelectronics* 26(4), 1178-1194.

van Eckert, R., Berghofer, E., Ciclitira, P.J., Chirido, F., Denery-Papini, S., Ellis, H.J., Ferranti, P., Goodwin, P., Immer, U., Mamone, G., Méndez, E., Mothes, T., Novalin, S., Osman, A., Rumbo, M., Stern, M., Thorell, L., Whim, A., Wieser, H., 2006. Towards a new gliadin reference material— isolation and characterisation. *Journal of Cereal Science* 43(3), 331-341.

van Heel, D.A., West, J., 2006. Recent advances in coeliac disease. *Gut* 55(7), 1037-1046.

van Hengel, A., 2007. Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and Bioanalytical Chemistry* 389(1), 111-118.

van Herpen, T., Goryunova, S., van der Schoot, J., Mitreva, M., Salentijn, E., Vorst, O., Schenk, M., van Veelen, P., Koning, F., van Soest, L., Vosman, B., Bosch, D., Hamer, R., Gilissen, L., Smulders, M., 2006. Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. *BMC Genomics* 7(1), 1.

van Koppen, E.J., Schweizer, J.J., Csizmadia, C.G.D.S., Krom, Y., Hylkema, H.B., van Geel, A.M., Koopman, H.M., Verloove-Vanhorick, S.P., Mearin, M.L., 2009. Long-term Health and Quality-of-Life Consequences of Mass Screening for Childhood Celiac Disease: A 10-Year Follow-up Study. *Pediatrics* 123(4), e582-e588.

Vautrin, S., Zhang, D., 2007. Real-Time Polymerase Chain Reaction Assay for Endogenous Reference Gene for Specific Detection and Quantification of Common Wheat-Derived DNA (*Triticum aestivum* L.). *Journal of AOAC International* 90(3), 794-801.

Ventura, A., Magazzù, G., Greco, L., 1999. Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. *Gastroenterology* 117(2), 297-303.

von Buren, M., Stadler, M., Luthy, J., 2001. Detection of wheat adulteration of spelt flour and products by PCR. *European Food Research and Technology* 212(2), 234-239.

Wang, W., Li, Y., Zhao, F., Chen, Y., Ge, Y., 2011. Optical thin-film biochips for multiplex detection of eight allergens in food. *Food Research International* 44(10), 3229-3234.

Wieser, H., 2001. Comparative investigations of gluten proteins from different wheat species. III. N-terminal amino acid sequences of alpha-gliadins potentially toxic for coeliac patients. *European Food Research and Technology* 213(3), 183-186.

Wieser, H., 2007. Chemistry of gluten proteins. *Food Microbiology* 24(2), 115-119.

Wieser, H., Antes, S., Seilmeier, W., 1998. Quantitative Determination of Gluten Protein Types in Wheat Flour by Reversed-Phase High-Performance Liquid Chromatography. *Cereal Chemistry Journal* 75(5), 644-650.

Wittwer, C.T., 2009. High-Resolution DNA Melting Analysis: Advancements and Limitations. *Human Mutation* 30(6), 857-859.

Wu, J., Campuzano, S., Halford, C., Haake, D., Wang, J., 2010. Ternary Surface Mono layers for Ultrasensitive (Zeptomole) Amperometric Detection of Nucleic Acid Hybridization without Signal Amplification. *Analytical Chemistry* 82(21), 8830-8837.

Xie, J., Jiao, K., Liu, H., Wang, Q., Liu, S., Fu, X., 2008. DNA electrochemical sensor based on PbSe nanoparticle for the sensitive detection of CaMV35S transgene gene sequence. *Chinese Journal of Analytical Chemistry* 36(7), 874-878.

Xu, G., Jiao, K., Fan, J., Sun, W., 2006. Electrochemical detection of specific gene related to CaMV35S using methylene blue and ethylenediamine-modified glassy carbon electrode. *Acta Chimica Slovenica* 53(4), 486-491.

Yamakawa, H., Akiyama, H., Endo, Y., Miyatake, K., Sakata, K., Sakai, S., Toyoda, M., Urisu, A., 2007. Specific detection of wheat residues in processed foods by polymerase chain reaction. *Bioscience Biotechnology and Biochemistry* 71(10), 2561-2564.

Zeltner, D., Glomb, M., Maede, D., 2009. Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and. *European Food Research and Technology* 228(3), 321-330.

Zhang, J., Song, S., Wang, L., Pan, D., Fan, C., 2007. A gold nanoparticle-based chronocoulometric DNA sensor for amplified detection of DNA. *Nature Protocols* 2(11), 2888-2895.

Zhang, W., Yang, T., Jiang, C., Jiao, K., 2008. DNA hybridization and phosphinothricin acetyltransferase gene sequence detection based on zirconia/nanogold film modified electrode. *Applied Surface Science* 254(15), 4750-4756.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic acids research* 31(13), 3406-3415.

2 OBJETIVOS

OBJETIVOS

La altísima incidencia de la enfermedad celíaca (1 de cada 100 personas), junto con las graves consecuencias que puede tener el consumo de gluten para individuos que padecen esta enfermedad ha obligado al desarrollo de métodos sensibles, selectivos y fiables para su detección y cuantificación en muestras alimentarias. Tradicionalmente los métodos ELISA son los más empleados, y recomendados por algunas organizaciones internacionales. Sin embargo, la reactividad cruzada y los falsos negativos debido a la desnaturalización de las proteínas durante el procesado de los alimentos, han impulsado el desarrollo de métodos alternativos que permitan, al menos, confirmar los resultados obtenidos con los métodos inmunoanalíticos anteriormente mencionados, y de esta forma verificar la seguridad del alimento para su consumo por el enfermo celíaco.

La aparición de los métodos basados en la cuantificación y detección de ADN, como la PCR o los genosensores, han supuesto un gran avance en la identificación y cuantificación de secuencias específicas de especies de todo tipo: patógenos, alérgenos y organismos modificados genéticamente. Los métodos basados en PCR aportan una elevada sensibilidad mientras que los genosensores constituyen una alternativa portátil, sencilla y de bajo coste para análisis de rutina por personal no especializado. A pesar de su versatilidad, en la actualidad no existen genosensores para la detección de gluten en matrices alimentarias.

En este sentido, el objetivo general del presente trabajo es el desarrollo y puesta a punto de métodos analíticos alternativos para la detección y cuantificación de gluten en alimentos, utilizando un analito diferente al empleado tradicionalmente: el ADN. Se pretende explorar el desarrollo de métodos de detección de gluten mediante genosensores y PCR.

Los objetivos concretos de esta Tesis Doctoral son:

1. Selección de secuencias de ADN específicas de gluten mediante análisis *in silico* de secuencias que codifiquen proteínas alergénicas del trigo. Verificación de la idoneidad de estas secuencias para su uso como dianas mediante técnicas de amplificación por PCR.
2. Diseño y desarrollo de métodos de cuantificación de gluten mediante amplificación por PCR en tiempo real de las secuencias seleccionadas, empleando sondas de hidrólisis con detección fluorescente. Evaluación de la selectividad respecto a cereales no tóxicos, sensibilidad y efectos de matriz.
3. La(s) secuencia(s) que mejor comportamiento analítico presenten en PCR en tiempo real serán utilizadas para el diseño de un genosensor

electroquímico sobre electrodos de oro. Para ello se explorarán diferentes fases sensoras basadas en la formación de monocapas autoensambladas, un formato de ensayo tipo sándwich para maximizar la selectividad y marcaje enzimático. Se compararán diferentes sondas de captura: lineales y estructuradas; se optimizará el diseño de los genosensores y se evaluarán comparativamente las características analíticas de los genosensores optimizados.

4. El genosensor electroquímico resultante se aplicará al análisis de muestras reales. Para ello será imprescindible una etapa de extracción y otra de amplificación previa del ADN, ya que la longitud del genoma y la presencia de la hebra complementaria impiden el análisis directo del ADN extraído. Se pondrá a punto un método de PCR convencional para su acoplamiento al genosensor en el análisis de harinas y alimentos procesados.
5. Dado que en algunas legislaciones es imperativo identificar la especie de la que proviene el gluten, se explorará un sistema de identificación más específico que la PCR, como es la técnica de análisis de curvas de disociación de alta resolución (HRM, *high resolution melting analysis*), que permita discriminar entre secuencias homólogas de cereales tóxicos para los enfermos celíacos.

3 CRIBADO DE SECUENCIAS Y EFECTO DE MATRIZ

3.3 Cribado de secuencias

Screening new gene markers for gluten detection in foods

3.4 Efecto de Matriz

Effect of matrix on the performance of quantitative real-time PCR of gluten-containing cereals

3.1 Cribado de secuencias

En la determinación de la presencia de trigo y cereales tóxicos en alimentos para celíacos a través del ADN es de capital importancia encontrar secuencias adecuadas para su detección. Como se ha expuesto con anterioridad, se han descrito diversos métodos que detectan tanto secuencias codificantes de proteínas en el vegetal, como secuencias intrónicas. También existen métodos para la detección de secuencias tanto multicopia como en bajo número de copias, siendo los primeros, en general, más sensibles, pero sus secuencias son, en general, poco conservadas a lo largo de la evolución, por lo que su especificidad debe ser demostrada experimentalmente.

La primera etapa de este trabajo consistió en evaluar el comportamiento de diferentes secuencias de ADN elegidas para la cuantificación de cereales tóxicos. Y en función de los datos recopilados, seleccionar aquella secuencia idónea para su uso como analito en la detección indirecta de gluten.

El estudio se centró en tres secuencias de ADN que codifican proteínas del gluten: α 2-gliadina, aglutinina isolectina y tiorredoxina h. Se evaluó la capacidad de amplificación en términos de límite de detección absoluto y relativo, reproducibilidad y eficiencia.



ELSEVIER

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Screening new gene markers for gluten detection in foods

Begoña Martín-Fernández^{a, b}, Joana Costa^a, M. Beatriz P.P. Oliveira^a, Beatriz López-Ruiz^b, Isabel Mafra^{a, *}^a REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal^b Sección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pz. Ramón y Cajal s/n, 28040 Madrid, Spain

ARTICLE INFO

Article history:

Received 7 January 2015

Received in revised form

23 February 2015

Accepted 25 February 2015

Available online 13 March 2015

Keywords:

Quantitative real-time PCR

Wheat

Gluten-containing cereals

Celiac patients

Wheat allergens

ABSTRACT

In the present work, three DNA sequences encoding wheat proteins (α 2-gliadin, agglutinin isolectin and thioredoxin h) were compared to trace gluten-containing cereals in food products. Quantitative real-time PCR methods using hydrolysis probes were successfully developed to target the three sequences for the detection of wheat DNA. The comparison of the three systems highlights the best sensitivity when tracing the α 2-gliadin marker sequence, showing an absolute limit of detection (LOD) of 2 pg of wheat DNA and a relative LOD of 0.005% (50 mg/kg) of wheat in soybean, which corresponds to 4.5 mg/kg of gluten. All the systems reveal high specificity for detecting other gluten-containing cereals, such as barley and rye. Therefore, the developed real-time PCR systems can be used as non-immunological tools to confirm the presence of gluten-containing cereals in foods, towards the safety of celiac patients and wheat allergic individuals.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

The celiac disease is defined as a common autoimmune inflammatory disorder with both genetic and environmental components, which is known to be elicited by gliadin and other related prolamines in genetically predisposed individuals. It is one of the most frequent chronic health conditions, being estimated to affect as much as 1% of the general population, although this number could be underestimated owing to difficulties in diagnosing this autoimmune disorder (Denham & Hill, 2013). Clinical manifestations of celiac disease are highly variable, including both gastrointestinal and non-gastrointestinal features, which may result in systemic manifestations (Reilly, Fasano, & Green, 2012). The major environmental trigger is the consumption of gluten, which corresponds to a significant portion of protein fraction in wheat, barley and rye (Denham et al., 2013). Due to the complexity of the disease, the only effective treatment consists of the avoidance of gluten-containing food. However, a minimal degree of gluten contamination is difficult to avoid (Fasano & Catassi, 2012). Although gluten threshold has not yet been defined, literature seems to suggest that small amounts of gluten, 10–50 mg per day (a slice of bread of 25 g

contains approximately 1.6 g of gluten) could be sufficient to cause damage to the celiac intestinal mucosa over time (Catassi et al., 2007). Therefore, specific legislation in European Union (EU) has been issued aiming at informing the consumer about the presence of gluten in food products by means of mandatory labelling of allergenic food ingredients (Directive 2007/68/EC; Regulation (EU) No. 41/2009; Regulation (EU) No. 1169/2011). The Codex Alimentarius (CODEX STAN 118, 1979) and the Regulation (EC) No 41/2009 endorse a maximum gluten contamination of 20 mg/kg in gluten-free labelled products as a safe threshold. Since the correct labelling of foods is the only effective way of protecting the celiac patients, it is essential to have an easy and reliable methodology to verify the content of gluten in foods. However, these regulations do not specify an analytical methodology for gluten analysis.

So far, most of the methods employed in the detection of gluten are based on protein detection, namely enzyme linked immunosorbent assays (ELISA) (Diaz-Amigo & Popping, 2013; Janssen, 2006), but false negative results, poor reproducibility or cross-reactions are frequently considered as their major drawbacks (Haraszi, Chassaingne, Maquet, & Ulberth, 2011; Mujico, Lombardia, Mena, Mendez, & Albar, 2011). DNA-based methods represent attractive options due to the high stability of DNA molecules upon food processing when compared with proteins (Mafra, Ferreira, & Oliveira, 2008). DNA amplification by means of polymerase chain reaction (PCR) offers a high sensitive and specific alternative to

* Corresponding author. Tel.: +351 220428640; fax: +351 226093390.
E-mail address: isabel.mafra@ff.up.pt (I. Mafra).

ELISA for screening or confirmation. Since PCR is completely orthogonal to ELISA, a positive result with both assays gives almost irrefutable proof that the offending cereal species is present (Janssen, 2006). The first PCR method to determine wheat in gluten-free products was described by Allmann, Candrian, Hofelein, and Luthy (1993). After that, several end-point PCR (Dahinden, von Buren, & Luthy, 2001; Debnath, Martin, & Gowda, 2009; Koppel, Stadler, Luthy, & Hubner, 1998; Maskova, Paulickova, Rysova, & Gabrovska, 2011; Olexová, Dovičovičová, Švec, Siekel, & Kuchta, 2006; Yamakawa et al., 2007) and real-time PCR-based methods (Mujico et al., 2011; Píknova, Brezna, & Kuchta, 2008; Sandberg, Lundberg, Ferm & Yman, 2003; Terzi, Malnati, Barbanera, Stanca, & Faccioli, 2003; Zeltner, Glomb, & Maede, 2009) were proposed for the detection of minute amounts of wheat in food products. The target sequences were selected among genes coding for allergenic proteins (glutenins, gliadins) or simply on a species-specific region of the genome of the allergenic food.

Wheat gluten is the cohesive mass that remains after washing dough. It consists of a complex mixture of storage proteins that can be classified into gliadin and glutenin polypeptides, according to the classical Osborne fractionation. Gliadins are monomers, whereas glutenins form large polymeric structures. On the basis of their amino-acid sequences, gliadins can be subdivided into α -, γ - and ω -gliadins, while glutenins can be classified into high-molecular-weight glutenins (~650–800 residues long) and low-molecular-weight glutenins (~270–330 residues long) (Haraszi et al., 2011; Sollid, 2002). Only few authors have selected sequences encoding these immunogenic proteins to detect gluten in foodstuffs probably because of their high variability. For the development of real-time PCR methods for wheat detection, DNA sequences encoding a ω -gliadin (Sandberg, Lundberg, Ferm, & Yman, 2003; Terzi et al., 2003) and high and low molecular glutenins (Debnath et al., 2009; Zeltner et al., 2009) were targeted.

We hereby report the comparison of three real-time PCR methods with TaqMan probes to detect gluten-containing cereals in foods based on the selective amplification of $\alpha 2$ -gliadin, agglutinin and thioredoxin gene sequences, which are involved in triggering immune responses in celiac (cell-mediated reaction) or wheat-allergic (IgE-mediated reaction) patients.

2. Materials and methods

2.1. Sample preparation

Flours from different plants (wheat, rye, barley, oat, maize, soybean and rice) were obtained at local markets. In the absence of certified or testing reference standards for wheat, binary model mixtures containing: 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1000 mg/kg and 5000 mg/kg, 10,000 mg/kg, 50,000 mg/kg, 100,000 mg/kg and 500,000 mg/kg of wheat flour in soybean material were prepared. The first sample containing 500,000 mg/kg (50%) of wheat was prepared by adding 50 g of wheat flour to 50 g of soybean flour. All the other model mixtures were prepared by successive additions of soybean flour until the spiked level of 1 mg/kg (0.0001%) in the equivalent proportion to a final weight of 100 g in a non-target plant matrix (soybean flour). All the mixtures were thoroughly homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and material, previously treated with a DNA decontamination solution. To avoid accidental cross-contaminations among samples, cereals and reference mixtures were prepared on different days. After preparation, all samples and reference mixtures were immediately stored at -20°C until further DNA extraction.

2.2. DNA extraction

DNA was extracted from 100 mg of sample using the commercial Nucleospin Food kit (Macherey–Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations as described by Costa, Mafra, Kuchta, and Oliveira (2012).

Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometric DNA quantification on a Synergy HT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

2.3. Target gene selection and oligonucleotide primers and probes

The initial objective of this study was searching for sequences that allow the detection of wheat DNA and other gluten-containing cereals in food. In the celiac disease, one of the most well studied peptides is the so-called 33-mer peptide, which is a fragment of the $\alpha 2$ -gliadin responsible for triggering the immune response associated with this disorder and playing a major role in the immunogenicity. Mutant peptides lacking this fragment lose their immunotoxicity. The immunogenicity of the referred peptide was attributed to its resistance to breakdown in the human intestine, mainly due to its high content of proline residues as well as high susceptibility to transglutaminase deamidation (Shan et al., 2002). DNA encoding this fragment has been recently used for gluten detection using a PCR-coupled electrochemical genosensor (Martín-Fernández et al., 2014).

As other potential DNA markers for gluten detection, nucleotide sequences encoding different allergenic proteins, namely Tri a 18 (agglutinin isolectin A) and Tri a 25 (thioredoxin h) were retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). The allergen Tri a 18 (agglutinin isolectin A) belongs to the pathogenesis-related (PR)-3 family of proteins or class I chitinases, being classified as an important family of allergenic proteins, whereas the Tri a 25 (thioredoxin h) is included in the super-family of enzyme and protease inhibitors (Breiteneder & Radauer, 2004). Primers and probes were specifically designed targeting the encoding sequences for $\alpha 2$ -gliadin, Tri a 18 and Tri a 25 using the Genbank accession numbers AJ133612.1, M25536.1 and AJ404845.1, respectively. Sets of primers with different annealing temperatures (Ta) were designed using the software Primer-BLAST designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), which are presented in Table 1.

The basic local alignment search tool BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was also used to identify regions of local similarity between the chosen nucleotide and homologue sequences of different species. *In silico* analysis estimated the statistical significance of the matches and confirmed the specificity of the designed primers with 100% of identity and 100% query cover for the wheat species. The software parameters were set to design a pair of primers (Gly-F/Gly-R, Tri18-F/Tri18-R and Tri25-F/Tri25-R) for each target sequence with an optimal annealing temperature of 61–63 $^{\circ}\text{C}$. For the application of real-time PCR and to increase the specificity of the proposed primers, dual labelled hydrolysis probes were also designed (Table 1). As a positive amplification control of end-point PCR and real-time PCR experiments, universal primers (18SRG-F/18SRG-R) targeting a conserved DNA region (nuclear 18S rRNA gene) were used. Primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany). Stock solutions were stored at -25°C .

Table 1

Primers and probes designed to specifically target sequences of *Triticum aestivum* (α 2-gliadin, agglutinin isolectin -Tri a 18, thioredoxin h -Tri a 25) and a conserved eukaryotic region (Nuclear 18S rRNA).

Nucleotide sequence (*Genbank accession no.)	Primers/probe sequences (5' → 3')	Amplicon (bp)	Reference
α 2-gliadin (*AJ133612.1)	Gly-F Gly-R Gly-P	CAACCATTTCCGCGAGCCGCAA TGCGAATACTGTGGTTGCGATTGTG FAM-ATCCGCGAGCCGCAACCATTTGACCACA-BHQ1	134 bp This work
Agglutinin isolectin (Tri a 18) (*M25536.1)	Tri18-F Tri18-R Tri18-P	CTGTTGTAGCAAGTGGGGATCCT ATTCTTGGAGAAGAGTGGAGTTGG FAM-CAGAGTGGCGGCTGCGATGGTGTCTT-BHQ1	125 bp This work
Thioredoxin h (Tri a 25) (*AJ404845.1)	Tri25-F Tri25-R Tri25-P	TGAACTGAAGTCCATTGCTGAGCA TGATTACTGGGCGCGTGTAG FAM-AACCCTGCTTCGACGTCTCCTTCAT-BHQ1	148 bp This work
Nuclear 18S rRNA (*HQ873432.1)	18SRG-F 18SRG-R	TCTGCCCTATCACTTCGATGG TAATTTGCGCGCTGCTG	113 bp Costa et al. (2013)

2.4. End-point PCR conditions

PCR amplifications were carried out in 25 μ L of total reaction volume containing 2 μ L of wheat DNA extract (200 ng), 67 mM of Tris–HCl (pH 8.8), 16 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.01% of Tween 20, 200 μ M of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 2.5 or 3.5 mM of MgCl_2 (α 2-gliadin, or Tri a 18/Tri a 25, respectively) and 160 nM or 120 nM of each set of primers (α 2-gliadin, or Tri a 18/Tri a 25, respectively). The reactions were performed in an MJ Mini thermal cycler (BioRad, Hercules, CA, USA) using the following program, with correction adjustment on the temperature of annealing according to target sequence: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, temperature of annealing (70 °C for α 2-gliadin, 62 °C for Tri a 18 and 61 °C for Tri a 25) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel containing 1 \times Gel Red (Biotium, CA, USA) for staining and carried out in 1 \times SGTB buffer (GRISP, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science™ DC120 (Rochester, NY, USA).

2.5. Real-time PCR conditions

Real-time PCR assays were performed in 20 μ L of total reaction volume, containing 2 μ L of DNA (200 ng), 1 \times of SsoFast Probes Supermix (BioRad, Hercules, CA, USA), 200 nM of each primer and 100 nM of hydrolysis probe (Table 1). The reactions were performed in a fluorimetric thermal cycler CFX96 real-time PCR detection system (BioRad, Hercules, CA, USA), whose optimised conditions were based on end-point PCR, following the temperature protocol: 95 °C for 5 min, 55 cycles at 95 °C for 15 s and temperature of annealing/extension of 61 °C (Tri a 25), 62 °C (Tri a 18) or 70 °C (α 2-gliadin) for 45 s with the fluorescence signal acquisition at the end of each cycle. Cycle threshold (Ct) values, which refer to the number

of cycles where the fluorescence significantly increases above the background level, were calculated using the software at automatic threshold setting. The continuous measurement of fluorescence is related to the amount of amplicon generated in the real-time PCR, yielding a quantitative result on the presence of the target species. Data were collected and analysed using the software Bio-Rad CFX Manager 3.0 (BioRad, Hercules, CA). Real-time PCR experiments were repeated in two independent assays using four replicates in each one.

3. Results

3.1. Specificity

To ensure the absence of any possible false-negative results, the DNA extracts from all cereals and model mixtures were previously evaluated for their amplification capacity using the primers 18SRG-F/18SRG-R (Costa, Oliveira, & Mafra, 2013), which target an universal eukaryotic region. All tested cereals and model mixtures amplified positively for the expected fragment of 113 bp, confirming that the DNA extracts had the adequate quality and purity for PCR amplification.

Afterwards, three genes encoding distinct wheat proteins were evaluated for their potential application as DNA markers for gluten detection. Since gluten is not exclusively present in wheat, its detection in foodstuffs should include the analysis of all toxic gluten-containing cereals. Owing to the existence of a close phylogenetic relationship among wheat and other cereal species (especially among members of the Poaceae family), homologous DNA sequences could be found in related plant species (e.g., wheat, barley, rye) (Hubner, Waiblinger, Pietsch, & Brodmann, 2001). Therefore, to assess the specificity of the designed primers for the α 2-gliadin, Tri a 18 and Tri a 25 sequences, some of the most important cereal species used in food production (rice, rapeseed, maize, oat, barley, rye, soybean and wheat) were tested. Summarised results of specificity and cross-reactivity of the three PCR systems are presented in Table 2. The sequence similarity between wheat and the closely related cereals, namely barley and rye, was confirmed since these species were positively amplified by the three primer sets. In opposition to the α 2-gliadin system, the Tri a 18 and Tri a 25 sequences were amplified in oat at optimised conditions (Table 2). Therefore, the latter two sequences allow the identification of all cereals involved in the celiac disease (wheat, barley, rye and oat). Unable to activate the celiac disease, rice and rapeseed are two plant-species more distantly related to wheat, which was also consistent with PCR results (Table 2). All the other plants did not amplify with the selected sets of primers, evidencing their adequacy for the detection of gluten-containing cereals.

Table 2

Results of PCR amplification of wheat and other related plant species targeting three different genes (α 2-gliadin, Tri a 18 and Tri a 25).

Sample name	Scientific name	α 2-gliadin	Tri a 18	Tri a 25
Wheat	<i>Triticum aestivum</i>	+	+	+
Barley	<i>Hordeum vulgare</i>	+	+	+
Rye	<i>Secale cereale</i>	+	+	+
Oat	<i>Avena sativa</i>	–	+	+
Rice	<i>Oryza sativa</i>	–	–	–
Maize	<i>Zea mays</i>	–	–	–
Rapeseed	<i>Brassica napus</i>	–	–	–
Soybean	<i>Glycine max</i>	–	–	–

3.2. Real-time PCR

Three real-time PCR systems were developed targeting each of the previously described wheat genes, using the primers Gly-F/Gly-R, Tri18-F/Tri18-R and Tri25-F/Tri25-R, for which specific hydrolysis probes (Gly-P, Tri18-P and Tri25-P) were designed to increase the specificity of the assays (Table 1). The systems were optimised using model mixtures of soybean material spiked with known amounts of wheat flour and serially diluted wheat DNA. For the comparison and the evaluation of the proposed real-time PCR assays, the prerequisites for method performance established in the available documents of MIQE guidelines (Bustin et al., 2009) and the definition of minimum performance requirements for analytical methods of genetically modified organism testing (Mazzara et al., 2008) were carefully considered.

3.2.1. Relative sensitivity

The application of real-time PCR systems targeting DNA sequences of α 2-gliadin, agglutinin isolectin (Tri a 18) and thio-redoxin h (Tri a 25) enabled the detection and quantification of wheat at different levels. The system targeting the α 2-gliadin sequence presented the best sensitivity results, which allowed detecting 0.002% (20 mg/kg) of wheat in soybean. Nevertheless, according to the guidelines for the development of quantitative real-time PCR assays (Bustin et al., 2009; Mazzara et al., 2008) the limit of detection (LOD) was considered as 0.005% (50 mg/kg) of wheat in soybean material (Fig. 1) since the amplification at the spiking level of 0.002% was achieved in only 50% of the replicates (should be in at least 95% of replicates). In this study, the LOD was always determined assuming the lowest concentration level with positive amplification in all replicates. The limit of quantification (LOQ) was found to be equal to the LOD since it was within the linear range of the calibration curve. Quantitative assays targeting the DNA sequences encoding Tri a 18 or Tri a 25 allergens enabled a relative LOD and LOQ of 0.05% (500 mg/kg) of wheat in soybean with both systems, thus presenting a 10-fold lower sensitivity than the α 2-gliadin real-time PCR assay (Fig. 1).

To correctly assess the performance of a real-time PCR method, several parameters have to comply with the acceptance criteria defined for this type of assays, namely correlation coefficient (R^2) above 0.98, PCR efficiency ranging from 90% to 110% and respective slope from -3.6 and -3.1 (Bustin et al., 2009; Mazzara et al., 2008). In general, the means of the performance parameters of the three

methods were in good agreement with the acceptance criteria defined for real-time PCR (Fig. 1). In terms of relative quantification, the method exhibiting the best performance parameters of PCR efficiency (100.3%), slope (-3.314) and correlation coefficient (0.9934) was the one targeting the α 2-gliadin sequence. Additionally, α 2-gliadin real-time PCR also evidenced the earliest amplification level since cycle threshold (Ct) values were two and three cycles earlier than the Tri a 18 and Tri a 25 systems, respectively for the same spiking level of wheat. In the systems targeting Tri a 18 and Tri a 25, a difference of approximately 1 Ct value could be observed for the amplification of the same spiking level of wheat (Fig. 1).

3.2.2. Absolute sensitivity

In order to establish the dynamic range and the absolute sensitivity of real-time PCR methods, wheat DNA extracts were 10-fold serially diluted (with PCR grade water) to cover 6 orders of magnitude of the analyte, ranging from 200 ng to 2 pg (Table 3). Each of the three assays exhibited high performance real-time PCR parameters, presenting near 100% PCR efficiency with approximately three additional cycles (~ 3 Ct) between two consecutive dilution levels and correlation coefficients above 0.99 (Table 3). The systems targeting Tri a 18 and Tri a 25 allowed amplifying wheat DNA until a dilution factor of 10,000, corresponding to a LOD of 20 pg and 1.1 genomic copies (Table 3). The number of amplified DNA copies was estimated according to the genome size of wheat (17.33 pg) and assuming the target sequences are single copy genes (<http://data.kew.org/cvalues/>). In the case of α 2-gliadin detection, a LOD of 2 pg (0.1 DNA copies) was achieved, being 10-fold lower than the other two systems. In terms of absolute quantification, the LOQ values were found to be equal to the respective LOD of each method.

Like for the relative quantification results, the system presenting the best performance was the real-time PCR targeting α 2-gliadin sequence, starting to amplify wheat approximately 2 and 3 cycles earlier than the systems for Tri a 18 and Tri a 25, respectively. Between the two last methods, an average difference of approximately 1 cycle in the amplifications for the same dilution level could be observed (Table 3).

3.2.3. Amplification of other gluten-containing cereals

Wheat, barley and rye, as cereal species containing gluten, together with oat, which previously showed positive amplification by end-point PCR, were analysed by the three proposed real-time PCR systems. Data showed positive amplification in all cases, after wheat (Fig. 2). For the same DNA amounts (200 ng), the amplification of rye evidenced a delay of approximately 6 cycles, barley of 8 cycles and oat of 11 cycles in relation to wheat, when using the α 2-gliadin system (Fig. 2A). In the same conditions, rye, barley and oat were amplified with delays of approximately 4, 5 and 11 cycles, in relation to wheat amplification using the system targeting the Tri a 18 sequence (Fig. 2B). With the method detecting Tri a 25 sequence, rye, barley and oat amplifications were delayed in approximately 8, 5 and 6 cycles, respectively, comparing to wheat amplification (Fig. 2C).

The comparison of results of the three target genes highlights that the sequences of α 2-gliadin and Tri a 18 are amplified in rye, barley and oat with similar delays in relation to wheat, while in Tri a 25 the pattern is changed for barley and rye (Fig. 2). This finding suggests close amplification behaviour of the four species in the cases of α 2-gliadin and Tri a 18 sequences. In general, the system amplifying α 2-gliadin gene allowed earlier amplifications for wheat and the other tested cereals, while the assay targeting the Tri a 25 sequence presented the latest amplifications for wheat and rye (Fig. 2C).

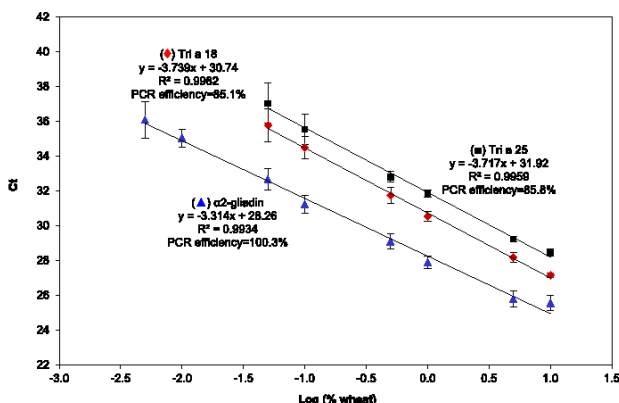


Fig. 1. Comparison of calibration curves obtained with three real-time PCR systems (α 2-gliadin, Tri a 18 and Tri a 25) of model mixtures containing 5%–0.005% of wheat in soybean material. Mean values and corresponding standard deviations of $n = 8$ replicates of two independent real-time PCR trials.

Table 3
Results of wheat detection by the three real-time PCR systems (α 2-gliadin, Tri a 18 and Tri a 25) for the absolute detection of wheat DNA.

Absolute quantity (pg)	α 2-gliadin		Tri a 18		Tri a 25	
	Ct \pm SD ^a	DNA copies ^b	Ct \pm SD ^a	DNA copies ^b	Ct \pm SD ^a	DNA copies ^b
200,000	21.29 \pm 0.24	11540.7	22.37 \pm 0.09	11540.7	23.58 \pm 0.10	11540.7
20,000	23.72 \pm 0.17	1154.1	25.76 \pm 0.16	1154.1	27.00 \pm 0.14	1154.1
2000	27.14 \pm 0.06	115.4	29.29 \pm 0.27	115.4	30.32 \pm 0.17	115.4
200	30.37 \pm 0.15	11.5	33.10 \pm 0.69	11.5	33.33 \pm 0.47	11.5
20	33.80 \pm 0.54	1.1	36.01 \pm 0.86	1.1	36.79 \pm 0.90	1.1
2	37.29 \pm 1.30	0.1	na	–	na	–
Slope	–3.241		–3.461		–3.272	
Correlation coefficient (R ²)	0.9975		0.9986		0.9996	
PCR efficiency	103.5%		94.5%		102.1%	

^a Mean cycle threshold (Ct) values \pm standard deviation (SD) for $n = 8$ replicates from two independent real-time PCR experiments.

^b Number of wheat haploid genome copies (17.33 pg) (<http://data.kew.org/cvalues/>). na – no amplification.

4. Discussion

The correct evaluation of the presence of gluten in food supplies is very important for the health of the celiac patients. In the last years, PCR-based methods have been applied owing to their high potential of being more specific than the current immunochemical methods. Allmann et al. (1993) evaluated the contamination of several foods with wheat based on the PCR detection of the intergenic region of 25S and 18S rRNA, which was in good agreement with the immunoenzymatic assay. Koppel et al. (1998), using the same PCR system, obtained a sensitivity of wheat in oats between 0.001% (10 mg/kg) and 0.01% (100 mg/kg). In the report of Dahinden et al. (2001), the intron of the chloroplast *trnL* gene was used as a PCR target to detect wheat with a sensitivity of 0.02% (200 mg/kg). The triticin gene of *Triticum aestivum* was used by Yamakawa et al. (2007) as a wheat specific target to develop a qualitative PCR assay able to detect 0.005% (50 mg/kg) of wheat in maize. Thereafter, a low molecular weight glutenin gene was amplified by PCR to detect wheat DNA down to 21.5 pg (Debnath et al., 2009). However, the lack of potential quantification remains a drawback since the relation between gluten and DNA is variable in grains of various species and cultivars. Considering that this variability does not exceed one order of magnitude, real-time PCR based methods have been regarded as excellent alternatives to ELISA in the analysis of gluten (Mafra et al., 2008; Piknova et al., 2008). The real-time PCR approaches based on melting curve analysis or hydrolysis probes have also demonstrated their applicability to detect cereals containing gluten (Mujico et al., 2011; Piknova et al., 2008; Sandberg et al., 2003; Terzi et al., 2003; Zeltner et al., 2009). The real-time PCR assay using the SYBR Green dye enabled the detection of wheat DNA between 5 and 50 pg (Sandberg et al., 2003) and 20 pg DNA/mg of sample (Mujico et al., 2011). Systems based on the use of TaqMan probes reached 26 pg of wheat DNA and relative sensitivities of 200 mg/kg of wheat flour in non-containing gluten flour (Piknova et al., 2008) and 2.5 mg/kg of wheat flour in rice flour (Zeltner et al., 2009).

In the present work, three specific sequences encoding allergenic proteins of wheat, namely α 2-gliadin, Tri a 18 and Tri a 25 were successfully targeted by real-time PCR to detect gluten-containing cereals in foods (wheat, barley, rye and oat), to our knowledge for the first time. Wheat, rye, and barley have a common ancestral origin in the grass family and can trigger the immune response in celiac patients. Oat has rarely been associated with celiac disease since its prolamins (avenins) are more distantly related to the analogous proteins in wheat, rye and barley (Tatham & Shewry, 2008). It is apparently more well-tolerated by the majority of the celiac patients, but a reduced number of individuals could still present adverse immunological reactions and

gastrointestinal symptoms to oat (Green & Jabri, 2006). The comparison of the three real-time PCR methods highlights the best sensitivity when tracing the α 2-gliadin marker sequence, showing an absolute LOD of 2 pg of wheat DNA and a relative LOD of 0.005% (50 mg/kg) of wheat in soybean. There are more than 100 α -gliadins encoded by three gene loci. The estimated copy number ranges from 25 to 150 (Anderson & Greene, 1997), but a significant number of them is considered pseudogene because of the presence of in-frame stop codons. It is worth mentioning that only few genes from D-genome contain the full sequence for the 33-mer immunodominant peptide (Molberg et al., 2005) that we have selected as PCR target. Although the precise copy number of α 2-gliadin is unknown, a copy number higher than 1 cannot be ruled out, which would explain the higher sensitivity of this gene when compared with that of other allergen genes. It is frequent that many cultivated crop plants contain a high number of gene duplication as a result of breeding processes (Hubner et al., 2001). Unfortunately, despite the advantages in terms of sensitivity arisen from the selection of a multicopy gene, the absence of the gene in most non-bread wheat (namely *Triticum durum*) obscures the usefulness of this gene for gluten detection in all wheat varieties that are still harmful for celiac patients. Nonetheless, promising results of real-time PCR were also recorded for Tri a 18 and Tri a 25, which enabled the detection of 500 mg/kg (0.05%) of wheat in soybean material. Taking into account that the gluten content is about 9% in wheat flour (Zeltner et al., 2009), this result corresponds to a detection of approximately 45 mg/kg (0.0045%) of gluten when using the sequences of Tri a 18 and Tri a 25, and 4.5 mg/kg (0.00045%) of gluten in the case of the α 2-gliadin marker. These sensitivity values are comparable with the above results reported in the literature, though Zeltner et al. (2009) reached an apparently higher sensitivity (2.5 mg/kg of wheat flour in rice) by real-time PCR. However, it is important to stress that those authors did not use the actual recommended guidelines (Bustin et al., 2009; Mazzara et al., 2008) to determine the LOD and used a different matrix. For a true comparison of data, the matrix or background in which the reference mixtures were prepared should be taken in consideration. A better DNA extraction yield from soybean, when compared to wheat or maize, has been reported (Sharma, Gill, & Singh, 2002), which might justify the differences. Therefore, the high content of soybean DNA versus wheat DNA or the high lipidic content of soybean could act as strong PCR inhibitors (Cankar, Stebih, Dreo, Zel, & Gruden, 2006), reducing the efficiency of the reaction. Furthermore, the sensitivity of a PCR method depends primarily on the genome size of the investigated plant species and on the copy number. Taking into account the large size of the wheat genome and the single-copy nature of Tri a 18 and Tri a 25, detection limits below 0.01% are very difficult to obtain.

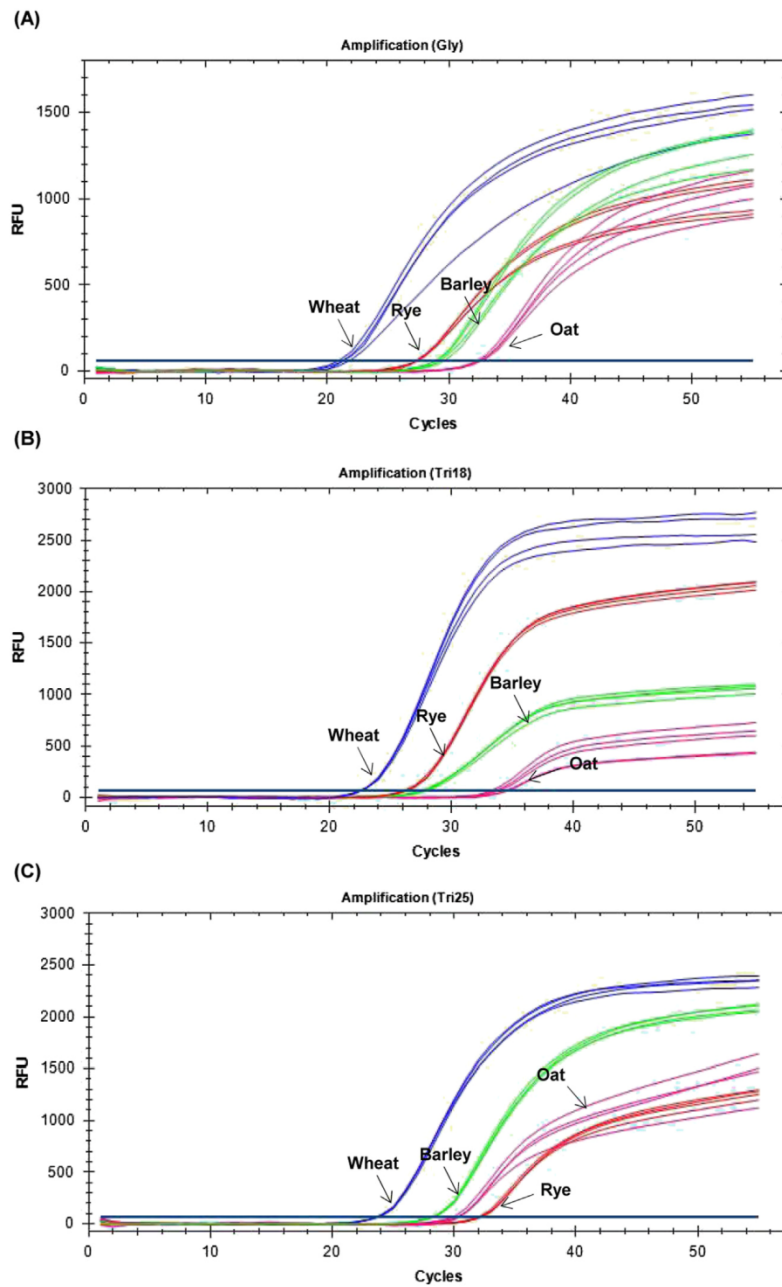


Fig. 2. Amplification curves obtained by real-time PCR systems targeting $\alpha 2$ -gliadin (A), Tri a 18 (B) and Tri a 25 (C) using 200 ng of DNA from wheat, other genetically related cereals containing gluten (barley and rye) and oat.

Presently, the number of commercial real-time PCR kits for the detection and quantification of allergens in foods is still limited. When compared to the commercial real-time PCR kit available for the detection of gluten in foods, the proposed quantitative method for $\alpha 2$ -gliadin evidenced a higher relative LOD (4.5 mg/kg of gluten) than the 0.4 mg/kg stated by SureFood® allergen gluten (r-Bio-pharm, Darmstadt, Germany). Regarding the absolute sensitivity, all the proposed real-time PCR methods presented lower LOD (<1.1 DNA wheat DNA copies) than that stated by the referred kit (5 DNA copies). However, it is important to refer that this commercial real-time PCR assay is not for quantification purposes since it does not

include a calibration curve, enabling only positive/negative responses until the referred limit of 0.4 mg/kg of gluten. Additionally, none of the herein proposed real-time PCR systems for the specific detection of gluten presented any cross-reactivity with safe cereals.

In summary, the developed real-time PCR methods presented in this work constitute adequate alternatives to detect toxic cereals for celiac patients at trace levels in foods. The detection of wheat DNA at minute amounts is a challenging aspect due to the complexity and large size of its genome (multiploidy). Unlike most of the PCR assays developed for gluten detection that usually target gliadin encoding sequences, in this work, the genomic regions encoding

the allergens Tri a 18 and Tri a 25 were also evaluated. Three real-time PCR methods were successfully developed allowing the detection down to 50 mg/kg of wheat flour or 4.5 mg/kg of gluten in soybean matrix. Additionally, all the systems enable detecting other gluten-containing cereals such as rye, barley and oat, which highlight the importance of this work for celiac patients. Further research work is still required to verify if there is a matrix effect that limits the sensitivity of the methods described herein.

Acknowledgements

This work was supported by Fundação para a Ciência e Tecnologia (FCT) through grant no. Pest-C/EQB/LA0006/2013 financed by POPH-QREN (subsidised by FSE and MCTES), the bilateral project Acções Integradas Luso-Espanholas/2012 between University of Porto (E-38/12) and University of Oviedo (Ministerio de Ciencia e Innovación, PRI-AIBPT-2011-0769), through Prof. Noemí Álvarez and Prof. M. Jesús Castañon. B.M.F is grateful to Complutense University PhD grant (BE45/10) and J.C. to FCT post-doctoral grant SFRH/BPD/102404/2014.

References

- Allmann, M., Candrian, U., Hofelein, C., & Luthy, J. (1993). Polymerase chain-reaction (PCR) – a possible alternative to immunochemical methods assuring safety and quality of food – detection of wheat contamination in non-wheat food-products. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 196, 248–251.
- Anderson, O. D., & Greene, F. C. (1997). The α -gliadin gene family. II. DNA and protein sequence variation, subfamily structure, and origins of pseudogenes. *Theoretical and Applied Genetics*, 95, 59–65.
- Breiteneder, H., & Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, 113, 821–830.
- Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55, 611–622.
- Cankar, K., Stebih, D., Dreo, T., Zel, J., & Gruden, K. (2006). Critical points of DNA quantification by real-time PCR – effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnology*, 6, 37.
- Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., et al. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *American Journal of Clinical Nutrition*, 85, 160–166.
- CODEX STAN 118. (1979). Amended in 1983 and 2008 for foods for special dietary use for persons intolerant to gluten. FAO/WHO Standards. Official Codex Standard. Available at <http://www.codexalimentarius.org/standards/list-of-standards/> Accessed 17.04.14.
- Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. B. P. P. (2012). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, 60, 8103–8110.
- Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Research International*, 54, 1722–1729.
- Dahinden, I., von Buren, M., & Luthy, J. (2001). A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for celiac patients. *European Food Research and Technology*, 212, 228–233.
- Debnath, J., Martin, A., & Gowda, L. (2009). A polymerase chain reaction directed to detect wheat glutenin: Implications for gluten-free labelling. *Food Research International*, 42, 782–787.
- Denham, J. M., & Hill, I. D. (2013). Celiac disease and autoimmunity: review and controversies. *Current Allergy and Asthma Reports*, 13, 347–353.
- Díaz-Amigo, C., & Popping, B. (2013). Accuracy of ELISA detection methods for gluten and reference materials: a realistic assessment. *Journal of Agricultural and Food Chemistry*, 61, 5681–5688.
- Directive 2007/68/EC of 27 November 2007 Amending annex IIIa to directive 2000/13/EC regarding certain food ingredients. *Official Journal of European Union*, L310, 11–14.
- Fasano, A., & Catassi, C. (2012). Celiac disease. *The New England Journal of Medicine*, 367, 2419–2426.
- Green, P., & Jabri, B. (2006). Celiac disease. *Annual Review of Medicine*, 57, 207–221.
- Haraszi, R., Chassaigne, H., Maquet, A., & Ulberth, F. (2011). Analytical methods for detection of gluten in food—method developments in support of food labeling legislation. *Journal of AOAC International*, 94, 1006–1025.
- Hubner, P., Waiblinger, H., Pietsch, K., & Brodmann, P. (2001). Validation of PCR methods for quantitation of genetically modified plants in food. *Journal of AOAC International*, 84, 1855–1864.
- Janssen, F. (2006). Detecting wheat gluten in food. In S. J. Koppelman, & S. L. Hefle (Eds.), *Detecting allergens in food* (pp. 244–272). Cambridge: Woodhead Publishing.
- Koppel, E., Stadler, M., Luthy, J., & Hubner, P. (1998). Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 206, 399–403.
- Mafra, I., Ferreira, I. M. P. L. V. O., & Oliveira, M. B. P. P. (2008). Food authentication by PCR-based methods. *European Food Research and Technology*, 227, 649–665.
- Martín-Fernández, B., Miranda-Ordieres, A. J., Lobo-Castañón, M. J., Frutos-Cabanillas, G., de-los-Santos-Álvarez, N., & López-Ruiz, B. (2014). Strongly structured DNA sequences as targets for genosensing: sensing phase desing and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment. *Biosensors and Bioelectronics*, 60, 244–251.
- Maskova, E., Paulickova, I., Rysova, J., & Gabrovská, D. (2011). Evidence for wheat, rye, and barley presence in gluten free foods by PCR method – comparison with ELISA method. *Czech Journal of Food Sciences*, 29, 45–50.
- Mazzara, M., Savini, C., Charles de Delobel, C., Broll, H., Damant, A., Paoletti, C., et al. (2008). *European network of GMO laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing*. Brussels: European Commission. Available online at <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm> Accessed 17.04.14.
- Molberg, O., Uhlen, A. K., Jensen, T., Flaete, N. S., Fleckenstein, B., Arentz-Hansen, H., et al. (2005). Mapping of gluten T cell epitopes in the bread wheat ancestors: implications for celiac disease. *Gastroenterology*, 128, 393–401.
- Mujico, J., Lombardia, M., Mena, M., Mendez, E., & Albar, J. (2011). A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chemistry*, 128, 795–801.
- Olexová, L., Dovičovičová, L., Švec, M., Siekel, P., & Kuchta, T. (2006). Detection of gluten-containing cereals in flours and “gluten-free” bakery products by polymerase chain reaction. *Food Control*, 17, 234–237.
- Piknova, L., Brezna, B., & Kuchta, T. (2008). Detection of gluten-containing cereals in food by 5′-nuclease real-time polymerase chain reaction. *Journal of Food and Nutrition Research*, 47, 114–119.
- Regulation (EU) No 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European parliament and of the council, and repealing commission directive 87/250/EEC, council directive 90/496/EEC, commission directive 1999/10/EC, Directive 2000/13/EC of the European parliament and of the council, commission directives 2002/67/EC and 2008/5/EC and commission regulation (EC) No 608/2004. *Official Journal of European Union*, L304, 18–63.
- Regulation (EU) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. *Official Journal of European Union*, L16, 3–5.
- Reilly, N. R., Fasano, A., & Green, P. H. R. (2012). Presentation of celiac disease. *Gastrointestinal Endoscopy Clinics of North America*, 22, 613–621.
- Sandberg, M., Lundberg, L., Ferm, M., & Yman, I. (2003). Real-time PCR for the detection and discrimination of cereal contamination in gluten free foods. *European Food Research Technology*, 217, 344–349.
- Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G., et al. (2002). Structural basis for gluten intolerance in celiac sprue. *Science*, 297, 2275–2279.
- Sharma, A. D., Gill, P. K., & Singh, P. (2002). DNA isolation from dry and fresh samples of polysaccharide-rich plants. *Plant Molecular Biology Reporter*, 20, 415a–415f.
- Sollid, L. (2002). Coeliac disease: dissecting a complex inflammatory disorder. *Nature Reviews Immunology*, 2, 647–655.
- Tatham, A., & Shewry, P. (2008). Allergens in wheat and related cereals. *Clinical and Experimental Allergy*, 38, 1712–1726.
- Terzi, V., Malnati, M., Barbanera, M., Stanca, A., & Faccioli, P. (2003). Development of analytical systems based on real-time PCR for *Triticum* species-specific detection and quantitation of bread wheat contamination in semolina and pasta. *Journal of Cereal Science*, 38, 87–94.
- Yamakawa, H., Hiroshi, A., Endo, Y., Miyatake, K., Sakata, K., Sakai, et al. (2007). Specific detection of wheat residues in processed foods by polymerase chain reaction. *Bioscience, Biotechnology, and Biochemistry*, 71, 2561–2564.
- Zeltner, D., Glomb, M., & Maede, D. (2009). Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and oat. *European Food Research Technology*, 228, 321–330.

3.2 Efecto de matriz

Tras el desarrollo de los tres métodos de PCR en tiempo real con sondas TaqMan para la amplificación de las tres secuencias que codifican α 2-gliadina, isolectina y tiorredoxina, se propuso la evaluación del comportamiento de éstas en función del tipo de matriz. Para ello se estudiaron comparativamente tres matrices inertes diferentes, soja, maíz y arroz, en forma de harina, donde se adicionaron cantidades conocidas de harina de trigo, para la preparación de muestras modelo, a falta de un material de referencia certificado. Una vez preparadas las muestras binarias, y extraídos los ADN de cada una de ellas se evaluó la sensibilidad de cada método de PCR desarrollado en cada matriz.

(submitted)



Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox



Effect of matrix on the performance of quantitative real-time PCR of gluten-containing cereals

Begoña Martín-Fernández^{a,b}, Joana Costa^a, M. Beatriz P.P. Oliveira^a, Beatriz López-Ruiz^b and Isabel Mafra^{a,*}

^a LAQV-REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

^b Sección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pz. Ramón y Cajal s/n, 28040 Madrid, Spain

* Corresponding author. Tel.: +351 220428640; fax: +351 226093390. E-mail address: isabel.mafra@ff.up.pt (I. Mafra)

ABSTRACT

The celiac disease is considered an autoimmune disorder caused by the ingestion of gluten in genetically susceptible individuals. Hence, gluten-containing foods cannot be consumed by celiac patients. DNA-based methods have been advanced as excellent alternatives for the detection and quantification of gluten-containing cereals, such as wheat in processed foods. In this paper, after the successful development of three real-time PCR approaches with TaqMan probes targeting α 2-gliadin, agglutinin isolectin and thioredoxin h genes, we intended to evaluate the performance of each method as influenced by the food matrix. For this purpose, soybean, maize and rice flours were used in model matrices spiked with known amounts of wheat flour. The results evidenced adequate performance parameters in terms of PCR efficiency and correlation coefficient, regardless the target gene and food matrix. However, sensitivity was considerably affected by both the food matrix and the target gene. Rice matrix allowed the highest sensitivity, while soybean was the most interfering one, with maize having an intermediate behaviour, but very close to the rice. The method using the α 2-gliadin sequence enabled the best sensitivity, especially when combined with rice matrix (5 mg/kg), followed by maize (10 mg/kg) and soybean (50 mg/kg). These results suggest that food matrix effects need to be carefully evaluated when developing real-time PCR assays for wheat detection and quantification, but without compromising their great effectiveness as tools to monitor gluten-containing cereals.

Keywords

Wheat, matrix-effect, real-time PCR, celiac disease, gluten-free food, food allergens.

INTRODUCTION

Gluten is classified as a complex mixture of storage proteins, mainly gliadins and glutenins, being commonly present in several cereals, namely wheat, rye, barley, kamut, spelt, and oat. So far, three pathologies have been associated with gluten intake, being the most relevant the celiac disease and wheat allergy. In these two pathologies, gluten reactivity is mediated by the activation of the T-cells in the gastrointestinal mucosa via different mechanisms. In wheat allergy, the cross-linking of immunoglobulin (Ig)-E to repeated regions of gluten fraction polypeptides (serine-glutamine-glutamine-glutamine-(glutamine-)proline-proline-phenylalanine) leads to the release of chemical mediators (e.g. histamine, cytokines) from basophils and mast cells (Tanabe, 2008). The celiac disease is considered an autoimmune disorder caused by the ingestion of gluten in genetically susceptible individuals (Fasano & Catassi, 2012) and the clinical symptoms are triggered by specific serologic autoantibodies, most notably serum anti-tissue transglutaminase and anti-endomysial antibodies (Sapone et al., 2012). Both conditions are highly relevant, especially in industrialised societies, since celiac disease and wheat allergy are estimated to affect up to 1% of the general population (children/adolescents and adults) (Denham & Hill, 2013). More recently, a third pathology associated with gluten consumption has been described as gluten sensitivity, but in which neither allergic nor autoimmune mechanisms are involved (Catassi et al, 2013, Sapone et al., 2012). Given the role of gluten in the referred pathologies, the cornerstone to safeguard the health of sensitised individuals is the total avoidance of gluten-containing cereals. Therefore, global legislation has been issued concerning the mandatory labelling of gluten-containing foods. In the European Union, gluten-containing foods above 20 mg/kg must be labelled (Regulation (EC) No. 41/2009; Regulation (EU) No. 1169/2011), which is in accordance with the Codex Alimentarius guidelines (CODEX-STAN118, 1979).

The detection and quantification of gluten in foods is currently of prime importance, relying on the accuracy, sensitivity and specificity of the available analytical methods. Independently on the chosen analytical technique (protein- or DNA-based) for the analysis of gluten in foods, two factors have gained special attention, namely the effect of food processing and the interference of the matrix (Kenk, Panter, Engler-Blum, & Bergemann, 2012). In the last years, owing to the high stability of DNA molecules upon processing, DNA-based methods have been advanced as excellent alternatives for the detection and

quantification of gluten in processed foods (Martín-Fernández, Costa, Oliveira, Lopez-Ruiz & Mafra, 2015; Mujico, Lombardia, Mena, Mendez, & Albar, 2011; Pegels, González, García, & Martín, 2015; Sandberg, Lundberg, Ferm, & Yman, 2003; Terzi, Caterina, Giovanardi, D'Egidio, Stanca, & Faccioli, 2004; Zeltner, Glomb, & Maede, 2009). As a consequence of unintended cross-contamination during food processing, gluten might be present at trace levels, which represents a major challenge for its detection/quantification in foods. Besides food processing that normally contributes to a general DNA degradation, the presence of components such as fats, carbohydrates or other plant metabolites might difficult the efficient extraction of DNA from foods (Fernandes et al., 2015; Kenk et al., 2012). Thus, DNA extracts have poor yields and low quality/purity, often presenting polymerase chain reaction (PCR) inhibitors (e.g. polyphenols) that might difficult the development of adequate methods for gluten analysis. Addressing this specific issue, few studies have been conducted to evaluate the effect of the matrix in quantitative/semi-quantitative real-time PCR methods (Olexová, Dovicovicova, Svec, Siekel, & Kuchta, 2006; Maskova, Paulickova, Rysova, & Gabrovska, 2011; Siegel, Mutschler, Boernsen, Pietsch, & Waiblinger, 2013; Zeltner et al., 2009).

Following the successful comparison of three real-time PCR methods with TaqMan probes to detect gluten-containing cereals in foods (Martín-Fernández et al., 2015), the present work intends to evaluate the performance of each real-time PCR system as affected by the type of matrix used to prepare model mixtures of wheat, namely soybean, maize and rice.

MATERIALS AND METHODS

Sample preparation

Flours from different plants (wheat, rye, barley, oat, maize, soybean and rice) and a wide range of plant species, namely peanut, pine nut, Brazil nut, almond, pecan nut, walnut, cashew, hazelnut, macadamia nut, peach, nectarine, apricot, mulberry, strawberry, cherry, plum, lupine, sunflower, potato, rapeseed and cassava used for cross-reactivity purposes, were obtained at local markets. In the absence of certified or testing reference materials for wheat, binary model mixtures, containing: 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1,000 mg/kg, 5,000 mg/kg, 10,000 mg/kg, 50,000 mg/kg, 100,000 mg/kg and 500,000 mg/kg of wheat flour in different flour matrices (soybean, maize or rice) were prepared, in a total of three independent sets of reference mixtures. The first mixture containing 500,000 mg/kg (50%) of wheat flour was prepared by adding 50 g of wheat flour to 50 g of soybean, maize or rice flour. All the other model mixtures were prepared by successive additions of soybean, maize or rice flours until the spiked level of 1 mg/kg (0.0001%) in the equivalent proportion to a final weight

of 100 g in a non-target plant matrix. All the mixtures were thoroughly homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and material, previously treated with a DNA decontamination solution. To avoid accidental cross-contaminations among samples, cereals and reference mixtures were prepared on different days (one set of model mixtures per day). After preparation, all cereals and reference mixtures were immediately stored at -20°C until further DNA extraction.

DNA extraction

DNA was extracted from 100 mg of sample using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations as described by Costa, Mafra, Kuchta and Oliveira (2012), using 2 µL of 10 mg/mL of RNase solution for 5 min at 37 °C, after lysis incubation.

Yield and purity of extracts were assessed by UV spectrophotometric DNA quantification on a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take 3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc, Vermont, USA). The quality of the extracts was also evaluated by electrophoresis in a 1.0% agarose gel containing 1× Gel Red (Biotium, CA, USA) for staining and carried out in 1× SGTB buffer (GRISP, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

Target gene selection and oligonucleotide primers

Three DNA sequences, namely α2-gliadin, agglutinin isolectin A (Tri a 18) and thioredoxin h (Tri a 25) were used to allow the detection of wheat DNA and other gluten-containing cereals in food by three distinct methods. Following our previous research, primers and probes were specifically designed targeting the encoding sequences for α2-gliadin, Tri a 18 and Tri a 25 (Martin-Fernandez et al., 2015), which are presented in Table 1. The universal primers (18SRG-F/18SRG-R) targeting a conserved DNA region (nuclear 18s rRNA gene) were used to assess amplifiability of extracts. Primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany). Stock solutions were stored at -25°C.

Table 1. Primers and probes designed to specifically target sequences of a conserved eukaryotic region (Nuclear 18S rRNA) and of *Triticum aestivum* (α 2-gliadin, agglutinin isolectin - Tri a 18, thioredoxin h - Tri a 25).

Nucleotide sequence (*Genbank accession no.)	Primers/probe sequences (5'→ 3')	Amplicon (bp)	Reference
Nuclear 18S rRNA (*HQ873432.1)	18SRG-F TCTGCCCTATCAACTTTTCGATGG 18SRG-R TAATTTGCGCGCCTGCTG	113 bp	Costa et al. (2013)
α 2-gliadin (*AJ133612.1)	Gly-F CAACCATTTCCGCAGCCGCAA Gly-R TGCGAATACTGTGGTTGCGATTGTG Gly-P FAM-ATCCGCAGCCGCAACCATTTTCGACCACA-BHQ1	134 bp	Martín-Fernandez et al. (2015)
Agglutinin isolectin (Tri a 18) (*M25536.1)	Tri18-F CTGTTGTAGCAAGTGGGGATCCT Tri18-R ATTCTTGGAGAAGAGTGGAGTTGG Tri18-P FAM-CAGAGTGGCGGCTGCGATGGTGTCTT-BHQ1	125 bp	Martín-Fernandez et al. (2015)
Thioredoxin h (Tri a 25) (*AJ404845.1)	Tri25-F TGAAGTGAAGTCCATTGCTGAGCA Tri25-R TGATTACTGGGCCGCGTGTAG Tri25-P FAM-AACCCTGTCCTTGACGTCTCCTTCCTTCA-T-BHQ1	148 bp	Martín-Fernandez et al. (2015)

End-point PCR

PCR amplifications were carried out in 25 μ L of total reaction volume containing 2 μ L of wheat DNA extract (100 ng), 67 mM of Tris–HCl (pH 8.8), 16 mM of $(\text{NH}_4)_2\text{SO}_4$, 0.01% of Tween 20, 200 μ M of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 2.5 or 3.5 mM of MgCl_2 (for α 2-gliadin, or Tri a 18/Tri a 25, respectively) and 160 nM or 120 nM of each set of primers (α 2-gliadin, or Tri a 18/Tri a 25, respectively). The reactions were performed in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) using the following program, with correction adjustment of the temperature of annealing according to target sequence: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, temperature of annealing (70 °C for α 2-gliadin, 63 °C for Tri a 18 and 61 °C for Tri a 25) for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel containing 1 \times Gel Red (Biotium, CA, USA) for staining and carried out in 1 \times SGTB buffer (GRISP, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (Bio-

Rad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time PCR assays

Real-time PCR assays were performed in 20 μ L of total reaction volume, containing 2 μ L of DNA (200 ng), 1 \times of SsoFast Probes Supermix (Bio-Rad, Hercules, CA, USA), 200 nM of each primer and 100 nM of hydrolysis probe (Table 1). The reactions were performed in a fluorimetric thermal cycler CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using the following temperature protocol: 95 °C for 5 min, 55 cycles at 95 °C for 15 s and temperature of annealing/extension of 61 °C (Tri a 25), 62 °C (Tri a 18) or 70 °C (α 2-gliadin) for 45 s with the fluorescence signal acquisition at the end of each cycle. Cycle threshold (Ct) values, which refer to the number of cycles where the fluorescence significantly increases above the background level, were calculated using the software at automatic threshold setting. The continuous measurement of fluorescence is related to the amount of amplicon generated in the real-time PCR, yielding a quantitative result on the presence of the target species. Data were collected and analysed using the software Bio-Rad CFX Manager 3.0 (Bio-Rad, Hercules, CA). Real-time PCR trials were performed in two independent assays using eight replicates in each one.

RESULTS

DNA extraction

The key feature for the successful PCR amplification is to obtain a high quality DNA extract since it is known that the efficiency of the DNA extraction clearly affects the quantitative result. This can also be influenced by matrix components that can affect either the extraction or the detection of the targets (van Hengel, 2007). The NucleoSpin Food kit with certain modified steps, in particular the period of incubation and concentration of RNase A solution used after lysis incubation, was critically chosen in order to obtain high DNA concentration, purity and quality, and, subsequently, high rate of PCR amplification. Especially in the case of binary mixtures prepared with rice matrix, both concentration of RNase A and respective incubation time had to be adjusted to obtain an adequate compromise between purity and yield. The results of DNA yield showed that wheat/soybean binary mixtures of 0.0001%-10% presented the highest values (197-262 ng/ μ L), while the other mixtures of wheat/maize and wheat/rice exhibited much lower and similar ranges (38-61 ng/ μ L and 37-87 ng/ μ L, respectively). The purity of extracts ranged, generally, between 1.8-1.9, with wheat/soybean extracts presenting all the ratios of 2.0. The quality of genomic DNA was checked after agarose gel (1%) electrophoresis, showing high molecular weight bands in all extracts from the

three different types of mixtures (data not shown). Therefore, the extracts presented adequate quality and purity for high rate of PCR amplification.

End-point PCR

Before proceeding to the specific PCR assays, to ensure the absence of any possible false-negative results, the DNA extracts from all model mixtures and plant species were evaluated for their capacity of amplification, using the primers 18SRG-F/18SRG-R, which targeted a conserved eukaryotic region (Costa et al., 2013). All DNA extracts amplified positively for the expected fragment of 113 bp, confirming that they had the adequate quality and purity for PCR amplification.

Previous studies were aimed at developing and optimising the PCR conditions targeting sequences of α 2-gliadin, agglutinin isolectin A and thioredoxin h genes of *Triticum aestivum* (Martín-Fernández et al., 2015). In the present work, specificity of the assays was further assessed by the analysis of DNA extracted from a wide range of plant species. Thus, some of the most important cereals, vegetables, fruits and nut species used in the food production (wheat, barley, rye, oat, rice, maize, soybean, rapeseed, lupine, pine nut, brazil nut, almond, pecan nut, walnut, hazelnut, macadamia nut, peach, nectarine, apricot, mulberry, strawberry, cherry, plum, cashew, sunflower, cassava, potato) were tested for cross-reactivity regarding each target gene. Results showed that all the non-cereal species tested negatively with the three sets of primers. The sequence similarity between wheat and the closely related cereals, namely barley and rye, was previously confirmed (Martín-Fernández et al., 2015). Then, the performance of end-point PCR assays was compared using model mixtures of known amounts of wheat flour (50,000 mg/kg down to 1 mg/kg) spiked in soybean, maize or rice matrix. The application of end-point PCR targeting α 2-gliadin enabled a relative sensitivity of 1,000 mg/kg (0.1%), 100 mg/kg (0.01%) and 50 mg/kg (0.005%), for soybean-, maize- and rice-based binary mixtures, respectively (data not shown). Tri a 18 PCR assay enabled amplification down to 1,000 mg/kg (0.1%), 500 mg/kg (0.05%) and 100 mg/kg (0.01%), for soybean-, maize- and rice-based binary mixtures, respectively (Figure 1). With the Tri a 25 assay, it was possible to detect 500 mg/kg (0.05%) for soybean- and maize-based matrices, and 100 mg/kg (0.01%) for rice-based matrix (data not shown).

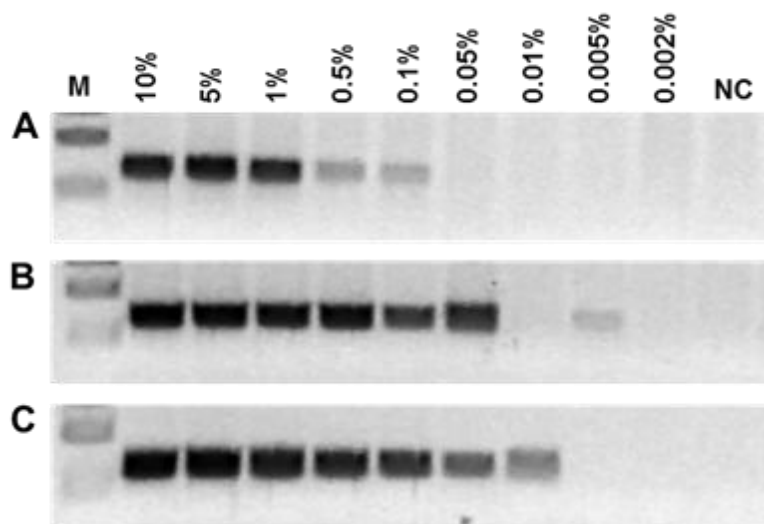


Figure 1. Agarose (1.5%) gel electrophoresis of PCR products targeting Tri a 18 sequence for known amounts of spiked wheat in (A) soybean; (B) maize and (C) rice matrices.

In order to establish the dynamic range and the absolute sensitivity of the method, wheat DNA was serially diluted from 200 ng to 20 pg and amplified targeting the three marker genes, for which 200 pg were detected, regardless the target gene.

Real-time PCR

The performance of the real-time PCR assays targeting the three DNA sequences of α 2-gliadin, Tri a 18 and Tri a 25 was then evaluated and compared for the different matrices. Several parameters have to comply with the acceptance criteria defined for real-time PCR of assays. Adequate performance parameters include a correlation coefficient (R^2) above 0.98, PCR efficiency ranging from 90% to 110% and respective slope from -3.6 and -3.1 (Bustin, Benes, Garson, Hellemans, Huggett, Kubista, et al., 2009; Mazzara et al., 2008). In order to establish the dynamic range and the absolute sensitivity of real-time PCR methods, wheat DNA extracts were 10-fold serially diluted to cover 6 orders of magnitude of the target, ranging from 200 ng to 2 pg. The assays exhibited high performance in terms of efficiency and correlation coefficients, according to guidelines. The systems targeting Tri a 18 and Tri a 25 allowed an absolute limit of detection (LOD) of 20 pg of wheat DNA, corresponding to 1.1 genomic copies (1 copy of wheat genome = 17.33 pg, retrieved from <http://data.kew.org/cvalues/>), obtained from a dilution factor of 10,000. A LOD of 2 pg (0.1 DNA copies) was achieved for the detection of α 2-gliadin, being 10-fold lower than the other two systems. In these real-time PCR

systems, the limit of quantification (LOQ) equalled the LOD, as the lowest amount of DNA target (20 pg or 2 pg, for Tri a 18/Tri a 25 or Gly, respectively) was within the linear range of the calibration curve.

Also, the system targeting the α 2-gliadin sequence presented the best results in terms of relative sensitivity, which allowed detecting 0.002% (20 mg/kg) of wheat in soybean. Nevertheless, the LOD was considered as 0.005% (50 mg/kg) of wheat in soybean material (Figure 2) since the amplification at the previous spiking level was achieved in only 50% of the replicates (data not shown). According to the guidelines for the development of quantitative real-time PCR assays (Bustin, et al., 2009; Mazzara et al., 2008) the LOD should be defined as the lowest concentration level with 95% of positive amplifications. In this study, the LOD was always determined assuming the lowest concentration level with positive amplification in all replicates. The LOQ was found to be equal to the LOD since it was within the linear range of the calibration curve. Quantitative assays targeting the DNA sequences encoding Tri a 18 or Tri a 25 allergens enabled a relative LOD and LOQ of 0.05% (500 mg/kg) of wheat in soybean with both systems (Figure 2B and 2C), thus presenting a 10-fold worse sensitivity than the α 2-gliadin real-time PCR assay (Figure 2A). For maize and rice matrices, the sensitivity was particularly increased, mainly using the α 2-gliadin system that reached the lowest LOD values of 0.001% (10 mg/kg) and 0.0005% (5 mg/kg), respectively (Figure 2A). Moreover, the sensitivities of the three sequences in maize and rice matrices were at least 5-fold better than with the soybean matrix, which can be highlighted in Table 2. The calibration curves for the three amplified sequences correlated well between spiked wheat and quantifiable DNA, resulting in comparable slopes in all three investigated matrices, respectively.

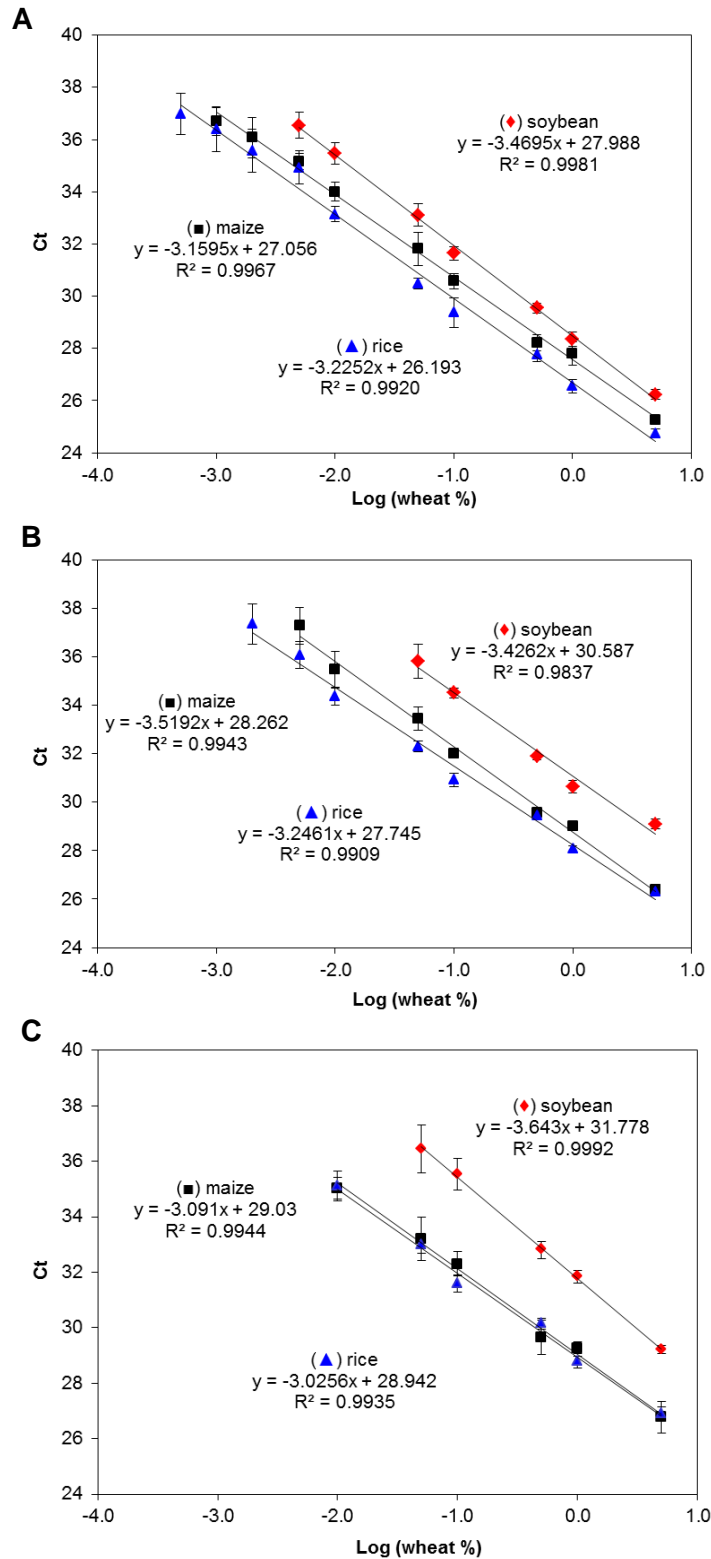


Figure 2. Comparison of calibration curves obtained with different real-time PCR systems targeting α -gliadin (A), agglutinin isolectin (Tri a 18) (B) and thioredoxin h (Tri a 25) (C) genes, using binary mixtures containing 5% to 0.0001% of wheat in soybean (\diamond), maize (\square) or rice (Δ) matrices. Mean values and corresponding standard deviations of $n=8$ replicates of two independent real-time PCR trials.

Table 2. Relative limits of detection of wheat in different matrices and correspondent gluten content.

Gene	Soybean			Maize			Rice		
	wheat	gluten	Wheat DNA copies	wheat	gluten	Wheat DNA copies	wheat	gluten	Wheat DNA copies
	mg/kg ^a			mg/kg ^b			mg/kg ^c		
α 2-gliadin	50	4.5	28.9	10	1.8	11.5	5	0.45	2.9
Tri a 18	500	45	289	50	4.5	28.9	20	1.8	11.5
Tri a 25	500	45	289	100	9	57.7	100	9	57.7

^a mg of wheat or gluten per kg of soybean flour; ^b mg of wheat or gluten per kg of maize flour; ^c mg of wheat or gluten per kg of rice flour.

In general, the mean performance parameters of PCR efficiency and correlation coefficient for the three methods were in good agreement with the acceptance criteria defined for real-time PCR, regardless the food matrix (Figure 2). However, the system of soybean-based matrix exhibit the worst sensitivity, evidenced by the latest amplification level since the cycle threshold (Ct) values were approximately one or two cycles later than in maize- and rice-based mixtures, irrespective of the target sequence (Figure 3). α 2-Gliadin was again the earliest amplified target compared to Tri a 18 and Tri a 25 systems for the same spiking level of wheat in the three different matrices. Between the maize and rice based matrices, similar Ct values could be observed for the same level of concentration (Figure 3). Comparing the systems based on Tri a 18 and the Tri a 25 sequences, it is clear that Tri a 25 presents the highest Ct values, which seriously affects sensitivity, mainly in maize- and rice-based matrices.

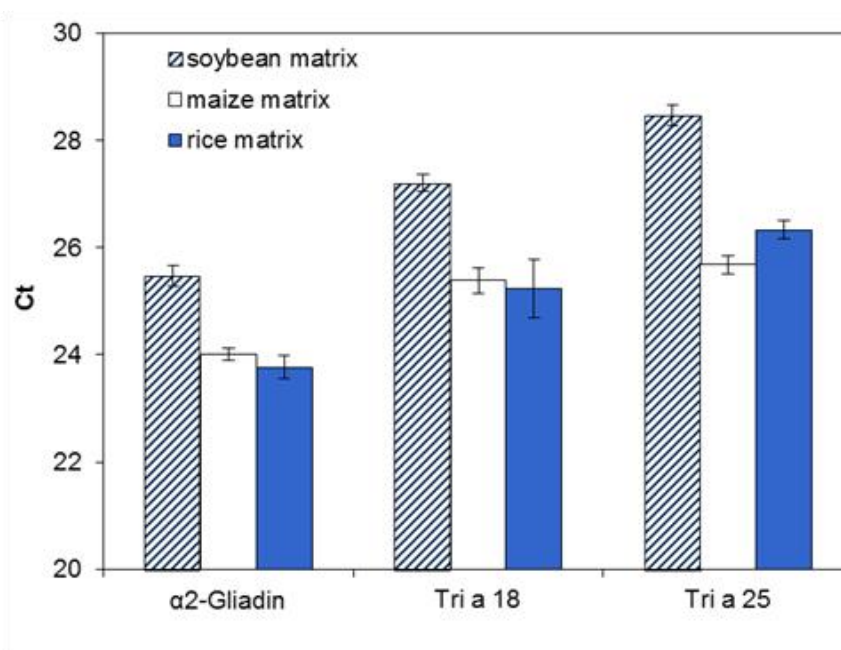


Figure 3. Comparison of cycle threshold values (Ct) of real-time PCR amplification assays targeting α2-gliadin, agglutinin isolectin (Tri a 18) and thioredoxin h (Tri a 25) genes, using the binary mixture containing 100,000 mg/kg wheat flour spiked in different matrices.

DISCUSSION

The only currently available treatment for celiac disease is the lifelong elimination of wheat and related cereals from the diet (Briani, Samaroo, & Alaedini, 2008). However, a minimal degree of gluten contamination is difficult to avoid. An alternative to the direct detection of proteins by ELISA is provided by DNA-based methods. The relation between gluten and DNA is not fixed in grains of various species, which may represent an obstacle for gluten quantification with DNA-based methods. Usually, this variation does not exceed one order of magnitude, allowing the correlation between DNA and amount of gluten (Luber et al., 2014), thus presenting excellent alternative methods to ELISA (Goesaert, Brijs, Veraverbeke, Courtin, Gebruers, & Delcour, 2005; Leszczyńska, Majak, & Bartos, 2014; Píknova, Brezna, & Kuchta, 2008). To determine wheat in gluten-free products, the selected target sequences could be both genes coding for an allergenic protein (glutenins, gliadins) (Debnath, Martin, & Gowda, 2009; Sandberg et al., 2003; Zeltner et al., 2009) or simply, a species-specific region in the genome of the allergenic food (Arlorio, Coisson, Cereti, Travaglia, Capasso, & Martelli, 2003; Dahinden, von Buren, & Luthy, 2001; Koppel, Stadler, Luthy, & Hubner, 1998; Maskova et al., 2011; Olexova et al., 2006; Pegels et al., 2015; Píknova et al., 2008; Tilley, 2004). Since there are no standard reference materials for wheat DNA analysis, for method development/optimisation and to determine the relative limits of detection,

reference mixtures must be prepared. Despite several efforts to detect trace amounts of wheat in food, very few studies reached the limit of 20 mg/kg set by the legislation to discriminate safe products for celiac patients (Regulation (EC) No. 41/2009).

The use of molecular methodologies is highly dependent on numerous factors, such as the type of food matrix, the allergens/DNA markers, the chosen methodology, genome size of the investigated species and on the copy number, among others (Costa et al., 2013; Scarafoni, Ronchi, & Duranti, 2009). In this work, we exploit the use of different real-time PCR systems, targeting both single and multi-copy genes (α 2-gliadin, agglutinin and thioredoxin) that encode allergenic proteins, in order to detect gluten-containing cereals as affected by food matrix. For any targeted sequence, rice and maize allowed a much higher sensitivity compared with the soybean-based matrix. Regardless the matrix, when comparing the three real-time PCR methods, the best sensitivity was obtained with α 2-gliadin marker sequence, reaching 0.005% (50 mg/kg), 0.001% (10 mg/kg) and 0.0005% (5 mg/kg) of wheat in soybean, in maize or in rice materials, respectively. The best sensitivity of the α 2-gliadin real-time PCR amplification system is consistent with previous findings (Martín-Fernández et al., 2015) and could be attributed to its multi-copy nature, being the copy number estimated to range from 25 to 150 (Anderson & Greene, 1997; van Herpen et al., 2006). Considering that the gluten content is about 9% in wheat flour (Zeltner et al., 2009), the sensitivity targeting the α 2-gliadin gene was as low as 0.45 mg/kg, 0.9 mg/kg and 4.5 mg/kg of gluten in rice, maize and soybean matrices, respectively (Table 2). The other target sequences of Tri a 18 and Tri a 25 allowed detecting 1.8 mg/kg and 9 mg/kg of gluten in rice matrix, respectively (Table 2).

The highlighted sensitivity values were generally in good agreement with the results reported in the literature. In the case of soybean matrix, the worst scenario, the obtained sensitivities of 0.005%, using the α 2-gliadin gene, and 0.05% with other genes, were much improved compared with the reported level of 0.1% (1,000 mg/kg) for wheat in soybean matrix (Maskova et al., 2011; Olexova et al., 2006). Concerning maize matrix, a better sensitivity was also obtained in the present work using the α 2-gliadin system (0.001%) than the reported level of 0.005% (50 mg/kg) (Yamakawa et al., 2007). Rice matrix exhibited the best sensitivity results, as emphasised by the lowest LOD obtained using α 2-gliadin gene (5 mg/kg), which is in good agreement with Zeltner et al. (2009) that reached 2.5 mg/kg of wheat flour in rice flour by real-time PCR. Although the reported sensitivity is apparently 2-fold higher than the obtained in the present work, it should be noted that the recommended guidelines for real-time PCR (Bustin et al., 2009; Mazzara et al., 2008) were taking in consideration, contrarily to the reported value of Zeltner et al. (2009).

A better DNA extraction yield from soybean, when compared to wheat or maize, has been reported (Sharma, Gill, & Singh, 2002; Zhu, Qu, & Zhu, 1993), which might justify these differences. The high content of soybean DNA versus wheat DNA or the high lipidic content of soybean could act as strong PCR inhibitors (Cankar, Stebih, Dreo, Zel, & Gruden, 2006), reducing the efficiency of the reaction. This might explain the poorer sensitivity of wheat in soybean-based mixtures observed in comparison with wheat in maize- and rice-based ones, which reached as less as 0.45 mg/kg of gluten for the α 2-gliadin system.

In this work, we demonstrate for the first time that there is a matrix effect on wheat detection/quantification by real-time PCR, which may result in a decrease of sensitivity, depending on whether the matrix is primarily made of soybean, maize or rice. Even though, some approaches have been recently proposed to compensate for the variability in DNA extraction and amplification in food materials (D'Andrea, Coisson, Travaglia, Garino, & Arlorio, 2009; Holzhauser, Kleiner, Janise, & Röder, 2014; Luber, et al., 2014), the detection of wheat gluten in food products is affected by the analysed matrices. Thus, the accurate detection/quantification of gluten would require different standard curves for different food matrices, according to their composition and processing conditions.

In summary, we reported the selective real-time PCR amplification of α 2-gliadin, agglutinin and thioredoxin gene sequences for wheat flour detection and quantification in different food matrices. The real-time PCR methods exhibited adequate analytical performance and successfully detected wheat at trace levels in all tested food matrices. Considering the need of verifying labelling compliance for gluten threshold (20 mg/kg of gluten), the α 2-gliadin real-time PCR assay showed the best performance since it allows detecting 0.45-4.5 mg/kg, depending on the food matrix. The other assays targeting Tri a 18 and Tri a 25 can also be useful, but only for gluten detection in maize or rice flours that still demonstrate adequate level of gluten detection (1.8-9.0 mg/kg) since soybean matrix seriously affected sensitivity. Therefore, it was demonstrated that the food matrix affects the detection and quantification of gluten by DNA-based approaches, but in spite of it, the proposed assays can be considered useful tools to verify labelling compliance. For the accurate quantification, the preparation of different standard curves for different matrices would be required, according to the main ingredient.

Acknowledgments

This work was supported by Fundação para a Ciência e Tecnologia (FCT) through grant no. UID/QUI/50006/2013 financed by POPH-QREN (subsidised by FSE and MCTES), the bilateral project Acções Integradas Luso-Espanholas/2012 between University of Porto (E-38/12) and University of

Oviedo (Ministerio de Ciencia e Innovación, PRI-AIBPT-2011-0769). B.M.F is grateful to Complutense University PhD grant (BE45/10) and J.C. to FCT post-doctoral grant SFRH/BPD/102404/2014.

REFERENCES

Anderson, O. D., & Greene, F. C. (1997). The α -gliadin gene family. II. DNA and protein sequence variation, subfamily structure, and origins of pseudogenes. *Theoretical and Applied Genetics*, 95, 59-65.

Arlorio, M., Coisson, J., Cereti, E., Travaglia, F., Capasso, M., & Martelli, A. (2003). Polymerase chain reaction (PCR) of puroindoline b and ribosomal/puroindoline b multiplex PCR for the detection of common wheat (*Triticum aestivum*) in Italian pasta. *European Food Research and Technology*, 216, 253-258.

Briani, C., Samaroo, D., & Alaedini, A. (2008). Celiac disease: From gluten to autoimmunity. *Autoimmunity Reviews*, 7, 644-650.

Bustin, S. A., Benes, V., Garson, J. A., Hellems, J., Huggett, J., Kubista, M. et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, 55(4), 611-622.

Cankar, K., Stebih, D., Dreo, T., Zel, J., & Gruden, K. (2006). Critical points of DNA quantification by real-time PCR - effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnology*, 6.

Catassi, C., Bai, J. C., Bonaz, B., Bouma, G., Calabrò, A., Carroccio, A. et al. (2013). Non-celiac gluten sensitivity: the new frontier of gluten related disorders. *Nutrients*, 5, 3839-3853.

CODEX-STAN118. (1979). Amended in 1983 and 2008 for foods for special dietary use for persons intolerant to gluten. FAO/WHO Standards. Available at: <http://www.codexalimentarius.org/standards/list-of-standards/>. Accessed 02.06.2015.

Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. (2012). Single-Tube Nested Real-Time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, 60, 8103-8110.

Costa, J., Oliveira, M., & Mafra, I. (2013). Novel approach based on single-tube nested real-time PCR to detect almond allergens in foods. *Food Research International*, 51, 228-235.

D'Andrea, M., Coïsson, J. D., Travaglia, F., Garino, C., & Arlorio, M. (2009). Development and validation of a SYBR-Green I real-time PCR protocol to detect hazelnut (*Corylus avellana* L.) in foods through calibration via plasmid reference standard. *Journal of Agricultural and Food Chemistry*, *57*, 11201-11208.

Dahinden, I., von Buren, M., & Luthy, J. (2001). A quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients. *European Food Research and Technology*, *212*, 228-233.

Debnath, J., Martin, A., & Gowda, L. (2009). A polymerase chain reaction directed to detect wheat glutenin: Implications for gluten-free labelling. *Food Research International*, *42*, 782-787.

Denham, J. M., & Hill, I. D. (2013). Celiac disease and autoimmunity: review and controversies. *Current Allergy and Asthma Reports*, *13*, 347-353

Fasano, A., & Catassi, C. (2012). Celiac Disease. *New England Journal of Medicine*, *367*, 2419-2426.

Fernandes, T. J. R., Costa, J., Plácido, A., Villa, C., Grazina, L., Meira, L. et al. (2015) GMO analysis as affected by DNA degradation. In R. Watson, V.R. Preedy (Ed.) *Genetically modified organisms in foods*, Oxford: Elsevier.

Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. M., Gebruers, K., & Delcour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends in Food Science & Technology*, *16*, 12-30.

Holzhauser, T., Kleiner, K., Janise, A., & Röder, M. (2014). Matrix-normalised quantification of species by threshold-calibrated competitive real-time PCR: allergenic peanut in food as one example. *Food Chemistry*, *163*, 68-76.

Kenk, M., Panter, S., Engler-Blum, G., & Bergemann, J. (2012). Sensitive DNA-based allergen detection depends on food matrix and DNA isolation method. *European Food Research and Technology*, *234*, 351-359.

Koppel, E., Stadler, M., Luthy, J., & Hubner, P. (1998). Detection of wheat contamination in oats by polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology*, *206*(6), 399-403.

Leszczyńska, J., Majak, I., & Bartos, A. (2014). Gluten testing in foods, pharmaceuticals and cosmetics. In G. Siragakis, & D. Kizis (Eds.) *Food Allergen*

Testing: Molecular, Immunochemical and Chromatographic Techniques (pp. 151-184), Chichester: John Wiley & Sons, Ltd.

Luber, F., Demmel, A., Herbert, D., Hosken, A., Hupfer, C., Huber, I. et al. (2014). Comparative assessment of DNA-based approaches for the quantification of food allergens. *Food Chemistry*, 160, 104-111.

Martín-Fernández, B., Costa, J., Oliveira, M. B. P. P., Lopez-Ruíz, B., & Mafra, I. (2015). Screening new gene markers for gluten detection in foods. *Food Control*, 56, 57-63.

Maskova, E., Paulickova, I., Rysova, J., & Gabrovska, D. (2011). Evidence for wheat, rye, and barley presence in gluten free foods by PCR method - comparison with ELISA method. *Czech Journal of Food Sciences*, 29, 45-50.

Mazzara, M., Savini, C., Charles de Delobel, C., Broll, H., Damant, A., Paoletti, C., et al. (2008). European Network of GMO Laboratories (ENGL). Definition of minimum performance requirements for analytical methods of GMO testing, Brussels: European Commission. Available online at: <http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm> . Accessed 03.03.2015.

Mujico, J., Lombardia, M., Mena, M., Mendez, E., & Albar, J. (2011). A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chemistry*, 128, 795-801.

Olexova, L., Dovicovicova, L., Svec, M., Siekel, P., & Kuchta, T. (2006). Detection of gluten-containing cereals in flours and "gluten-free" bakery products by polymerase chain reaction. *Food Control*, 17, 234-237.

Pegels, N., González, I., García, T., & Martín, R. (2015). Authenticity testing of wheat, barley, rye and oats in food and feed market samples by real-time PCR assays. *LWT - Food Science and Technology*, 60, 867-875.

Piknova, L., Brezna, B., & Kuchta, T. (2008). Detection of gluten-containing cereals in food by 5'-nuclease real-time polymerase chain reaction. *Journal of Food and Nutrition Research*, 47, 114-119.

Regulation (EC) No. 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. *Official Journal of European Union*, L16, 3-5.

Regulation (EU) No. 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC,

Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004, *Official Journal of European Union*, L304, 18-63.

Sandberg, M., Lundberg, L., Ferm, M., & Yman, I. (2003). Real Time PCR for the detection and discrimination of cereal contamination in gluten free foods. *European Food Research and Technology*, 217, 344-349.

Sapone, A., Bai, J., Ciacci, C., Dolinsek, J., Green, P., Hadjivassiliou, M., Kaukinen, K., Rostami, K., Sanders, D., Schumann, M., Ullrich, R., Villalta, D., Volta, U., Catassi, C., & Fasano, A. (2012). Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Medicine*, 10shan, 13.

Scarafoni, A., Ronchi, A., & Duranti, M. (2009). A real-time PCR method for the detection and quantification of lupin flour in wheat flour-based matrices. *Food Chemistry*, 115(3), 1088-1093.

Sharma, A. D., Gill, P. K., & Singh, P. (2002). DNA isolation from dry and fresh samples of polysaccharide-rich plants. *Plant Molecular Biology Reporter*, 20, 415a–415f.

Siegel, M., Mutschler, A., Boernsen, B., Pietsch, K., & Waiblinger, H.-U. (2013). Food matrix standards for the quantification of allergenic food ingredients using real-time PCR. *European Food Research and Technology*, 237, 185-197.

Tanabe, S. (2008). Analysis of food allergen structures and development of foods for allergic patients. *Bioscience Biotechnology and Biochemistry*, 72, 649-659.

Terzi, V., Caterina, M., Giovanardi, D., D'Egidio, M., Stanca, A., & Faccioli, P. (2004). DNA-based analysis for authenticity assessment of monovarietal pasta. *European Food Research and Technology*, 219, 428-431.

Tilley, M. (2004). PCR amplification of wheat sequences from DNA extracted during milling and baking. *Cereal Chemistry*, 81, 44-47.

van Hengel, A. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and Bioanalytical Chemistry*, 389, 111-118.

van Herpen, T., Goryunova, S., van der Schoot, J., Mitreva, M., Salentijn, E., Vorst, O. et al. (2006). Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. *BMC Genomics*, 7, 1.

Yamakawa, H., Akiyama, H., Endo, Y., Miyatake, K., Sakata, K., Sakai, S. et al. (2007). Specific detection of wheat residues in processed foods by polymerase chain reaction. *Bioscience Biotechnology and Biochemistry*, 71, 2561-2564.

Zeltner, D., Glomb, M., & Maede, D. (2009). Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and. *European Food Research and Technology*, 228, 321-330.

Zhu, H., Qu, F., & Zhu, L. (1993). Isolation of genomic DNAs from plants, fungi and bacteria using benzyl-chloride. *Nucleic Acids Research*, 21(22), 5279-5280.

4 GENOSENSORES ELECTROQUÍMICOS

4.1 Genosensor para la detección de gliadina con sonda de captura lineal

Strongly structured DNA sequences as targets for genosensing: Sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment

4.2 Acoplamiento del genosensor con PCR para el análisis de muestras alimentarias procesadas

Challenging genosensors in food samples: the case of gluten determination

4.3 Genosensor para la detección de gliadina con sonda de captura estructurada

Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a gliadin fragment

4.4 Genosensor para la detección de Tri a 18

4.1 Genosensor para la detección de gliadina con sonda de captura lineal

El análisis de las secuencias de ADN llevó a la conclusión de que el sistema más sensible para la detección de cereales tóxicos en diferentes matrices alimentarias era el basado en la amplificación y detección de la secuencia de ADN que codifica la α 2-gliadina, probablemente debido a su naturaleza multicopia, frente a la presencia en una o pocas copias de las otras dos secuencias de ADN que codifican respectivamente aglutinina isolectina y tiorredoxina h. Debido a la mayor sensibilidad del método de amplificación de la secuencia de ADN que codifica gliadina, se desarrolló un sensor electroquímico para la detección de esta secuencia en muestras alimentarias. Para ello se diseñó un genosensor basado en un ensayo tipo sándwich y un marcaje enzimático, utilizando como sistema de inmovilización monocapas autoensambladas compuestas por una sonda de captura lineal y un diluyente, quimisorbidos sobre la superficie electródica de oro.

Se procedió a la optimización y caracterización del genosensor utilizando oligonucleótidos sintéticos. Posteriormente se aplicó a la detección de muestras reales, previa extracción del ADN y amplificación por PCR convencional.

Un problema adicional encontrado al trabajar con esta secuencia codificante de gliadinas fue la naturaleza repetitiva y fuertemente estructurada que presenta esta cadena oligonucleotídica. Este hecho obligó a rediseñar las sondas que componen el sensor con el objetivo de minimizar los citados efectos adversos sobre la respuesta del genosensor.



Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Strongly structured DNA sequences as targets for genosensing: Sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment



Begoña Martín-Fernández^{a,b}, Arturo J. Miranda-Ordieres^b, María Jesús Lobo-Castañón^b, Gloria Frutos-Cabanillas^a, Noemí de-los-Santos-Álvarez^{b,*}, Beatriz López-Ruiz^{a,*}

^a Sección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pz. Ramón y Cajal s/n, 28040 Madrid, Spain

^b Departamento de Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain

ARTICLE INFO

Article history:

Received 14 January 2014

Received in revised form

2 April 2014

Accepted 17 April 2014

Available online 30 April 2014

Keywords:

Electrochemical genosensor
Self-assembled monolayers
Screen printed gold electrodes
 α 2-gliadin
PCR
Celiac disease

ABSTRACT

Electrochemical genosensors are becoming cost-effective miniaturizable alternatives to real-time PCR (RT-PCR) methods for the detection of sequence-specific DNA fragments. We report on the rapid detection of PCR amplicons without the need of purification or strand separation. A challenging target sequence for both PCR amplification and electrochemical detection allowed us to address some difficulties associated to hybridization on electrode surfaces. The target was a highly specific oligonucleotide sequence of wheat encoding the most immunogenic peptide of gliadin that triggers the immune response of celiac disease (CD), the 33-mer. With a sandwich assay format and a rational design of the capture and tagged-signaling probes the problems posed by the strong secondary structure of the target and complementary probes were alleviated. Using a binary self-assembled monolayer and enzymatic amplification, a limit of detection of 0.3 nM was obtained. The genosensor did not respond to other gluten-containing cereals such as rye and barley. Coupling to PCR to analyze wheat flour samples required tailoring both the capture and signaling probes. This is the first time that deleterious steric hindrance from long single-stranded regions adjacent to the electrode surface is reported for relatively short amplicons (less than 200 bp). The importance of the location of the recognition site within the DNA sequence is discussed. Since the selected gene fragment contains several repetitions of short sequences, a careful optimization of the PCR conditions had to be performed to circumvent the amplification of non-specific fragments from wheat flour.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

DNA hybridization is one of the most selective reactions known and the strategy of choice to unequivocally identify a specific DNA fragment in multiple areas of interest from diagnostics to food safety. In many applications such as DNA chips (Southern et al., 1999), DNA strip assays (Hillemann et al., 2005; Mao et al., 2009) or more generally, in genosensors (Miranda-Castro et al., 2009a), this recognition strategy requires a surface hybridization between a short complementary DNA sequence and the target.

Among the diversity of material supports proposed and transduction techniques, gold electrode surfaces are advantageous because

of the easiness of DNA self-assembly to develop miniaturizable, portable and cost-efficient electrochemical devices. Practical aspects of the probe design and the challenges faced when performing hybridization on solid surfaces, found scattered in the literature, have been compiled in two noticeable reviews (Lucarelli et al., 2008; Miranda-Castro et al., 2009a). While optimization of the surface probe density (Kjallman et al., 2008; Peterson et al., 2001; Watterson et al., 2000), concentration and selection of co-adsorbed diluent thiol (Campuzano et al., 2011; Wu et al., 2010), or the length and nature of spacer to avoid steric hindrance from surface (Shchepinov et al., 1997) have been extensively studied, other issues have comparatively attracted less attention.

Geometry of the capture probe (Miranda-Castro et al., 2007), length and structure of the real target (Mir and Southern, 1999) or the relative position of the recognition sequence within the target are also important factors (Del Giallo et al., 2005) that are often set aside in the discussion. This is especially true when shifting from synthetic to real DNA fragments. Current hybridization technology cannot reach the extremely demanding levels of sensitivity

* Corresponding authors.

E-mail addresses: begonamartin@ucm.es (B. Martín-Fernández), amir@uniovi.es (A.J. Miranda-Ordieres), mjlc@uniovi.es (M.J. Lobo-Castañón), gloriafr@ucm.es (G. Frutos-Cabanillas), santosnoemi@uniovi.es (N. de-los-Santos-Álvarez), bealopru@ucm.es (B. López-Ruiz).

<http://dx.doi.org/10.1016/j.bios.2014.04.033>

0956-5663/© 2014 Elsevier B.V. All rights reserved.

required for some applications such as genotyping, pathogen detection or food contamination/authentication. For that reason pre-amplification steps coupled to hybridization-based assays are still regarded as unavoidable. PCR amplicons are frequently much longer than the initially selected target, which are usually designed shorter than 52nt to minimize stable autohybridization (Lucarelli et al., 2008). The length and the double stranded structure of amplicons pose additional challenges to surface hybridization.

In this work, the detection of specific sequences of wheat encoding the immunodominant peptide 33-mer involved in triggering celiac disease (CD) (Shan et al., 2002, 2005), allow us to study the influence of strong secondary structures as well as long amplicons on the hybridization efficiency. Disposable Au screen-printed electrodes modified with an optimized mixed self-assembled monolayer were employed to build the sandwich assay. The enzymatic label was introduced into the signaling probe through a monovalent system because of the previously reported improved sensitivity in comparison with the multivalent (strept) avidin–biotin system (González-Álvarez et al., 2013; Gonzalez-Fernandez et al., 2013). The 99nt DNA target selected belongs to a gene expressing the alpha type of gliadin, the protein storage of wheat. This protein along with glutenin constitutes gluten, the injurious agent in CD. A strict control of gluten content in food is essential for the safety of celiac patients that must pursue a long-life gluten-free diet. Gliadin has a high content of glutamine and proline amino acids, so its sequence is highly repetitive, which complicates the selection of suitable targets for PCR amplification. When analyzing wheat samples, the PCR amplification designed yielded an amplicon longer than the 99nt target. The implications of single-stranded DNA overhangs on the hybridization efficiency on sandwich DNA schemes are discussed. The PCR-coupled chronoamperometric genosensor developed was sensitive enough to detect wheat in real samples.

2. Experimental

2.1. Reagents

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H₂O₂), Tween 20 and reagents for buffer preparation (10 × phosphate buffer (10 × PBS) and saline sodium phosphate–EDTA (20 × SSPE) pH 7.4), were purchased from Sigma–Aldrich (Spain). Ethanol was purchased

from Panreac (Spain). 1% Casein buffer in 1 × PBS was obtained from Fischer Scientific (Spain). Anti-fluorescein–HRP Fab fragments (antiFITC–HRP) were received from Roche Diagnostics GmbH (Mannheim, Germany). NAP-10 columns of Sephadex G-25 were obtained from Life Technologies (Spain). Water was purified with a Milli-Q system (Millipore, Spain).

Oligonucleotide sequences used are shown in Table 1. Oligomers were obtained as lyophilized desalted salts from Sigma–Life Sciences. All stock solutions were prepared in MilliQ water without any treatment except for thiolated capture probes (CP and CPA) that were deprotected as indicated below. The concentration of all stock solutions was checked spectrophotometrically before stored at –20 °C until use.

2.2. Instrumentation

Electrochemical measurements were carried out with screen-printed gold electrodes (SPEAu, DropSens-220BT, Spain), connected to a µ-AutoLab type II potentiostat with GPES 4.9 software (EcoChemie, The Netherlands). The layout of the disposable planar screen-printed gold electrodes includes three electrodes in the same alumina sheet: a working gold electrode (Ø ≈ 4 mm), a Ag pseudo-reference electrode and a gold counter electrode, all of them screen-printed on a ceramic substrate.

PCR amplification was performed in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Spain) and PCR amplicons were analyzed by electrophoresis using a 2% agarose gel stained with ethidium bromide and visualized through an UV transilluminator (VilberLourmat, France). Amplicon concentrations were quantified fluorimetrically using Quant-iT™ PicoGreen kit (Life Technologies, Spain) in a Minifluorometer TBS-380 (Turner Biosystems, CA). Spectrophotometric measurements were carried out with a UV260 Shimadzu spectrophotometer (Japan).

2.3. Sensing phase preparation

SPEAu were washed with ethanol and water, and dried with nitrogen. The electrodes were conditioned in 0.5 M H₂SO₄ solution by sweeping the potential between 0 and 1.25 V at a scan rate of 100 mV/s until a stable cyclic voltammogram was obtained. Finally, electrodes were washed with water and dried with nitrogen immediately before adding on the working electrode 15 µL of 2 µM of the corresponding capture probe in 2 × SSPE pH 7.4 solution, previously deprotected with DTT as indicated elsewhere (Miranda-Castro et al., 2007). Chemisorption proceeded

Table 1
Synthetic oligonucleotides, probes, target sequences and primers.

	Oligonucleotide sequences 5' → 3'
Target (wheat)	CTG CAG CTG CAA CCA TTT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CCA TTT
SP-FITC	CTG CGG ATA TGG TAG TTG CCG CTG CGG ATA TGG TAG TTG CCG CTG CGG AAA TGG TTG CAG CTG CAG-FITC
SPa-FITC	CTG CGG ATA TGG TAG TTG CCG CTG CGG ATA TGG TAG TTG CCG CTG CGG AAA TGG TTG CAG CTG-FITC
CP	HS-(CH₂)₆-AAA TGG TTG CCG CTG CGG ATA TGG TAG TTG CCG
CPa	HS-(CH₂)₆-TAC TGT GGT TGC GAT TGT GGA TAT GGT TGT TGT GGT CGA AAT GGT TGC GGC TGC GGA TAT GGT AGT TGC GG
Fully mismatched	GAC GTC GAC GTT GGT AAA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GGT AAA
Rye	GCA ACC ATT TCC CCA ACC CCA ACA ACC AAC CCC CAT ACA ACC ACA ACA ACC ATT CCC CCA GCG ACC CCA ACA ACC TTT CCC CCA GCC CCA ACA
Barley	AAC AAC CTT TTC CTC AGC CCC AAC AAC CAT TCC CCT GGC AAC CAC AAC AAC CAT TTC CCC AGC CTC AAC AAC CAT TTC CCC TGC AAC CAC AAC
Forward primer	CAG CTG CAA CCA TTT CCG CAG
Reverse primer	TAC TGT GGT TGC GAT TGT GGA TA
Amplicon	CAG CTG CAA CCA TTT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CCA TTT CGA CCA CAA CCA CCA TAT CCA CAA TCG CAA CCA CAG TA

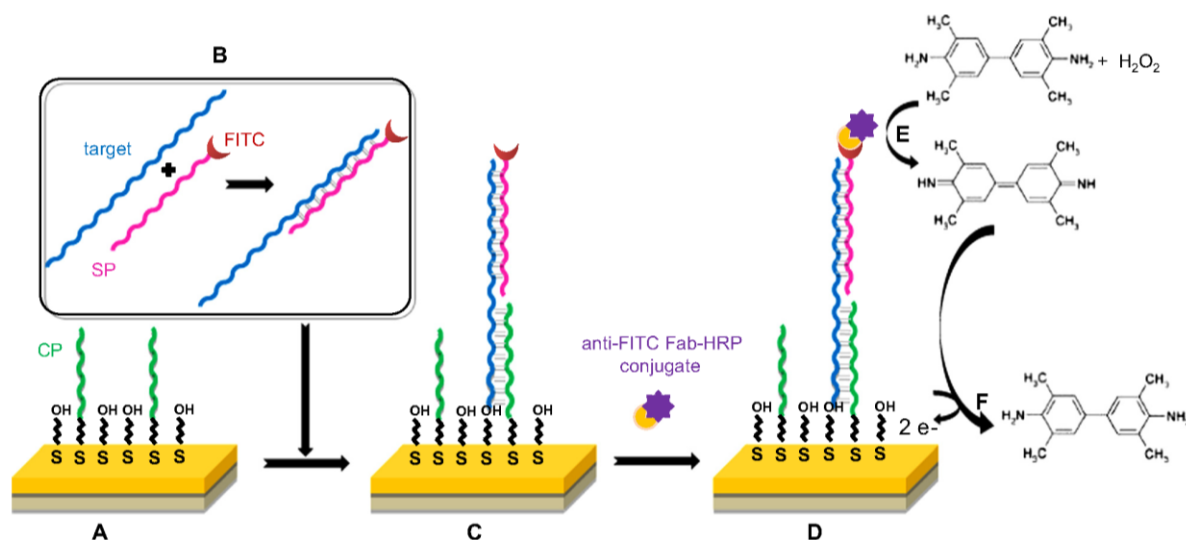


Fig. 1. Schematic drawing of the setup for the recognition and electrochemical detection of DNA coding for 33-mer immunodominant peptide.

overnight and after washing with $2 \times$ SSPE buffer in order to displace the unspecifically adsorbed probe, $10 \mu\text{L}$ of 0.5 mM solution of MCH in $2 \times$ SSPE was placed onto the probe modified surface for 30 min (unless otherwise indicated). The modified electrodes were then washed with buffer and dried with nitrogen before carrying out the sandwich assay (Fig. 1A).

2.4. Sandwich assay and measurement

Hybridization experiments were carried out in a sandwich-like format through two consecutive steps: homogeneous (Fig. 1B) and heterogeneous hybridization reactions (Fig. 1C).

For homogeneous hybridization, the target was added to a $2 \times$ SSPE solution containing $1.25 \mu\text{M}$ of the corresponding signaling probe labeled with FITC (SP- or SPa-FITC). DNA strands were thermally treated by using a heating block for 1.5 mL vial tubes (5 min at $98 \text{ }^\circ\text{C}$), and cooled down by placing in ice-water bath for 5 min . Then, the mixture was brought to room temperature over 30 min . Immediately after, $15 \mu\text{L}$ aliquot of this solution was placed onto the sensing phase for 2 h (heterogeneous hybridization). Afterwards, the working electrode surface was washed and covered with $1 \times$ PBS solution containing 1% casein (w/v) and 0.05% Tween 20 (w/v) pH 7.4 (blocking buffer) for 2 min in order to prevent enzyme conjugate non-specific adsorption. $15 \mu\text{L}$ of a solution of the anti-FITC Fab-HRP conjugate in the blocking buffer (0.5 U/mL) was added to the electrode surface. After 30 min , the sensing phase was washed, first with blocking buffer and then with $2 \times$ SSPE, and dried with nitrogen (Fig. 1D). The cell was then covered with $40 \mu\text{L}$ of TMB and after 1 min of reaction (Fig. 1E), the enzymatically oxidized TMB was chronoamperometrically measured at -0.2 V for 60 s (Fig. 1F). All measurements were carried out in three replicates.

2.5. DNA isolation from flour samples and PCR amplification protocol

Genomic DNA was extracted from cereal flour samples (wheat, rye, barley, oats, soy and rice) using commercial Wizard SV genomic DNA purification system (Promega, Madison) according to manufacturer instructions. The A_{260}/A_{280} ratio was determined, and values near to 1.8 were obtained, indicating the adequate quality of DNA. The concentration was estimated from the 260 nm UV-vis readout.

The amplification reactions were performed using a total volume of $20 \mu\text{L}$. The reaction mixtures contained $2 \mu\text{L}$ of $10 \times$ PCR buffer (670 mM Tris-HCl, pH 8.3, 160 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween-20; supplied with polymerase); variable volumes of 50 mM MgCl_2 in order to obtain different concentrations of this cofactor; $4 \mu\text{L}$ of 50 mM dNTPs (ACGT dNTP mix); $1 \mu\text{M}$ forward primer; $1 \mu\text{M}$ reverse primer, $0.5 \mu\text{L}$ of $5 \text{ U}/\mu\text{L}$ hot-start immobilase™ DNA polymerase (Bioline, Spain) and 40 ng genomic DNA previously extracted. A negative control containing all the reagents except the genomic DNA was included in each series. PCR amplification was performed using: 1 cycle of 15 s at $95 \text{ }^\circ\text{C}$ and 35 cycles composed by 15 s at $95 \text{ }^\circ\text{C}$, 15 s at a fixed temperature within the range $59\text{--}66 \text{ }^\circ\text{C}$ and 30 s at $72 \text{ }^\circ\text{C}$. The reaction was subjected to a post-amplification step of 7 min at $72 \text{ }^\circ\text{C}$ to extend any incomplete product. In order to confirm the reliability of the reaction, PCR products were analyzed by 2% (w/v) agarose gel electrophoresis using $1 \times$ TBE buffer (89 mM Tris-borate, and 2 mM EDTA, pH 8.3) at 90 V for 90 min . DNA bands were visualized by UV light and their size was compared to a known PCR low ladder marker set ($20\text{--}1000 \text{ bp}$, Sigma-Aldrich). PCR products were stored frozen. Concentration was estimated with PicoGreen system and then analyzed with the genosensor without any purification protocol.

3. Results and discussion

3.1. Genosensor design

The scheme of the approach is depicted in Fig. 1. A binary self-assembled monolayer of a capture probe and mercaptohexanol on SPEAu constitutes the sensing phase on which a sandwich binding assay is built.

First of all, the specificity of the wheat target sequence must be ensured. Multicopy genes such as chloroplast genes are in principle more sensitive because of the high copy number associated to the high chloroplast content per cell (Dahinden et al., 2001; Olexova et al., 2006). However, this copy number is variable and the sequence is highly conserved among species, complicating precise quantitation and increasing the probability of false positive results, respectively (Koppelman and Hefle, 2006; Zeltner et al., 2009). In general, single-copy allergen encoding genes are more specific. Then, we select the DNA coding for this specific trait of the

plant, the allergen. Explicitly, the peptide from $\alpha 2$ -gliadin that plays a major role in the immunogenicity, the so-called 33-mer. Mutant peptides lacking this fragment are no longer immunotoxic. The immunogenicity was attributed to its resistance to further breakdown in the human intestine due to its high proline content as well as susceptibility to transglutaminase deamidation (Shan et al., 2002, 2005). From Genbank database we selected as the genosensor target the 99 nucleotide sequence (accession number AJ133612.1) that encodes this protein fragment. The BLAST software was applied to ensure the specificity of the selected sequence¹. No homology in yeast and environmental microorganisms was found.

Using Mfold web Server (Zuker, 2003) the target is predicted to have a very stable secondary structure ($\Delta G = -12.32$ kcal/mol) under the assay conditions at 20 °C (Fig. S1). In general, target sequences shorter than 50nt are preferable for genosensing to minimize the risk of strong secondary structures that lowers the hybridization efficiency at the electrode surface. The complementary strand would suffer even a stronger autohybridization (-19.68 kcal/mol). A very difficult, if possible, hybridization between both strands is anticipated. Hybridization in two steps (sandwich format) has the advantage of splitting the overall complementary strand into two fragments reducing the chances of strong autohybridization. Additionally, it also permits heat denaturation of the stable autohybridized target before the surface hybridization (heterogeneous hybridization step). Capture probe (CP) and signaling probe (SP) sequences were designed to minimize these competing secondary structures prioritizing the linearity of the CP because the anchored probe cannot be thermally denatured without disrupting the SAM architecture. A 33-nt long CP and a 66-nt long SP with ΔG values of -2.21 and -13.17 kcal/mol, respectively, were finally selected as a compromise solution. In Table 1 the regions of the target that hybridize with SP and CP are indicated in bold and italics, respectively. They form a perfect duplex with the target without single-stranded fragments in the middle, which were shown to be deleterious for genosensor performance (Miranda-Castro et al., 2007). The high content of guanine in SP might also favor the formation of G-quartets. This would imply additional stable 3D conformations that further prevent the hybridization. The SP sequence was checked for G-quartets using available free online resources.² As anticipated several G-quartet forming regions were found. As a consequence of the strong stability of autohybridization in both SP and target, a denaturing step prior hybridization was carried out. Preliminary experiments using this set of sequences indicated that the denaturing step coupled to a standard 1-h heterogeneous hybridization was not enough to obtain suitable analytical signals. Extending this step to two hours was the effective way of recovering proper intensity currents. This effect might be also related to the length of the target as previously reported (Miranda-Castro et al., 2009b).

Composition of the sensing phase was subsequently optimized by varying the CP concentration from 0.1 μM to 2 μM and MCH between 0.5 mM and 4.5 mM and carrying out the sandwich assay format depicted in Fig. 1. The SP is labeled with a fluorescein molecule, so a monovalent enzymatic labeling step was accomplished with anti-FITC-Fab-HRP conjugate. The enzymatic activity of the conjugate immobilized on the SAM architecture is then directly related to the amount of target hybridized and was chronoamperometrically measured. Lower MCH concentrations are not recommended because they cannot effectively displace laid-down CPs (Kuralay et al., 2012). While the lowest MCH concentration yielded the best signal-to blank ratio for a fixed CP

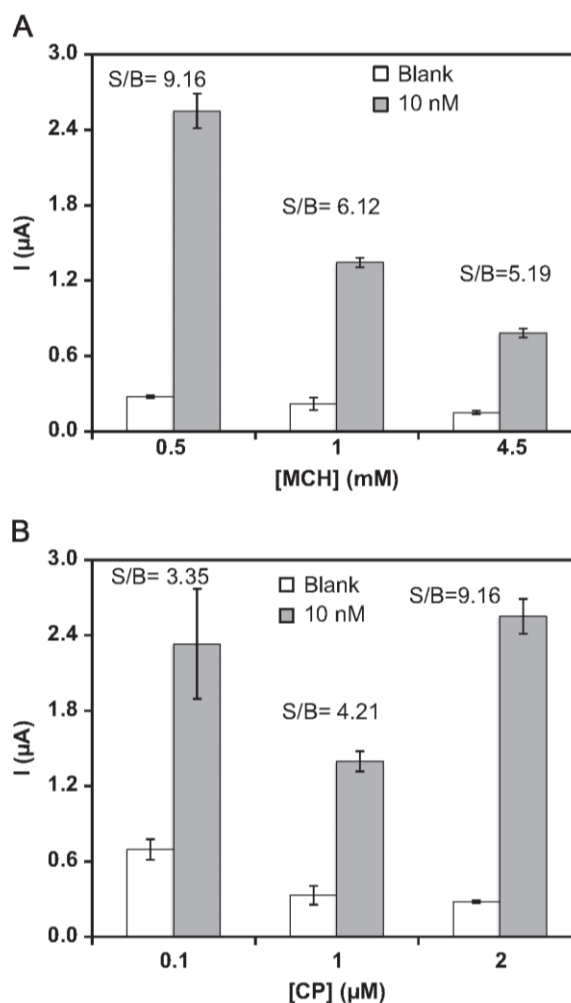


Fig. 2. Effect of MCH (A) and CP (B) concentrations used during the preparation of the sensing phase on analytical signal for the blank (white bars) and 10 nM of 99nt target (gray bars). (A) $[\text{CP}] = 2 \mu\text{M}$ (B) $[\text{MCH}] = 0.5 \text{ mM}$. Signal to blank ratios (S/B) are also indicated for each experiment.

concentration (2 μM) (Fig. 2A), the highest CP concentration provided the highest analytical signals for a fixed MCH concentration of 0.5 mM (Fig. 2B) due to an increase in the number of binding sites. Proper probe spacing is essential for maximizing the hybridization efficiency and minimizing nonspecific adsorption events. The double function of MCH, refilling pinholes and preventing multipoint attachment of thiolated CP molecules through their nucleobases, has contrary effects. Excessive dilution of the CP contributes to low specific currents but also to low background currents due to a better coverage of the Au surface with diluent thiol. In our case the optimal relationship for the sensing phase was found to be 2 μM of CP and 0.5 mM of MCH. These experiments additionally confirm that the use of highly-structured signaling probes and long synthetic targets is feasible by using a sandwich strategy with previous thermal denaturation.

3.2. Analytical performance of genosensor

Under the optimized experimental conditions, the response of the genosensor to increasing concentrations of target was studied in the range from 1 nM to 150 nM. In order to account for electrode-to-electrode variation, the current intensity was normalized and

¹ <http://blast.ncbi.nlm.nih.gov/Blast.cgi> (last accessed 12.01.13).

² <http://quadbase.igib.res.in/> (accessed 27.11.13).

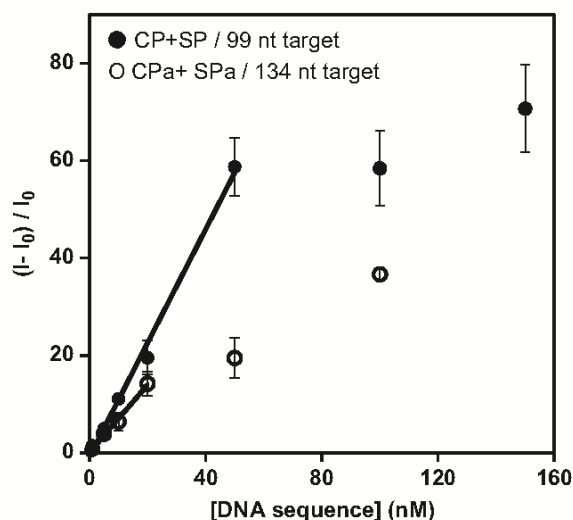


Fig. 3. Variation of the normalized current measured by chronoamperometry at -0.2 V with the concentration of 99nt target using the CP+SP set of probes (●) and 134nt target (○) using the CPa+SPa set of probes under the optimized conditions (see Experimental Section for details).

expressed as $(I - I_0)/I_0$ where I and I_0 denoted the current intensity measured with each target concentration and in the absence of target, respectively. As it can be observed in Fig. 3 filled circles, a linear dependence was obtained up to 50 nM where saturation of the recognition layer was achieved. The corresponding regression equation was $(I - I_0)/I_0 = 1.16 (\pm 0.04) [\text{Target}] (\text{nM}) - 0.6 (\pm 0.8)$ with a correlation coefficient of 0.998. The limit of detection (LOD), calculated as three times the standard deviation of the blank divided by the slope of the regression equation, was estimated to be 0.3 nM. It is worth noting that analytical features are comparable to other similar approaches (Miranda-Castro et al., 2009b, 2007) in spite of using a SP and a target with much stronger secondary structures. This verifies the effectiveness of the denaturing step to favor strongly impeded hybridization without loss in sensitivity. The reproducibility of the proposed sensor was assessed by measuring five replicates of target DNA at 5 and 50 nM. Relative standard deviations (RSD) for both concentrations were 3.5% and 4.3%, respectively, which indicates a satisfactory reproducibility of the designed DNA sensor.

The specificity of the proposed genosensor was evaluated with a fully non-complementary oligonucleotide, and oligonucleotide sequences that encode related cereals proteins such as hordeins and secalins (Shan et al., 2002). These CD-triggering proteins contain important homologies as indicated by *in silico* analysis of the 33-mer amino acid sequence. Non-toxic food grain proteins, such as orzeins (rice), and zeins (maize) and the controversial avenins (in oats), do not contain homologous sequences to the 33-mer from gliadin (Shan et al., 2002).

Intensity currents for the fully non-complementary sequence and hordein DNA are indistinguishable from the blank up to 10 nM within the experimental error (Fig. 4A for 10 nM of each sequence). Partial recognition of the secalin sequence was observed. Alignment of secalin and hordein sequences to gliadin target revealed similar level of homologies; 69/99 for hordein and 67/99 for secalin. Using a two-state melting hybridization modeling (Markham and Zuker, 2005), the structure of the most stable duplex formed between CP+SP and each sequence was calculated (Fig. S2). The duplex with hordein DNA was 14 kcal/mol more stable than that with secalin but none of them can form a complete duplex leaving several single stranded regions at both ends in the case of hordein DNA. According to this the opposite behavior would be expected. However, such

hybridization only would take place if a one-step hybridization were carried out in the presence of the three sequences (SP, CP and the corresponding target). For this reason, heterogeneous and homogeneous hybridization were analyzed separately. Duplexes formed between SP and each sequence still indicates higher stability of the hordein helix. Interestingly, the key information is extracted from the structural analysis (Fig. 4B). Regions involved in hybridization are clearly distinct in both sequences. While the most stable duplex with barley starts at 10 position from the 3' end, leaving a short fragment that cannot hybridize with CP (Fig. 4B, upper panel), the most stable duplex with rye starts at 32 position from the 3' end, leaving this fragment to be captured by CP (Fig. 4B, lower panel). The subsequent heterogeneous hybridization of CP with the remaining 31 nt single-stranded rye fragment would lead to a non-perfect duplex with a fringe region of three unpaired nucleotides (in red in Fig. 4B) just in the junction between CP and SP probes helices. The duplexes depicted in Fig. 4B can satisfactorily explain the experimental results. The heterogeneous hybridization with hordein DNA is not possible under the assay conditions, so signals comparable to blank assays are obtained. In contrast, a duplex with rye DNA can be built on the surface but its stability is poor due to the high number of mismatches. Additionally the presence of a single-stranded region in the middle can contribute to further diminish the analytical signal by providing more flexibility to the duplex allowing the FITC tag to reach the SAM making less efficient the labeling step (Miranda-Castro et al., 2007).

3.3. Detection of genomic wheat DNA

Although low DNA concentrations can be measured using this genosensor, the genomic DNA extracted from wheat consists of 16,000 Mbp (Gill et al., 2004), the length and double stranded nature preclude its direct application to the assay. Consequently, a PCR was designed to shorten and amplify a specific region containing the selected target. The selection of primers was not straightforward given the repetitive nucleotide sequence of the DNA encoding $\alpha 2$ -gliadin, as a result of the high content of proline and glutamine in the immunotoxic fragments of gliadin. Using the primer 3 software,³ a search for the shortest amplicon that includes the 99nt sequence was carried out. Finally, a 134-bp amplicon was selected (Table 1) although three nucleotides of the 99nt target at the very 5' end are not included and amplified. Taking into account the melting temperature of primers, 60 °C of annealing temperature and 3 mM of MgCl_2 were applied as starting PCR conditions. After extraction of genomic DNA from wheat flour with a commercial kit, the PCR amplicons were visualized by gel electrophoresis. Positive amplification was observed but more than one band appeared because of the extension of spurious products (Fig. S3, lane 4). As a control, a pure synthetic 134 bp amplicon was also amplified yielding a single band at the expected size (Fig. S3, lane 7). Sequencing of the three bands obtained for genomic extracts confirmed that only the largest band contained the desired sequence of the $\alpha 2$ -gliadin.

The PCR conditions were then optimized to amplify only the desired sequence. As magnesium ions bind tightly to the phosphate sugar backbone of nucleic acids, variations of the Mg^{2+} concentration below 4 mM can improve the performance of PCR by affecting the specificity (Blanchard et al., 2006). Also, increasing the annealing temperature can enhance discrimination against incorrectly annealed primers and reduce miss-extension of incorrect nucleotides at the 3' end of primers (Innis and Gelfand, 1990). Annealing temperature effect was studied from 60 to 66 °C. To examine the effect of magnesium on PCR yield, its

³ <http://bioinfo.ut.ee/primer3-0.4.0/> (accessed 13.05.13).

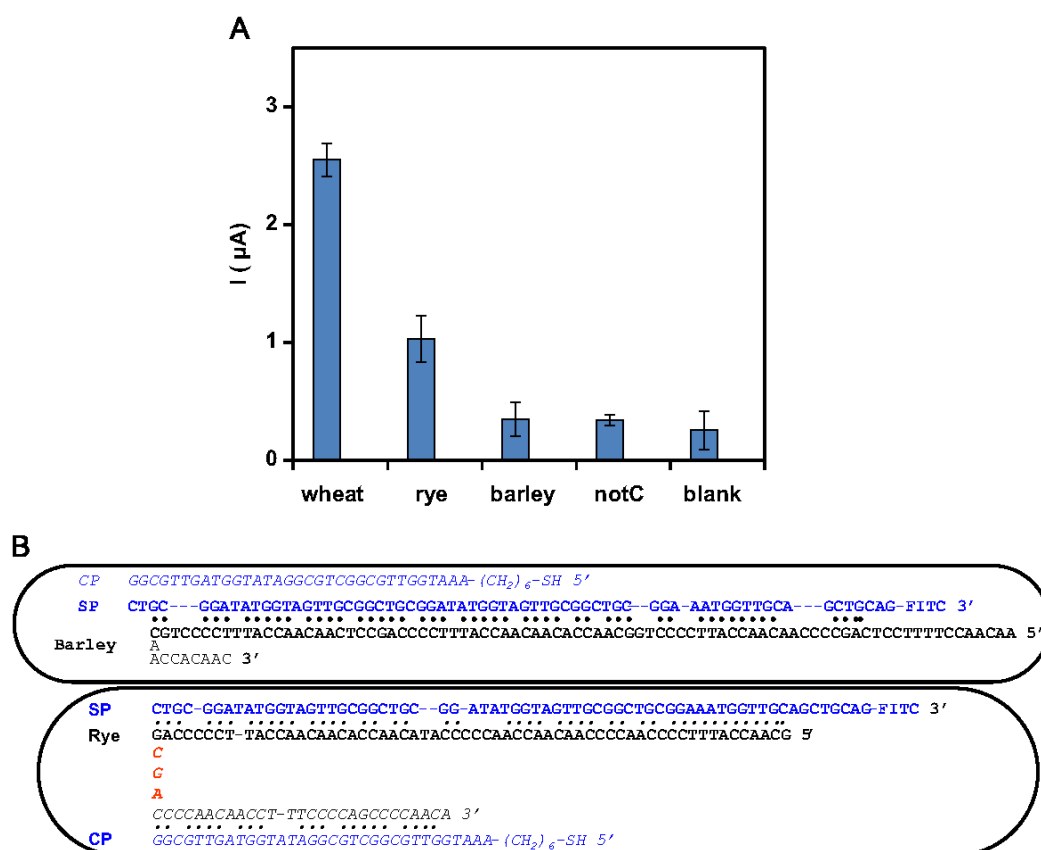


Fig. 4. (A) Intensity currents at a concentration of 10 nM of target (wheat), rye, barley and fully non-complementary (notC) DNA sequences and for the blank experiment using the CP+SP set of probes. Same conditions as in Fig. 3. (B) Base pairing of the thermodynamically most stable duplex formed in two hybridization steps between barley-SP+CP (upper panel) and rye-SP+CP (lower panel) obtained using DINAMelt Web Server. Bold letters indicates the barley or rye regions involved in hybridization with SP. CP and the rye region that hybridizes with it is written in italics. Red letters are used to stand out the fringe region. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration was varied between 1.5 and 3 mM at the two extreme temperatures, 60 °C (Fig. S3, lanes 1–4) and 66 °C (Fig. S3, lanes 8–11) and compared to the positive control (Fig. S3, lane 7). As anticipated, specificity is increased at low levels of MgCl₂ and at high annealing temperatures, reducing the presence of secondary PCR-amplified structures (Fig. S3; lane 8, at 1.5 mM Mg²⁺ and 66 °C).

The specificity of the primer pair to amplify the 134 bp fragment under the optimized conditions (MgCl₂ 1.5 mM and 66 °C for annealing step) was evaluated using genomic DNA extracted from CD-triggering cereals (rye, barley and possibly oat flours) as well as non-related CD grains (soybean and rice). No amplification was observed with any of them even for rye and barley, those containing 99nt target analogs. This result is not unexpected. The high degree of homology among proteins decreases at DNA level because of the degeneration of the genetic code. So, small differences in amino acid sequences among 33-mer analogs multiply in nucleotide sequences, which prevent the primer annealing on the related sequences under these stringent PCR conditions.

Once satisfactory PCR conditions were obtained, wheat amplicons were used without purification as a target of the proposed genosensor. The PCR blank (negative control) was prepared by mixing all PCR reagents except the cereal sample. A serial dilution of the PCR amplified mixture was carried out considering that all DNA comes from the 134 bp amplicon, which is an acceptable assumption after PCR optimization as it is shown below. Chronoamperometric currents obtained with the PCR negative control

were similar to the buffer blank, which demonstrated the absence of any nonspecific interaction of PCR reagents with neither the probes nor the modified electrode at optimized conditions. A dependence between the current intensity measured and the amplicon concentration was observed (Fig. 5A). However, the analytical signals are clearly lower than those obtained with the 99nt synthetic target. The lower efficiency of hybridization with the long amplicon was attributed to steric hindrance due to all extra (134–99) nucleobases, located at the 3' end of the target, that is, at the proximity of the electrode surface when the duplex is formed (Fig. 5B, C). Consequently, the CP recognizes an inner region of the amplicon instead of the terminal region as in the case of the 99nt target. The relative position of the recognition site within the target has been previously shown to affect the magnitude of the electrochemical signal when testing long amplicon (600 bp) (Del Giallo et al., 2005). In that work, a displacement of 21nt towards the inner region of the target accounted for an analytical signal that was one-third of the one obtained when the CP hybridizes at the very end of the target. This factor was not found significant for sequences shorter than 200 bp. Our results clearly indicate that overhangs of 32nt facing the electrode surface from small amplicons (less than 150 bp) also cause steric hindrance and decrease the hybridization efficiency. In addition to this, a short single-stranded fragment of three bases is present in the SP due to the primer design that trimmed the target sequence at the 5' end. The presence of flexible fragments could also make difficult the labeling step reducing further the analytical signal.

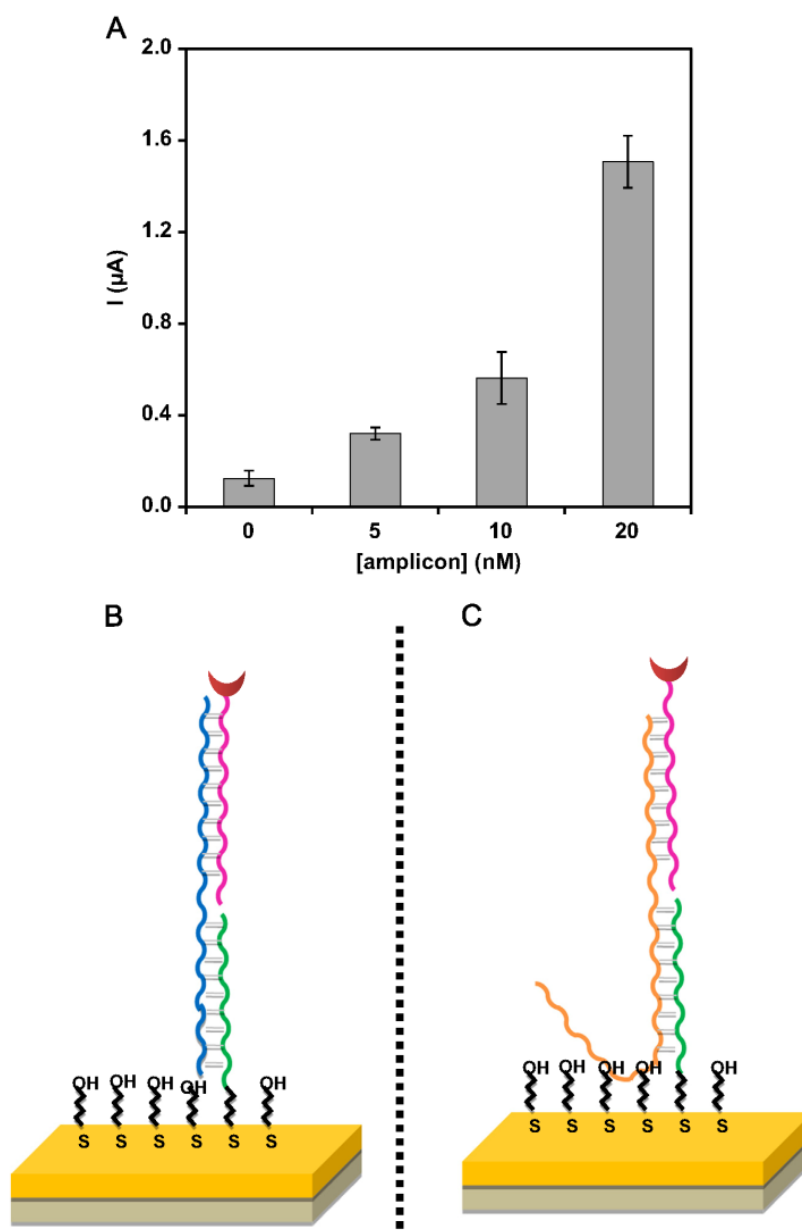


Fig. 5. (A) Variation of the current intensity with concentration of wheat amplified fragments under optimum PCR conditions without further purification using the CP+SP set of probes. Schematic representation of the duplex formed (B) with the synthetic target and (C) with the PCR amplicon on the electrode surface modified with CP.

To verify our hypothesis, the CP and SP probes were redesigned to form a perfect duplex with the aim of maximizing the analytical features of the sensor in presence of the long amplicon. Capture probe was extended at its 5' end to form a perfect duplex with the sequence of the amplicon (CPa), and signaling probe was cut down at its 3' end to remove non-hybridized oligonucleotides (SPa) (Table 1, extended region in CPa is underlined). Although the new CPa contains 71nt, the stability of the secondary structure in terms of Gibbs energy is kept at a safe level (-4.28 kcal/mol). The shortened SPa has a less strong secondary structure than the previous one (-9.74 kcal/mol). The extension of the target, however, did not imply a stronger structure (-11.59 kcal/mol), so this set of sequences are overall thermodynamically more favorable than the initial one. The structures are depicted in Fig. S1.

A synthetic 134 bp amplicon at 10 nM was assayed both with the redesigned set of probes and the previous one. The current intensity measured with the new set that forms a perfect duplex is much higher ($2.65 \mu\text{A}$) than that obtained with the set that leaves overhangs ($0.64 \mu\text{A}$). This result confirms the deleterious effect of single stranded fragments on the electrode side even when using short amplicons. It is worth noting that using the CP+SP set of probes the signal obtained with the synthetic amplicon is identical, within the experimental error, to that obtained with PCR mixture at the same concentration (Fig. 5A), which confirms that our hypothesis that all DNA is from the amplicon is reasonable.

The analytical signal was linearly dependent on the concentration of the 134 bp synthetic amplicon between 1 and 20 nM (Fig. 3, open circles) with the following equation: $(I - I_0)/I_0 = 0.69 (\pm 0.03) [134 \text{ bp amplicon}] (\text{nM}) + 0.05 (\pm 0.3)$, $r = 0.992$. Above this

concentration, the current levels off because of the saturation of the binding sites. The limit of detection was 0.2 nM, slightly lower than that estimated using shorter target. When testing PCR amplicons with the new set of probes, both blanks and signals were higher but similar signal to blank ratios were obtained. This indicates that purification of PCR products is not needed with our sensing phase.

Recently a 326 bp amplicon was successfully electrochemically detected using Ru complexes bound by electrostatic attraction. The benefit of designing a CP directed to the very end of the amplicon (the primer region) is recognized to keep the low limit of detection when shifting from synthetic to amplified DNA targets. The approach could also take advantage of the larger number of negative charges when using long sequences that permits higher accumulation of the redox probe (García-Mendiola et al., 2013). Comparatively, our approach has similar detectability without the need for surface nanostructuring or asymmetric PCR.

4. Conclusions

From results shown in this work, it is apparent that genosensors for detecting complicated DNA sequences are feasible upon a careful design of the DNA probes involved. In this context complicated means highly structured DNA sequences belonging to genes with a great number of repetitive fragments, which strongly hampered the search for a specific PCR. A sandwich assay format is advantageous because it permits to split the complementary probe into two DNA fragments of variable length. The selection of the capture and signaling probes, that is, the cutting point chosen in the complementary probe, is far from trivial. Two requirements were imposed and shown to be successful: minimization of the thermodynamic stability of secondary structures and preference of higher stability on the signaling probe versus the capture probe. Even with Gibbs energies as high as -13 kcal/mol of the SP and the potential formation of G-quartets the hybridization could be accomplished using a previous denaturation step, although a penalty in reaction time was paid. The construction of perfect duplex without fringe single-stranded regions and/or overhangs especially at the end adjacent to the electrode surface is essential for adequate performance of the genosensor. This holds true when analyzing real amplicons that are usually longer than the initial targets. The position of the recognition site within the amplicon is decisive to maximize hybridization efficiency and so, the analytical signal, even with short amplicons. Consequently, redesign of the original probes to fulfill all the requirements is recommended. In this work, a highly specific DNA fragment of gliadin protein was detected. The repetitive characteristics of the immunotoxic peptides added a problem to design a specific PCR. Under the optimized conditions the desired amplicon was preferentially amplified but not exclusively. Nonetheless, the selectivity of the genosensor developed allowed the detection of this fragment without the need for purification of both DNA spurious products and PCR reagents. The successful detection of DNA extracted from flour samples verifies the suitability of the described PCR-coupled genosensor to complement the immunochemical methods to detect gluten for CD patients' safety.

Acknowledgments

B.M.F. thanks to Universidad Complutense for a predoctoral grant and N.S.A, thanks to Spanish Government for a Ramón y Cajal contract. Authors also thank Dr. José Luis Martínez Fernández for his advice in PCR optimization. This work has been cofinanced by Project CTQ2008-02429/BQU granted to "Grupos Consolidados" and the European Regional Development Fund.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2014.04.033>.

References

- Blanchard, M.M., Taillon-Miller, P., Nowotny, P., Nowotny, V., 2006. *PCR Meth. Appl.* 2, 234–240.
- Campuzano, S., Kuralay, F., Lobo-Castañón, M.J., Bartosik, M., Vyavahare, K., Palecek, E., Haake, D.A., Wang, J., 2011. *Biosens. Bioelectron.* 26, 3577–3583.
- Dahinden, I., von Buren, M., Luthy, J., 2001. *Eur. Food Res. Technol.* 212, 228–233.
- Del Giallo, M.L., Lucarelli, F., Cosulich, E., Pitarino, E., Santamaria, B., Marrazza, G., Mascini, M., 2005. *Anal. Chem.* 77, 6324–6330.
- García-Mendiola, T., Gamero, M., Campuzano, S., Revenga-Parra, M., Alonso, C., Pedrero, M., Pariente, F., Pingarrón, J.M., Lorenzo, E., 2013. *Anal. Chim. Acta* 788, 141–147.
- Gill, B.S., Appels, R., Botha-Oberholster, A.-M., C.R., B., Bennetzen, J.L., Chalhou, B., Chumley, F.J.D., Iwanaga, M., Keller, B., Li, W., McCombie, W.R., Ogihara, Y., Quetier, F., Sasaki, T., 2004. *Genetics* 168, 1087–1096.
- González-Álvarez, M.J., Pérez-Ruiz, E., Miranda-Castro, R., de-los-Santos-Álvarez, N., Miranda-Ordieres, A.J., Lobo-Castañón, M.J., 2013. *Electroanalysis* 25, 147–153.
- Gonzalez-Fernandez, E., de-los-Santos-Alvarez, N., Miranda-Ordieres, A.J., Lobo-Castanon, M.J., 2013. *Sens. Actuat. B—Chem.* 182, 668–674.
- Hillemann, D., Weizenegger, M., Kubica, T., Richter, E., Niemann, S., 2005. *J. Clin. Microbiol.* 43, 3699–3703.
- Innis, M.A., Gelfand, D.H. (Eds.), 1990. *PCR Protocols, A Guide to Methods and Applications*, First ed. Academic Press, Inc., San Diego.
- Kjallman, T.H.M., Peng, H., Soeller, C., Travas-Sejdic, J., 2008. *Anal. Chem.* 80, 9460–9466.
- Koppelman, S.J., Hefle, S.L. (Eds.), 2006. *Detecting Allergens in Food*, First ed. Woodhead Publishing Ltd and CRC Press LLC, Boca Raton, FL (USA).
- Kuralay, F., Campuzano, S., Wang, J., 2012. *Talanta* 99, 155–160.
- Lucarelli, F., Tombelli, S., Minunni, M., Marrazza, G., Mascini, M., 2008. *Anal. Chim. Acta* 609, 139–159.
- Mao, X., Ma, Y.Q., Zhang, A.G., Zhang, L.R., Zeng, L.W., Liu, G.D., 2009. *Anal. Chem.* 81, 1660–1668.
- Markham, N.R., Zuker, M., 2005. *Nucleic Acids Res.* 33, W577–W581.
- Mir, K.U., Southern, E.M., 1999. *Nat. Biotechnol.* 17, 788–792.
- Miranda-Castro, R., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2009a. *Electroanalysis* 21, 2077–2090.
- Miranda-Castro, R., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2009b. *Biosens. Bioelectron.* 24, 2390–2396.
- Miranda-Castro, R., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2007. *Anal. Chem.* 79, 4050–4055.
- Olexova, L., Dovicovicova, L., Svec, M., Siekel, P., Kuchta, T., 2006. *Food Control* 17, 234–237.
- Peterson, A.W., Heaton, R.J., Georgiadis, R.M., 2001. *Nucleic Acids Res.* 29, 5163–5168.
- Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M., Khosla, C., 2002. *Science* 297, 2275–2279.
- Shan, L., Qiao, S.W., Arentz-Hansen, H., Molberg, O., Gray, G.M., Sollid, L.M., Khosla, C., 2005. *J. Proteome Res.* 4, 1732–1741.
- Shchepinov, M.S., Udalova, I.A., Bridgman, A.J., Southern, E.M., 1997. *Nucleic Acids Res.* 25, 4447–4454.
- Southern, E., Mir, K., Shchepinov, M., 1999. *Nat. Genet.* 21, 5–9.
- Watterson, J.H., Piunno, P.A.E., Wust, C.C., Krull, U.J., 2000. *Langmuir* 16, 4984–4992.
- Wu, J., Campuzano, S., Halford, C., Haake, D.A., Wang, J., 2010. *Anal. Chem.* 82, 8830–8837.
- Zeltner, D., Glomb, M., Maede, D., 2009. *Eur. Food Res. Technol.* 228, 321–330.
- Zuker, M., 2003. *Nucleic Acids Res.* 31, 3406–3415.

**STRONGLY STRUCTURED DNA SEQUENCES AS TARGETS FOR
GENOSENSING: SENSING PHASE DESIGN AND COUPLING TO PCR
AMPLIFICATION FOR A HIGHLY SPECIFIC 33-MER GLIADIN DNA FRAGMENT**

Begoña Martín-Fernández^{a,b}, Arturo J. Miranda-Ordieres^b, María Jesús. Lobo-Castañón^b, Gloria Frutos-Cabanillas^a, Noemí de-los-Santos-Álvarez^{*b}, Beatriz López-Ruiz^{*a}

Supplementary information: Structural analysis obtained from The mfold Web Server and gel electrophoresis of PCR amplicons.

Figure S1: Most stable structures of 99-nt target, 33-nt capture probe (CP) and 66nt-signalling probe (SP) (upper panel) and 134-nt amplicon, 71-nt capture probe (CPa) and 51-nt signaling probe (SPa).

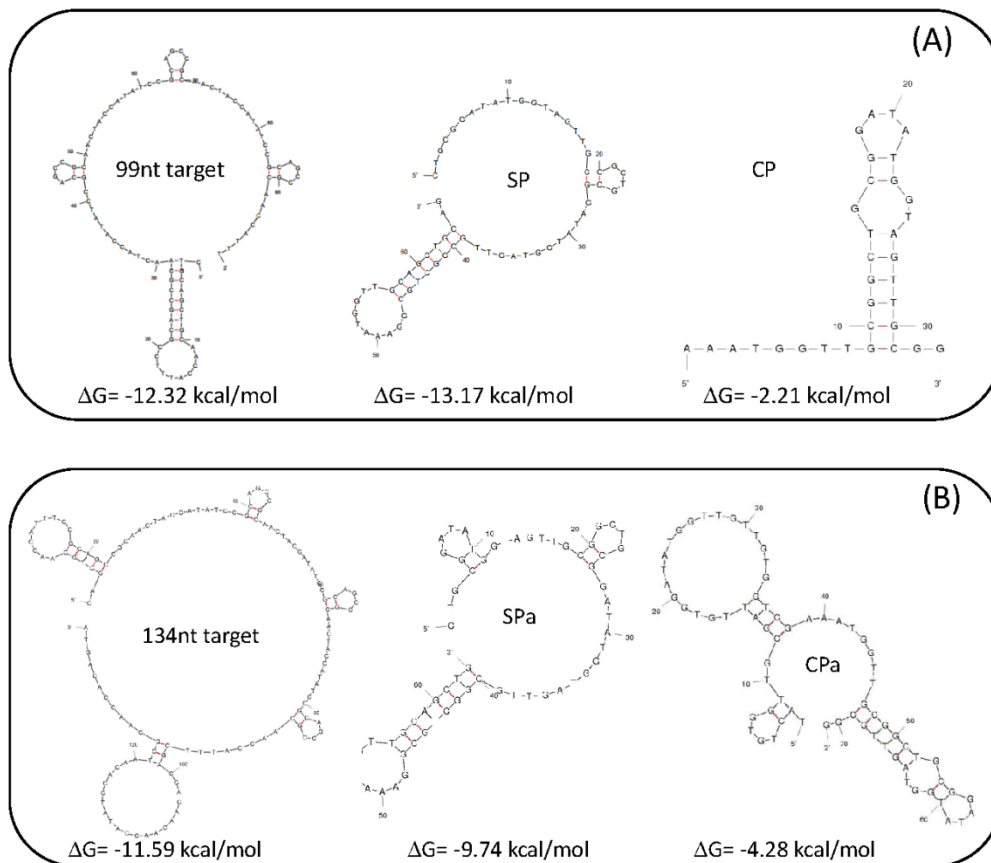


Figure S2: Most stable base pairing of one-step hybridization between CP+SP and barley (A) or rye (B) DNA sequences.

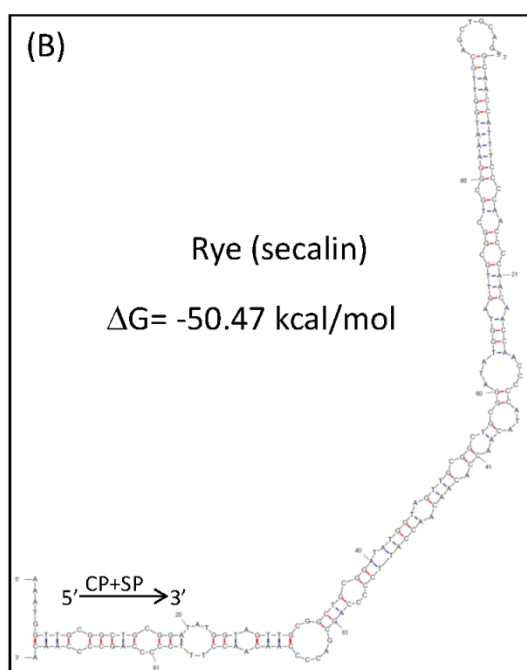
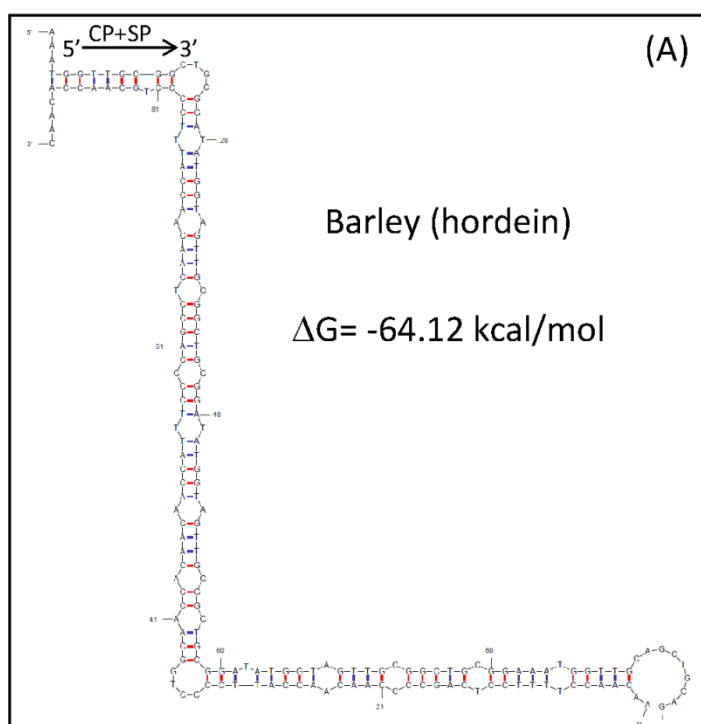
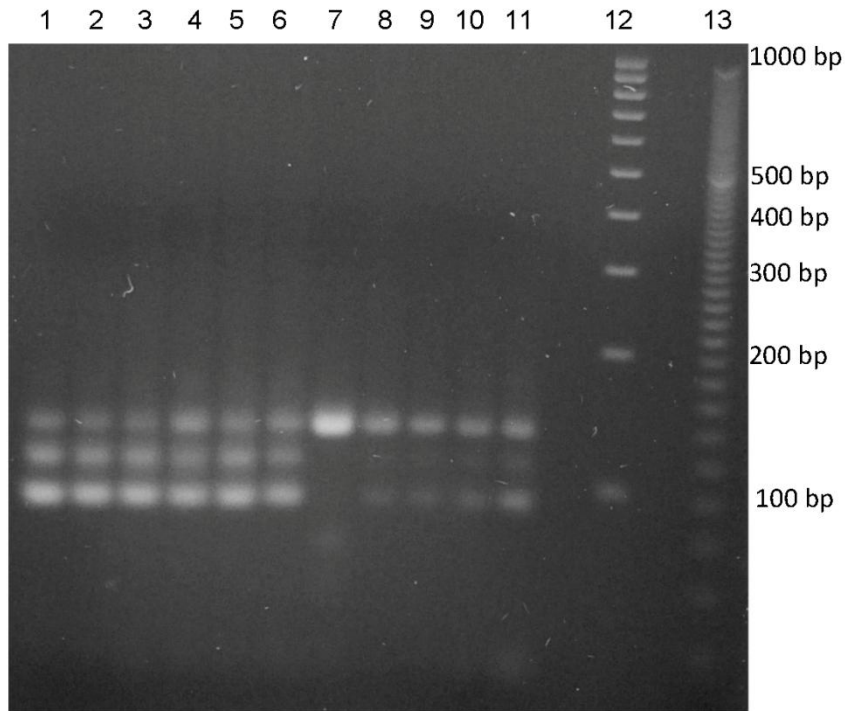


Figure S3. Effect of PCR annealing temperature and [MgCl₂] on the amplification of wheat genomic DNA revealed at agarose 2% ethidium bromide gel electrophoresis. Lanes 1-4, 60 °C and 1.5 mM, 2 mM, 2.5 mM and 3 mM of MgCl₂, respectively; lane 5, 62 °C and 3 mM; lane 6, 64 °C and 3 mM; lane 7, synthetic amplicon 60 °C and 3 mM as a positive control; lanes 8-11, 66 °C and 1.5 mM, 2 mM, 2.5 mM and 3 mM of MgCl₂, respectively; lane 12, 100 bp size ladder; lane 13, 20 bp size ladder.



4.2 Acoplamiento del genosensor con PCR para el análisis de muestras alimentarias procesadas

Si bien se han descrito infinidad de genosensores electroquímicos para la detección de diversos analitos en muestras alimentarias, la cuantificación de alérgenos es una tarea difícil que pocas veces se hay llevado a cabo con estos dispositivos. En la mayor parte de los casos, los resultados obtenidos con estos dispositivos se expresan en en términos de concentración de ADN, que carecen de interés para los consumidores alérgicos. Por este motivo los métodos basados en ADN (PCR y genosensores) han quedado relegados a simples herramientas cualitativas, únicamente capaces de proporcionar un resultado positivo o negativo y, las pocas veces que se ha alcanzado la cuantificación de las muestras, los datos no se han podido transformar a unidades que realmente tengan sentido para los consumidores o que permitan verificar el cumplimiento con la legislación vigente.

Teniendo en cuenta estas limitaciones, la siguiente fase de este trabajo de investigación fue dirigida a analizar y cuantificar muestras reales. Con este objetivo, se estudió a fondo el acoplamiento del genosensor a una etapa de amplificación por PCR previa, para maximizar la selectividad del dispositivo sin la pérdida de su capacidad cuantitativa. Por primera vez se consiguió obtener una calibración con un genosensor que dio lugar a la cuantificación del gluten en alimentos, incluso en aquellos que presentan mayores problemas durante el análisis: los altamente procesados térmica y enzimáticamente, los malteados y los que contienen chocolate. Además, los resultados obtenidos se correlacionaron adecuadamente con los obtenidos con el inmunoensayo considerado oficial, destacando la idoneidad de los genosensores como una herramienta de monitorización asequible y simple.

(submitted)



Challenging genosensors in food samples: the case of gluten determination

Begoña Martín-Fernández^a, Noemí de-los-Santos-Álvarez^b, Juan Pedro Martín-Clemente^c, María Jesús Lobo-Castañón^b, Beatriz López-Ruiz^a

^aSección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pz. Ramón y Cajal s/n, 28040 Madrid, Spain. E-mails: begonamartin@ucm.es (B.M.F), bealopru@quim.ucm.es (B.L.R)

^bDepartamento de Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain. E-mails: santosnoemi@uniovi.es (N.S.A), mjlc@uniovi.es (M.J.L)

^cDepartamento de Biotecnología - Biología Vegetal, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain. E-mail: juanpedro.martin@upm.es (J.P.M.C.)

ABSTRACT

Electrochemical genosensors have undergone an enormous development the last decades, but only very few have achieved a quantification of target content in highly processed food samples. The detection of allergens, and particularly gluten, is challenging because legislation establishes a threshold of 20 ppm for labelling as gluten-free but most genosensors expresses the results in DNA concentration or DNA copies. This paper describes the first attempt to correlate the genosensor response and the wheat content in real samples, even in the case of highly processed food samples. Binary model mixtures, as a reference material, and real samples have been analyzed. DNA from food was extracted and a fragment encoding the immunodominant peptide of α 2-gliadin amplified by a tailored PCR. The sensor was able to selectively detect toxic cereals for celiac patients, such as different varieties of wheat, barley, rye and oats, from non-toxic plants. As low as 0.001% (10 mg/kg) of wheat flour in an inert matrix was reliably detected, which directly compete with the current method of choice for DNA detection, the real-time PCR. A good correlation with the official immunoassay was found in highly processed food samples.

Keywords: gluten quantification, wheat, celiac disease, highly processed food, electrochemical genosensor, disposable electrodes

1. INTRODUCTION

Celiac disease is an autoimmune disorder of the small intestine that occurs in genetically predisposed people. The inflammatory response is triggered by the gluten fraction, present in wheat, a staple food which belongs to the Poaceae family [1], but also in closely phylogenetically related cereals such as rye and barley [2], which have a common ancestral origin in the grass family [3]. Oats, more distantly related to wheat, could also trigger the typical symptoms, although its harmful role is actually a term of debate and celiac patients are advised to avoid its consumption [4, 5]. It is also possible to find gluten in the durum wheat, primarily used in the manufacture of pasta products, and in more ancient and less frequently consumed species, such as spelt, whose market is booming with the increasing demand of organic and natural products. Considering their harmful effect in celiac patients, the complete avoidance of gluten-containing cereals in their diet is desirable [6], but extremely difficult. For this reason, the term “gluten-free” is presently applicable to food products containing less than 20 mg/kg of gluten [7], as a tradeoff between the concentration reliably detected by the current analytical methodology and the safety of most celiac individuals.

Despite the legislation, no specific analytical methodology has been recommended for gluten monitoring yet. Most employed methods are based on enzyme linked immunosorbent assays directed to different gluten protein fractions [8, 9]. Recently, aptamer based approaches have appeared with increased sensitivity [10].

Due to the high stability of DNA molecules compared to proteins, methods relying on DNA have also attracted great attention. Several articles have appeared in the last two decades to quantify gluten content in foodstuff based on the real-time polymerase chain reaction (PCR) [11-16]. Despite the sensitivity and fast-analysis of real-time PCR methods in gluten analysis, the need for expensive, complex and delicate optical instruments precludes its wide implementation in medium and small lab facilities as those available in food industry. Aiming at developing a portable, cheap and simple technology for DNA analysis, efforts have been done to replace the optical detection by non-optical techniques, which are more robust, less expensive and easier to miniaturize.

As alternative systems to detect DNA, electrochemical genosensors have arisen. They rely on a hybridization recognition reaction between two DNA complementary strands: the target and a recognition element, called probe. These devices use an electrode-based platform as transducer and, in order to convert this highly specific event into a measurable signal, a reporter molecule is usually incorporated, e.g. redox enzymes. After more than a decade of development, few electrochemical genosensors have been challenged to real food samples with quantitation purposes [17]. Analyzing DNA in food samples

requires a mandatory pretreatment, which typically includes extraction of DNA, amplification, usually by PCR, and a subsequent post-PCR purification step [18]. In addition to this, the usual way of expressing the result is in DNA concentration units, which is meaningless for allergic consumers, and for verifying the compliance with the legislation.

In this paper, the challenge of facing an electrochemical genosensor to food samples is first addressed. The analytical performance under realistic conditions is evaluated in term of wheat percentage from which a correlation with legal threshold can be obtained to allow the verification of the labeling. Detection of other toxic cereals for the determination of total gluten content was also evaluated along with cross-reactivity against non-toxic rice and soy. Finally, processed food samples, with a wide range of expected gluten content were analyzed and validated against the Codex recommended method [19].

2. EXPERIMENTAL

2.1. Reagents

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H₂O₂) and saline sodium phosphate-EDTA (20×SSPE pH 7.4) were purchased from Sigma-Aldrich (Spain). 1% Casein buffer in 1×PBS was obtained from Fischer Scientific (Spain). Anti-fluorescein-HRP Fab fragments (antiFITC-HRP) were received from Roche Diagnostics GmbH (Mannheim, Germany). Ethanol was purchased from Panreac (Spain). NAP-10 columns of Sephadex G-25 were obtained from Life Technologies (Spain). Water was purified with a Milli-Q system (Millipore, Spain).

Oligonucleotide sequences used are shown in Table 1. Oligomers were obtained as lyophilized desalted salts from Sigma-Life Sciences. These sequences were selected to detect a sequence of 134-nt that encodes a highly toxic protein from wheat for celiac patients, the α 2-gliadin, Gli [20], a multi-copy gene, which could provide a better performance in terms of sensitivity, compared to single-copy genes. Two probes, CP-Gli and SP-Gli, which hybridize with the entire target sequence were previously designed [21]. To ensure the quality of the extracted DNA for PCR amplification, the nuclear 18S rRNA gene was amplified as a universal control [22]. All stock solutions were prepared in MilliQ water and all reagents were used without any treatment except for CP-Gli that was unprotected as indicated elsewhere [21].

Table 1. Target, probes and primers sequences used.

Sequence name	Length	Oligonucleotide sequences 5' → 3'
Signaling probe (SP-Gli)	63 nt	CTG CGG ATA TGG TAG TTG CGG CTG CGG ATA TGG TAG TTG CGG CTG CGG AAA TGG TTG CAG CTG -FITC
Capture probe (CP-Gli)	71 nt	HS-(CH ₂) ₆ - TAC TGT GGT TGC GAT TGT GGA TAT GGT TGT TGT GGT CGA AAT GGT TGC GGC TGC GGA TAT GGT AGT TGC GG
Gli forward primer (Gli-F)	21 nt	CAG CTG CAA CCA TTT CCG CAG
Gli reverse primer (Gli-R)	23 nt	TAC TGT GGT TGC GAT TGT GGA TA

2.2. Instrumentation

Electrochemical measurements were carried out with screen-printed gold electrodes (SPEAu, DropSens-220BT, Spain), connected to a PGSTAT101 potentiostat with NOVA 1.9 software (EcoChemie, The Netherlands). The layout of the disposable planar screen-printed gold electrodes includes three electrodes in the same alumina sheet: a working gold electrode ($\varnothing \sim 4$ mm), an Ag pseudo-reference electrode and a gold counter electrode, all of them screen-printed on a ceramic substrate. The PCR reactions were performed in an Eppendorf Mastercycler thermocycler (Eppendorf, Hamburg, Germany).

2.3. Sample preparation

Wheat flour was mixed with rice flour to obtain different percentages of wheat from 50% down to 0.0001%. The most concentrated mixture (50%) was prepared by mixing 50 g of rice flour with 50 g of wheat flour. The rest of mixtures were prepared by successive dilution with rice flour to obtain a final amount of 100 g in separated recipients ensuring that no carry-over DNA was present from previous experiments by using a mixture of bleach and detergent and hot water, and thoroughly homogenized in a mixer.

Flours from different plants (bread wheat, durum wheat, spelt, barley, rye, oat, soybean and rice) were obtained at local markets. To minimize cross-contaminations among samples, the preparation of cereals, food samples and reference mixtures were carried out on different days and immediately stored at -20°C until its use for DNA extraction.

2.4. DNA extraction

DNA was extracted from 200 mg of sample using the Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations [16]. UV spectroscopy measurements (UV260 Shimadzu, Japan) were used to control the yield and purity of extracts (260/280

nm ratio around 1.8) and to estimate the DNA concentration extracted from which the working solution (50 ng/ μ L) was prepared.

2.5. PCR amplification conditions

Unless otherwise indicated, the end-point PCR amplifications were carried out in 25 μ L of total reaction volume containing 100 ng of DNA (2 μ L), 200 μ M of each dNTP, 200 nM of each Gli primer (Gli-F/Gli-R), 1.5 mM of $MgCl_2$, 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% Tween 20, and 1.0 U SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany). Amplification conditions were: initial step at 95 $^{\circ}C$ for 10 min; 40 cycles at 95 $^{\circ}C$ for 45 s, 67 $^{\circ}C$ for 30 s and 72 $^{\circ}C$ for 30 s. A final extension at 72 $^{\circ}C$ for 5 min was also carried out. PCR amplifications targeting the universal region (18S rRNA) were performed as described by Martín-Fernández et al. [16].

This protocol generated amplified fragments that were analyzed in a 1.5% (w/v) agarose gel run in 1 \times SGTB buffer (GRISP, Porto, Portugal) at 150 V for 20 min using a 100 bp ladder (Sigma-Aldrich) as a molecular size marker. The amplicons were visualized with 1 \times Gel Red (Biotium, CA, USA) a digital image was obtained using a Kodak Digital ScienceTM (Rochester, NY, USA).

2.6. Sensing phase preparation and genosensor assay

The genosensor was prepared on SPEAu previously washed with ethanol and water, and dried with nitrogen. After conditioning, a binary self-assembled monolayer composed of the CP-Gli and MCH was prepared as described elsewhere [21].

Hybridization experiments were carried out in a sandwich-like format to avoid deleterious effects associated to the strong secondary structure of the long target containing a significant percentage of C/G bases [21]. First, amplicons were added to a hybridization buffer (2 \times SSPE) solution containing 1.25 μ M of the SP-Gli, heated at 98 $^{\circ}C$ for 5 min and cooled down in ice-water bath for 5 min. After bringing the mixture to room temperature (30 min), 15 μ L of this solution was placed onto the sensing phase for 2 h. Afterwards, the working electrode surface was washed and covered with 1 \times PBS solution containing 1% (w/v) casein and 0.05% (w/v) Tween 20 pH 7.4 (blocking buffer). Then 15 μ L of a solution of the anti-FITC Fab-HRP conjugate in the blocking buffer (0.5 U/mL) was added to the electrode surface for 30 min. After a washing step with blocking buffer and then with 2 \times SSPE, the entire cell was covered with 40 μ L of TMB and after 1 min of reaction, the enzymatically oxidized TMB was reduced at -0.2 V recording the current during 60 s.

3. RESULTS AND DISCUSSION

The α 2-gliadin is an immunotoxic wheat protein for celiac patients that contain a high percentage of prolines and glutamines, so its sequence is highly repetitive. This fact imposes certain constraints when designing a suitable genosensor targeting a fragment of this protein, the 33-mer immunodominant peptide [21]. Nevertheless, its use is advantageous over other DNA fragments codifying for other allergenic proteins because it is present in several copies in the wheat genome. Since DNA directly extracted can not be analyzed with genosensor due to the length and double stranded nature, a step of length restriction is currently unavoidable. This is usually assessed by PCR amplification because not only shortens the genome but also amplifies the fragment of interest. In highly repetitive genes, careful design of this step is critical for the analytical performance of the genosensor.

End-point PCR was optimized using different wheat species, all of them toxic in a greater or lesser extent for celiac patients; specifically, common wheat (*Triticum aestivum* L.), durum wheat (*T. durum* L.) and spelt wheat (*T. spelta* L.). The annealing temperature and $MgCl_2$ concentration were varied (62 - 70°C and 1.5 - 2.5 mM, respectively) in order to amplify only the desired sequence, and obtain the best sensitivity of the assay. To verify the absence of false positive results, all amplifications were carried out using a negative control or blank (wherein water was used instead of template DNA).

Gli primers amplified several bands at high concentrations of $MgCl_2$ and low annealing temperatures, being the second one at about 130 bp size the fragment of interest, so restrictive conditions were necessary (Fig. 1 A). DNA polymerase activity is dependent on the presence of free divalent cations, such as $MgCl_2$. On one hand, an excessive concentration of this ion can lead to non-specific amplification as polymerization activity increases but specificity can fall. On the other hand, lower concentrations reduce polymerization but increase accuracy [23, 24]. To increase the selectivity lower concentrations of $MgCl_2$ (1.5 mM) and higher annealing temperature were needed. Although the best discrimination was achieved at 68 °C, the gain in sensitivity achieved at lower temperature, 67 °C (Fig. 1 B) was relevant and therefore this temperature was selected for further experiments with the genosensor, that is expected to provide extra selectivity.

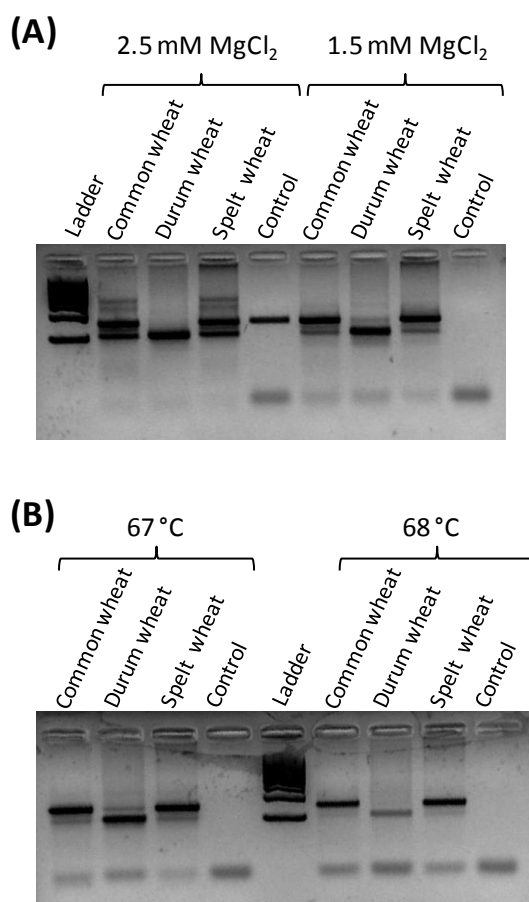


Figure 1. Profiles obtained on 1.5 % agarose gel electrophoresis of PCR amplicons of different wheat species (common, durum and spelt) obtained using Gli primers under selected conditions: (A) varying MgCl₂ concentrations (1.5 and 2.5 mM) at 66 °C of annealing temperature; (B) varying annealing temperature (67 and 68 °C) at 1.5 mM MgCl₂.

3.1. Detection of DNA from pure cereal flours

Using the designed PCR, 100 ng of genomic DNA from wheat were PCR amplified and 10 µL of the amplification product were directly analyzed with the genosensor, according to the protocol described in the experimental section. A current intensity of about 12.6 µA was obtained, but the blank (no DNA) was very high (about 1 µA) in comparison with a blank obtained in the absence of PCR mix (about 0.3 µA). This indicates a high nonspecific contribution to the current due to the adsorption of primers, polymerase enzyme or buffer components of PCR reaction to the genosensor surface [25]. Therefore, prior to detection with the genosensor, a sample dilution was performed at different ratios. As expected, the dilution of all reagents coming from the amplification reaction resulted in a significant decrease in the current intensity of the blank, which remained low for all dilutions ratios compared with undiluted blank sample (Fig. 2). The dilution of the target did not cause the decrease of the

signal as much as in the case of blank sample. This verifies the effectiveness of the dilution step to favor the detection of amplified fragments with a slightly reduction of sensitivity. Considering that the most favorable ratio was obtained for the lower dilution (1:5), this was selected for real samples analysis.

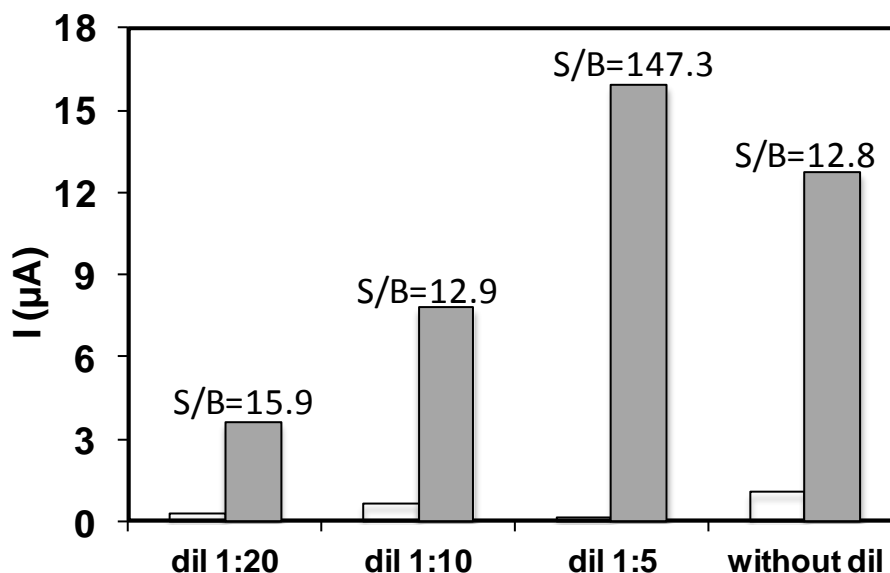


Figure 2. Variation of the current intensity obtained in the absence (blank, white bars) and in the presence of 100 ng of wheat DNA (black and grey bars, respectively) with the dilution of the amplified sample. The signal/blank ratio (S/B) is also indicated.

Finally, the influence of the number of PCR cycles on the electrochemical signal was studied. Serial dilutions of genomic DNA of common wheat were amplified by PCR and measured using the electrochemical genosensor. The current intensity values were plotted versus the initial genomic amount of wheat DNA and the results are presented in Fig. 3A. After 40 cycles of PCR-amplification, the continuous increase in current intensity with the initial DNA amount is leveled off at about 10 ng while an increase at slower pace is observed after 39 cycles so a plateau is not even achieved at the highest DNA amount tested. Interestingly, the lowest detectable amount of DNA was 15 pg and 420 pg after 40 and 39 cycles, respectively calculated as the DNA amount corresponding to three times the blank signal. These results match those obtained by gel electrophoresis (Fig. 3B), which suggests that the saturation of the analytical signal is due to depletion of the DNA amplification. The number of amplified DNA copies can be estimated according to the genome size of wheat (17.33 pg) (<http://data.kew.org/cvalues/>). On the basis of a single copy, as low as 0.86 genomic copies of wheat can be detected with this method. Considering

that the estimated copy number ranges from 25 to 150 [26, 27], the number of amplified copies might varies from 22 to 129.

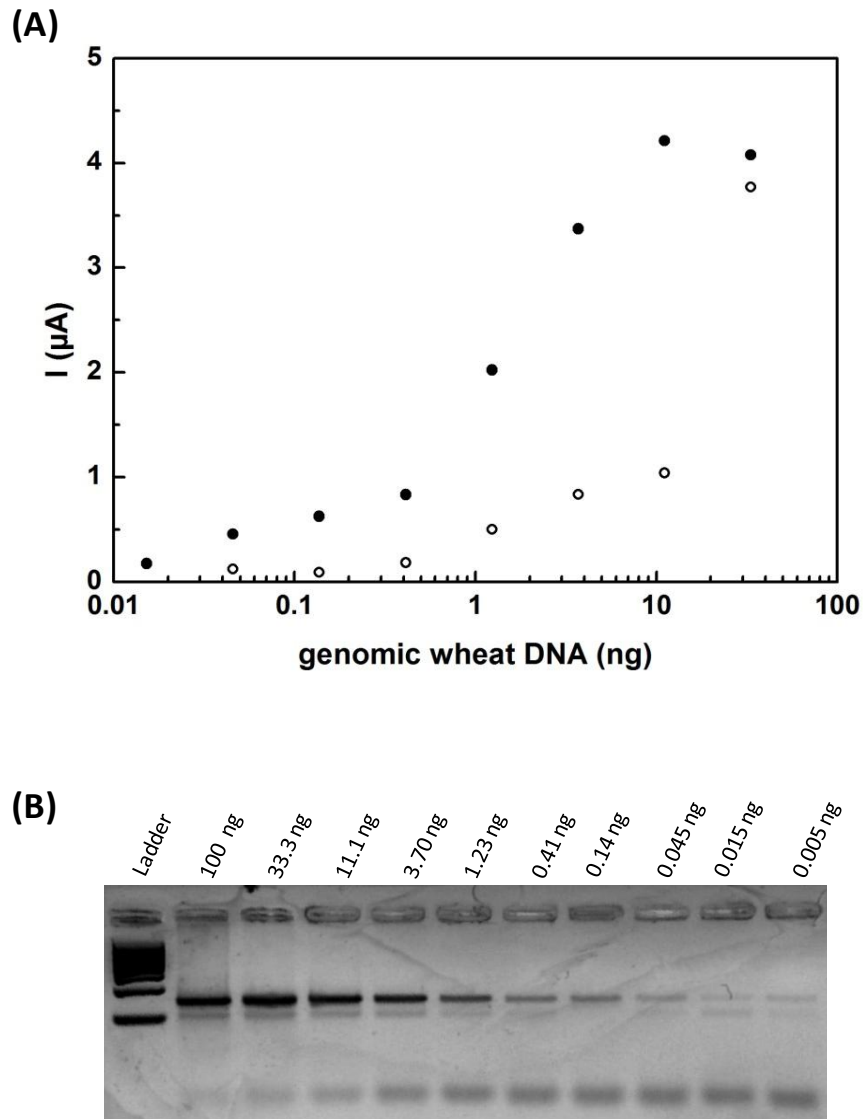


Figure 3. (A) Electrochemical detection of amplified products obtained varying the amount of genomic common wheat DNA with (o) 39 or (●) 40 cycles of PCR. (B) Profiles obtained on 1.5 % agarose gel electrophoresis of PCR amplicons from different starting amounts of wheat DNA for 40-cycles of PCR amplification.

Amplified DNA extracts of species that trigger celiac disease symptoms, namely, common wheat, durum wheat, spelt wheat, barley, rye and oats, and two safe species, soy and rice, were diluted according to the optimized protocol and analyzed with genosensor. Using 100 ng of genomic DNA of each sample, a positive response for all studied wheat species was observed (Fig. 4A). Higher current intensities are measured in the case of common wheat compared to durum and spelt wheat. These results are in accordance to the lower toxicity of the two latter wheat species for celiac patients. The sensor also responded positively towards closely related cereals, such as barley, rye and oats (Fig. 4B). On the contrary, no false positive results were obtained from non-toxic widely consumed species, such as rice and soy as expected. The electrochemical hybridization sensor was therefore selective for toxic plant species, and do not respond to those that are safe for celiac patients

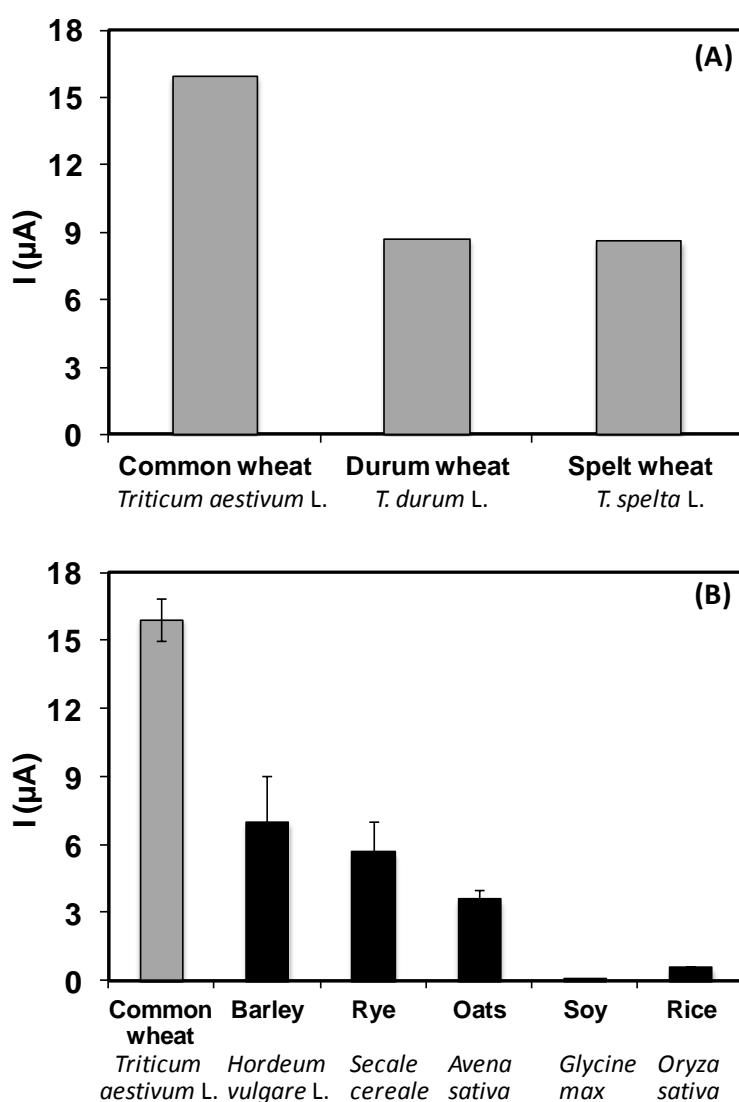


Figure 4. Genosensor selectivity evaluated as the current intensity measured after amplification of 100 ng of genomic DNA of: (A) different wheat species, and

(B) several related cereal and leguminous species, under the conditions previously optimized.

3.2. Detection of DNA from mixtures of cereals and processed food

Most electrochemical genosensors applied to food samples are capable of detecting the presence of the target, but the result is usually expressed in DNA concentration, which did not give a precise idea of the real amount of the species. This holds true when dealing with allergens, and more specifically with gluten, the only allergen with a legal threshold associated in Europe for labeling.

For this reason, the performance of the genosensor assay was further evaluated in flour mixtures. In the absence of certified reference materials for wheat gluten, binary mixtures of wheat flour in an inert matrix, rice flour with a similar granulometry were prepared in a wide range of known weight percentages, from 50% to 0.0001%. These samples were extracted, amplified and then diluted according to the protocol described above, before the analysis with the electrochemical genosensor. An increase of current intensity with the % w/w of wheat at the started material was observed (Fig. 5). The data was adjusted to a four parameters logistic model (equation 1)

$$y = A + \frac{(B-A)}{1 + \left(\frac{x}{x_0}\right)^p} \quad (1)$$

where A is the response at saturation concentration of analyte, B is the response at a zero concentration of analyte, x_0 is the percentage of analyte that corresponds to the 50 % of the maximum signal and p is the Hill slope that represents the slope at the inflexion point of the sigmoidal curve. The equation of the best fitting is the following:

$$I (\mu A) = 9.81 + (0.31 - 9.81) / (1 + ([\% \text{ wheat}] / 0.015)^{0.66}); r = 0.92.$$

The lowest concentration that could be detected was 0.001% (10 ppm or 10 mg/kg) of wheat flour in rice flour, three times higher than the blank signal.

Taking into account that the gluten content is about 9% in wheat flour [14], this result corresponds to a detection of approximately 0.9 mg/kg (0.00009 %) of gluten. It is worth noting the detectability obtained with the developed DNA sensor was similar or even better than other DNA approaches directed to detect wheat in food samples, such as real-time PCR approaches which relied on an optical detection of the amplified fragments [28-30] or microarrays [31]. This advantageous feature was attributed to the multi-copy nature of the target gliadin sequence that can provide methods 10-100 times more sensitive than those based on the detection of sequences found only in one copy, as the

gibberellin response gene [32] or puroindoline-b gene [33-35], that showed sensitivities around to 0.1% -1% of wheat in an inert matrix.

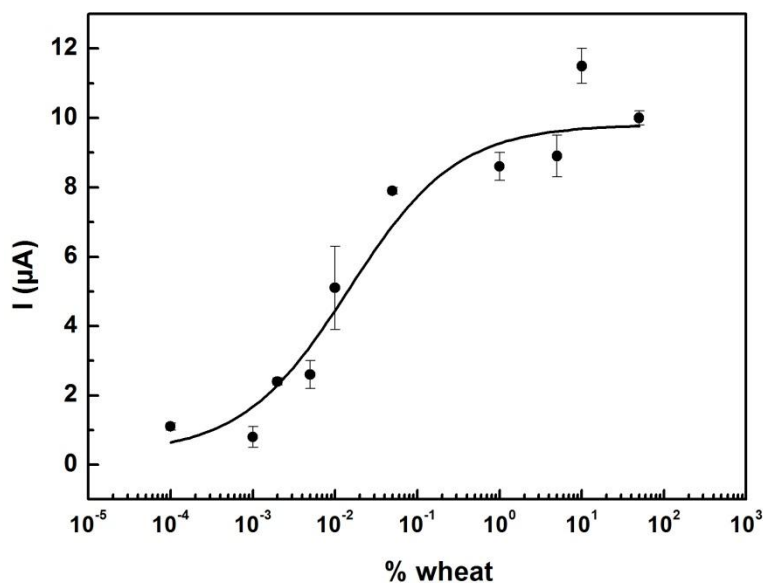


Figure 5. Relationship between the current intensity and the % wheat (w/w) under the optimized conditions.

The designed genosensor was finally applied for gluten screening in a set of commercial food products of different brands and characteristics (three replicates of each sample). Both gluten-containing foods and gluten-free foods were analyzed. Representative samples were chosen to cover the main aspects considered difficult. The higher the severity of the processing the higher the difficulty of DNA extraction, so thermal, mechanical and chemical processed samples were included (Table 2). Those containing or that may contain gluten (G1-3) resulted in currents higher than 2.5 μA that correspond to a content of wheat estimated between 1.4 and 0.003%. In Table 2 the calculated content in ppm of gluten is shown. Cookies contained high amount of gluten but the snack could be labeled as gluten-free. Beer is also expected to contain gluten although some degree of DNA degradation could be expected [12]. The rest of samples (Gf1-4) were labeled as safe for celiac patients, so they should have < 20 ppm of gluten, according to the legislation in force. Gf3 has chocolate, a matrix difficult for DNA extraction. Very low currents undistinguishable from the blank were recorded for all of them except for the snack Gf1. The analysis by gel electrophoresis of the amplicons revealed a total agreement with the genosensor results indicating that the Gf1 sample it is not a false positive of the genosensor.

There are only a few attempts to correlate PCR-based results with immunoassay results. All of them are qualitative since PCR analysis is presented as positive/negative. Only one of them tried to establish a correlation

between ppm of gluten measured by R5 ELISA and pg of DNA /mg of food, with some limited success especially in hydrolyzed samples [12]. Herein, the samples were analyzed by an independent laboratory certified for gluten analysis using the official method, the R5 ELISA sandwich (Table 2). All gluten-free samples contained undetectable amounts of gluten, which confirms the readout obtained by DNA analysis except for the Gf1 sample. Recently slight positive results by PCR in samples negative by R5 were reported [12] but a contamination with DNA during sample processing can not be completely ruled out. Alternatively, degradation of proteins preserving the integrity of DNA in baked products is considered unlikely.

The agreement between both methods is verified in the gluten-containing food subgroup. The low gluten content of the snack is confirmed. This food has a precautionary label indicating that was manufactured in a factory handling gluten-containing ingredients but cross-contamination is undetectable by protein analysis and only traces of DNA were found, which ensures its safety for celiac patients. Beer is a hydrolyzed product so sandwich format assays are not intended for them. In spite of it, a small quantity of gluten was detected suggesting that not fully degraded fragments are still present in the final product. The detection of DNA from toxic cereals settles the presence of gluten. This is the type of samples where DNA genosensors can show their potential as confirmatory methods.

Table 2. Estimated content of gluten of commercial food products (with gluten – G1 to G3– and gluten-free –Gf1 to Gf4–) using the developed electrochemical genosensor and R5 immunoassay.

Sample code	Sample type (trademark)	Gluten content (ppm)	
		Genosensor	R5
G1	Cookies (Fontaneda)	> 100	> 80
G2	Beer (Heineken)	20	15.6
G3	Snack (Bicentury)	7.5	< 5
Gf1	Snack (natuchips)	> 100	< 5
Gf2	Cookies (Celibene)	<0.9	< 5
Gf3	Bread (Schär)	<0.9	< 5
Gf4	Grissini (Schär)	<0.9	< 5

4. CONCLUSIONS

Quantification of allergens in food samples using DNA as target and genosensors is a challenging task that only rarely devices have addressed. In addition results in terms of DNA concentration are meaningless for consumers so they are often used as yes/no tools. In this work, combining the PCR amplification stage with genosensor measurements, a sensitivity matching that of the real-time PCR technology was achieved without complicated

pretreatment protocols. Only a single dilution of the amplicon is required to obtain an improved blank signal without impairing the specific current signals. For the first time, a genosensor calibration in wheat percentage allowed us to quantify the gluten content of food products. The lowest detectable amount was 0.001% of wheat that corresponds to 0.9 ppm of gluten. All toxic cereals can be readily detected without interferences from safe grains. Analysis of highly processed food products revealed a good correlation with immunoassay results, highlighting the suitability of genosensors as an affordable and simple monitoring tool.

5. ACKNOWLEDGEMENTS

B.M.F. thanks to Universidad Complutense de Madrid for a predoctoral grant. This work has been supported by Project CTQ2012-31157 and the European Regional Development Fund.

6. REFERENCES

[1] R. Drikvand, M.R. Bihamta, G. Najafian, A. Ebrahimi, Investigation of genetic diversity among bread wheat cultivars (*Triticum aestivum* L.) using SSR markers, *J. Agr. Sci.*, 5 (2013) 122-129.

[2] R. Haraszi, H. Chassaing, A. Maquet, F. Ulberth, Analytical methods for detection of gluten in food-method developments in support of food labeling legislation, *J. AOAC Int.*, 94 (2011) 1006-1025.

[3] M. Kagnoff, Celiac disease: pathogenesis of a model immunogenetic disease, *J. Clin. Invest.*, 117 (2007) 41-49.

[4] C. Briani, D. Samaroo, A. Alaedini, Celiac disease: From gluten to autoimmunity, *Autoimmun. Rev.*, 7 (2008) 644-650.

[5] P. Green, B. Jabri, Celiac disease, *Annu. Rev. Med.*, 57 (2006) 207-221.

[6] G.J. Tack, W.H.M. Verbeek, M.W.J. Schreurs, C.J.J. Mulder, The spectrum of celiac disease: epidemiology, clinical aspects and treatment, *Nat. Rev. Gastroenterol. Hepatol.*, 7 (2010) 204-213.

[7] Regulation (EC) 41/2009, of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten, *Off. J. Eur. Union*, L16, pp. 3-5.

[8] C. Diaz-Amigo, B. Popping, Accuracy of ELISA Detection Methods for Gluten and Reference Materials: A Realistic Assessment, *J. Agric. Food Chem.*, 61 (2013) 5681-5688.

[9] F. Janssen, Detecting wheat gluten in food, in: S.J. Koppelman, S.L. Hefle (Eds.) *Detecting Allergens in Food*, Woodhead Publishing, Boca Raton, 2006, pp. 244-272.

[10] S. Amaya-Gonzalez, N. de-los-Santos-Alvarez, A.J. Miranda-Ordieres, M.J. Lobo-Castanon, Aptamer Binding to Celiac Disease-Triggering Hydrophobic Proteins: A Sensitive Gluten Detection Approach, *Anal. Chem.*, 86 (2014) 2733-2739.

[11] M. Sandberg, L. Lundberg, M. Ferm, I. Yman, Real Time PCR for the detection and discrimination of cereal contamination in gluten free foods, *Eur. Food Res. Technol.*, 217 (2003) 344-349.

[12] J. Mujico, M. Lombardia, M. Mena, E. Mendez, J. Albar, A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients, *Food Chem.*, 128 (2011) 795-801.

[13] M. Hernandez, T. Esteve, M. Pla, Real-time polymerase chain reaction based assays for quantitative detection of barley, rice, sunflower, and wheat, *J. Agric. Food Chem.*, 53 (2005) 7003-7009.

[14] D. Zeltner, M. Glomb, D. Maede, Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and, *Eur. Food Res. Technol.*, 228 (2009) 321-330.

[15] N. Pegels, I. González, T. García, R. Martín, Authenticity testing of wheat, barley, rye and oats in food and feed market samples by real-time PCR assays, *LWT - Food Sci. and Technol.*, 60 (2015) 867-875.

[16] B. Martín-Fernández, J. Costa, M.B.P.P. Oliveira, B. López-Ruiz, I. Mafrá, Screening new gene markers for gluten detection in foods, *Food Control*, 56 (2015) 57-63.

[17] J. Tosar, G. Branas, J. Laiz, Electrochemical DNA hybridization sensors applied to real and complex biological samples, *Biosens. Bioelectron.*, 26 (2010) 1205-1217.

[18] C.L. Manzanares-Palenzuela, B. Martín-Fernández, M. Sánchez-Paniagua López, B. López-Ruiz, Electrochemical genosensors as innovative tools for detection of genetically modified organisms, *Trends Analyt. Chem.*, 66 (2015) 19-31.

[19] CODEX-STAN118, Amended in 1983 and 2008 for foods for special dietary use for persons intolerant to gluten. FAO/WHO Standards. Available at: <http://www.codexalimentarius.org/standards/list-of-standards/>. Accessed June 25, 2015, 1979.

[20] L. Shan, O. Molberg, I. Parrot, F. Hausch, F. Filiz, G. Gray, L. Sollid, C. Khosla, Structural basis for gluten intolerance in Celiac sprue, *Science*, 297 (2002) 2275-2279.

[21] B. Martín-Fernández, A.J. Miranda-Ordieres, M.J. Lobo-Castanon, G. Frutos-Cabanillas, N. de-los-Santos-Alvarez, B. Lopez-Ruiz, Strongly structured DNA sequences as targets for genosensing: Sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment, *Biosens. Bioelectron.*, 60 (2014) 244-251.

[22] J. Costa, M. Oliveira, I. Mafra, Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes, *Food Res. Int.*, 54 (2013) 1722-1729.

[23] G. Slack, J. Low, G. Kaminski, D. Yirrell, Optimising PCR reactions; remember magnesium?, *J. Clin. Virol.*, 52 (2011) 63-64.

[24] M.M. Blanchard, P. Taillon-Miller, P. Nowotny, V. Nowotny, PCR buffer optimization with uniform temperature regimen to facilitate automation, *Genome Res.*, 2 (1993) 234-240.

[25] B. Martín-Fernández, N. de-los-Santos-Álvarez, M. Lobo-Castañón, B. López-Ruiz, Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a gliadin fragment, *Anal. Bioanal. Chem.*, 407 (2015) 3481-3488.

[26] O.D. Anderson, F.C. Greene, The α -gliadin gene family. II. DNA and protein sequence variation, subfamily structure, and origins of pseudogenes, *Theor. Appl. Genet.*, 95 (1997) 59-65.

[27] T. van Herpen, S. Goryunova, J. van der Schoot, M. Mitreva, E. Salentijn, O. Vorst, M. Schenk, P. van Veelen, F. Koning, L. van Soest, B. Vosman, D. Bosch, R. Hamer, L. Gilissen, M. Smulders, Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes, *BMC Genomics*, 7 (2006) 1.

[28] A. Pasqualone, C. Montemurro, A. Grinn-Gofron, G. Sonnante, A. Blanco, Detection of Soft Wheat in Semolina and Durum Wheat Bread by Analysis of DNA Microsatellites, *J. Agric. Food Chem.*, 55 (2007) 3312-3318.

[29] J. Debnath, A. Martin, L. Gowda, A polymerase chain reaction directed to detect wheat glutenin: Implications for gluten-free labelling, *Food Res. Int.*, 42 (2009) 782-787.

[30] G. Sonnante, C. Montemurro, A. Morgese, W. Sabetta, A. Blanco, A. Pasqualone, DNA Microsatellite Region for a Reliable Quantification of Soft Wheat Adulteration in Durum Wheat-Based Foodstuffs by Real-Time PCR, *J. Agric. Food Chem.*, 57 (2009) 10199-10204.

[31] T.W. Prins, J.P. van Dijk, A.M. Angeline Van Hoef, M.M. Voorhuijzen, S. Broeders, S. Trapmann, R. Seyfarth, A. Pardigol, C.D. Schoen, H.J.M. Aarts, E.J. Kok, Towards a multiplex cereal traceability tool using padlock probe ligation on genomic DNA, *Food Chem.*, 118 (2010) 966-973.

[32] M. Allmann, U. Candrian, J. Lüthy, Detection of wheat contamination in dietary non-wheat products by PCR, *The Lancet*, 339 (1992) 309.

[33] R. Alary, A. Serin, M. Duviou, P. Joudrier, M. Gautier, Quantification of common wheat adulteration of durum wheat pasta using real-time quantitative polymerase chain reaction (PCR), *Cereal Chem.*, 79 (2002) 553-558.

[34] R. Alary, C. Buissonade, P. Joudrier, M.-F. Gautier, Detection and discrimination of cereal and leguminous species in chestnut flour by duplex PCR, *Eur. Food Res. Technol.*, 225 (2007) 427-434.

[35] M. Arlorio, J. Coisson, E. Cereti, F. Travaglia, M. Capasso, A. Martelli, Polymerase chain reaction (PCR) of puroindoline b and ribosomal/puroindoline b multiplex PCR for the detection of common wheat (*Triticum aestivum*) in Italian pasta, *Eur. Food Res. Technol.*, 216 (2003) 253-258.

4.3 Genosensor para la detección de gliadina con sonda de captura estructurada

Una vez resueltos los problemas encontrados con la secuencia oligonucleotídica codificante de la $\alpha 2$ -gliadina debido a su carácter estructurado y a la repetición de las bases en su secuencia y su relativa gran longitud, y verificada la adecuación del genosensor al análisis de muestras reales con fines cuantitativos, se buscó mejorar su selectividad frente a la detección de trigo, respecto de otros cereales filogenéticamente estrechamente relacionados. En Europa, la legislación vigente exige el etiquetado de los productos alimenticios que contengan gluten, independientemente del cereal de origen (Regulation (EC) 41/2009). Sin embargo, en algunos países el etiquetado debe especificar el cereal en concreto que puede ser nocivo para el enfermo celíaco, por lo que se hace necesario aumentar la selectividad de los métodos de detección de gluten, para que sean capaces de discriminar entre uno u otro cereal. El problema radica en que, especies que presentan una alta homología, como los cereales tóxicos para los enfermos celíacos, son muy difíciles de distinguir entre sí (Bendich and McCarthy 1970; Cahoon et al. 2010), debido a la proximidad filogenética entre estas especies (Figura 11).

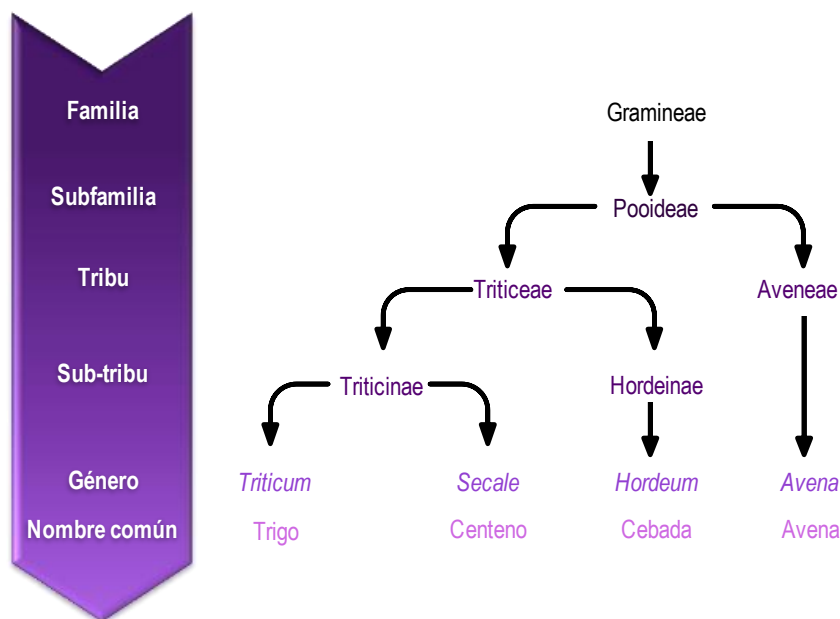


Figura 11. Árbol filogenético de las especies tóxicas para los pacientes celíacos.

Para la detección de ADN, los genosensores pueden aportar dicha selectividad. La reacción de hibridación es una de las reacciones más selectivas que se conocen, ya que se fundamenta en el reconocimiento entre bases complementarias, que en el caso de los genosensores se trata de la hibridación entre las sondas y el analito específico. Esta hibridación es perfecta

(todas las bases que componen el dúplex), mientras que secuencias similares presentan diferencias en la secuencia oligonucleotídica que hacen del dúplex una estructura no perfectamente complementaria, y por tanto, menos estable termodinámicamente. Estos errores de emparejamiento de bases entre el analito y las sondas que componen el genosensor pueden afectar en diferente grado a la estabilidad del dúplex, según sea su posición, su número total y su número relativo en la longitud total de la secuencia, por lo que su discriminación frente al dúplex perfecto no es en absoluto sencilla. Por todo ello, se han explorado diversas formas de poner de manifiesto los dúplex no perfectos.

La primera de ellas consiste en aplicar condiciones extremas durante la hibridación, como el aumento de temperatura o el uso de determinados agentes químicos. Otro tipo de aproximación más elegante es el uso de sondas estructuradas de captura en el diseño del genosensor (Miranda-Castro et al. 2009). La sonda puede presentar, por ejemplo, una estructura tipo horquilla, que provoca la competición con la hibridación sonda-analito, y que aumenta la selectividad de la sonda y limita la extensión relativa de las reacciones de hibridación no deseadas. En esta sección se exploró de forma comparativa el efecto del tipo de sonda, no estructurada o tipo horquilla, en la selectividad del genosensor.

Bibliografía

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic acids research* 31(13), 3406-3415

Regulation (EC) 41/2009, of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. *Official Journal of European Union*, pp. 3-5.

Miranda-Castro, R., de-los-Santos-Álvarez, N., Lobo-Castañón, M.J., Miranda-Ordieres, A.J., Tuñón-Blanco, P., 2009. Structured nucleic acid probes for electrochemical devices. *Electroanalysis* 21(19), 2077-2090.

Cahoon, A.B., Sharpe, R.M., Mysayphonh, C., Thompson, E.J., Ward, A.D., Lin, A., 2010. The complete chloroplast genome of tall fescue (*Lolium arundinaceum*; Poaceae) and comparison of whole plastomes from the family Poaceae. *American Journal of Botany* 97(1), 49-58

Bendich, A.J., McCarthy, B.J., 1970. DNA Comparisons among Barley, Oats, Rye, and Wheat. *Genetics* 65(4), 545-565.

Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a gliadin fragment

Begoña Martín-Fernández · Noemí de-los-Santos-Álvarez ·
María Jesús Lobo-Castañón · Beatriz López-Ruiz

Received: 17 December 2014 / Revised: 5 February 2015 / Accepted: 10 February 2015 / Published online: 25 February 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract High selectivity of genosensors is crucial for certain applications such as those involving species with high genetic variability. This is an unresolved problem when dealing with long target sequences that is further complicated when the target contains repetitive sequence domains. As a model for this situation, the problem of detecting gluten in food with identification of the source is studied. In order to discriminate the specific DNA sequence that encodes the wheat prolamins (gliadin) from rye and barley prolamins, the exquisite selectivity of a rationally designed hairpin capture probe is proposed and compared to a nonstructured capture probe. An electrochemical sandwich assay is proposed, involving capture probes chemisorbed on Au surfaces and biotinylated-signaling probes in combination with streptavidin-peroxidase labeling conjugates. As a result, a genosensor with similar sensitivity to that observed with linear probes but with complete specificity against closely related species was achieved. The surface-attached DNA stem-loop yields a device capable of accurately discriminating wheat DNA from rye and barley with a limit of detection of 1 nM.

Keywords Hairpin-DNA probe · Electrochemical genosensor · Self-assembled monolayers · Enzyme amplification · Screen-printed gold electrodes · Specificity

Introduction

Genetic analysis at point of need using genosensors, a class of biosensors that makes use of the hybridization event between complementary DNA strands, is one of the most active areas of electrochemical biosensing not only for clinical or diagnostic purposes but also for food safety surveillance. To this aim, the hardest efforts are focusing on achieving the extremely low detectability sometimes required. Ingenious but often complicated amplification schemes are approaching limits of detection that make it feasible to avoid PCR preamplification. DNA hybridization is one of the most selective recognition events known, so selectivity is regarded to be not as challenging as sensitivity except for single-nucleotide polymorphisms (SNP) detection where it becomes a major concern [1]. All genosensor devices claim a clear difference in response between the target and mismatched sequences. However, a substantial number of them fail to unambiguously discriminate closely related DNA sequences because a lower analytical signal can be misinterpreted as a low concentration of the intended target.

Hybridization selectivity relies on the destabilization mismatches caused in the duplex structure that depends on their location, their number, and the total length of the double helix. In general, short oligonucleotides are better suited than long sequences for mismatch discrimination probably because of the greater stability of the longer duplexes formed [2]. Although the selection of a specific target is essential as a starting point in the design of genosensors, the choices can be limited by other considerations, e.g., the detection of species subjected to high genetic variability, naturally or through breeding programs, such as certain grains. When faced with highly similar and long oligonucleotide target sequences, a selective recognition is not straightforward. Fortunately, there are tools to assist the best discrimination of nonperfect duplexes. In this way, stringent conditions are usually applied (temperature,

B. Martín-Fernández · B. López-Ruiz (✉)
Sección Departamental de Química Analítica, Facultad de Farmacia,
Universidad Complutense de Madrid, Pz. Ramon y Cajal s/n,
28040 Madrid, Spain
e-mail: bealopru@ucm.es

N. de-los-Santos-Álvarez · M. J. Lobo-Castañón
Departamento de Química Física y Analítica, Universidad de
Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain

chemical agents, etc.) [3–8]. In more challenging applications, another way to improve the selectivity of the genosensor recognition layer is the use of structured probes [9]. This strategy is being extensively exploited in SNP detection, typically targeting short oligonucleotides (<35 nt) [10]. The hairpin structure provides a competition reaction with probe-target hybridization that increases probe specificity and limits the relative stability of undesired hybridization events [11]. The combination of hairpin capture probes with a sandwich-type assay can further increase the discrimination ability due to the two recognition events involved [12]. Besides, it overcomes the large background currents caused by the variable population of hairpin in its opened state due to thermal fluctuations and dynamic equilibrium. This could become important when labeled hairpin probes, the electrochemical counterpart of molecular beacons, are used. These self-reported probes can originate significant signals in the absence of the target [11]. Impedimetric measurements avoid the use of labels but the redox probe in solution is sensitive to the folding or unfolding state of the hairpins contributing to make difficult the interpretation of the results [13].

We have recently reported a DNA sensor for electrochemical detection of a gliadin specific fragment [14]. The sensitive, rapid and simple detection of a sequence coding for an immunotoxic wheat protein for celiac patients, was achieved by a sandwich assay using a linear capture probe, and signaling linear probe both hybridizing with the target. But close related sequences from cereals also triggering the celiac disease provided low but distinguishable signals from the blank. Although under the European Regulations the detection of species containing gluten as a group is demanded [15], in other countries the identification of the phylogenetic origin is compulsory [16]. In the present work, we have judiciously optimized the described approach making use of a structured capture probe with the aim of obtaining a more selective genosensor for the highest discrimination of wheat sequence from other cereals. All the variables involved in genosensor

design were carefully studied for this relatively complex target, which presents a long, strongly structured and repetitive DNA sequence.

Experimental

Reagents

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), KCl, tetramethylbenzidine (TMB) in a ready-to-use reagent format (K-blue enhanced activity substrate, also containing H₂O₂), streptavidin-peroxidase (HRP-Strp), bovine serum albumin (BSA), Tween 20, formamide, dextran sulfate from *Leuconostoc mesenteroides*, and saline sodium phosphate-EDTA (20× SSPE) (pH 7.4) were purchased from Sigma-Aldrich (Spain). Ethanol was purchased from Panreac (Spain) and potassium ferrocyanide was from Probus (Spain). NAP-10 columns of Sephadex G-25 were obtained from Life Technologies (Spain). Water was purified with a Milli-Q system (Millipore, Spain).

Oligonucleotide sequences used are shown in Table 1. Oligomers were obtained as lyophilized desalted salts from Sigma-Life Sciences. These sequences were selected to detect a sequence of 99-nt that encodes a highly toxic protein from wheat for celiac patients, the α2-gliadin [17]. The target presents a three-repeated domain of 21 bp that is underlined. The structured probe acquires a hairpin conformation. The regions that form the stem and that hybridize with the target are indicated in italics and in bold, respectively. To test the selectivity of the genosensor noncomplementary targets that encode gliadins from barley and rye and fully noncomplementary sequences were employed. All stock solutions were prepared in Milli-Q water without any treatment except for thiolated hairpin and linear capture probes that were deprotected as indicated below. The concentration of all stock solutions was

Table 1 Probes and target sequences

	Oligonucleotide sequences 5'→3'
Target (wheat, 99 nt)	CTG CAG CTG CAA CCA TTT <u>CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CTA CCA TAT CCG CAG CCG CAA CCA TTT</u>
Signaling probe (66 nt)	CTG CGG ATA TGG TAG TTG CGG CTG CGG ATA TGG TAG TTG CGG CTG CGG AAA TGG TTG CAG CTG CAG-FITC
Hairpin capture probe (43 nt)	HS-(CH ₂) ₆ - <i>GTT CCG CAA ATG GTT GCG GCT GCG GAT ATG GTA GTT GCG GAA C</i>
Linear capture probe (33 nt)	HS-(CH ₂) ₆ - AAA TGG TTG CGG CTG CGG ATA TGG TAG TTG CGG
Fully mismatched (99 nt)	GAC GTC GAC GTT GGT AAA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GAT GGT ATA GGC GTC GGC GTT GGT AAA
Rye (93 nt)	GCA ACC ATT TCC CCA ACC CCA ACA ACC AAC CCC CAT ACA ACC ACA ACA ACC ATT CCC CCA GCG ACC CCA ACA ACC TTT CCC CCA GCC CCA ACA
Barley (93 nt)	AAC AAC CTT TTC CTC AGC CCC AAC AAC CAT TCC CCT GGC AAC CAC AAC AAC CAT TTC CCC AGC CTC AAC AAC CAT TTC CCC TGC AAC CAC AAC

checked with a UV260 Shimadzu spectrophotometer (Japan) before stored at $-20\text{ }^{\circ}\text{C}$ until use.

Instrumentation

Electrochemical measurements were carried out with screen-printed gold electrodes (SPEAu, DropSens-220BT, Spain), connected to a μ -AutoLab type II potentiostat with GPES 4.9 software (EcoChemie, The Netherlands). The layout of the disposable planar screen-printed gold electrodes includes three electrodes in the same alumina sheet: a working gold electrode ($\varnothing \sim 4\text{ mm}$), an Ag pseudo-reference electrode and a gold counter electrode, all of them screen-printed on a ceramic substrate. The pH measurements were performed on a Crison micropH 2001 pHmeter (Spain).

Sensing phase preparation

SPEAu were washed with ethanol and water, and dried with nitrogen. The electrodes were conditioned in $0.5\text{ M H}_2\text{SO}_4$ solution by sweeping the potential between 0 and 1.25 V at a scan rate of 100 mV/s until a stable cyclic voltammogram was obtained. Finally, electrodes were washed with water and dried with nitrogen immediately before adding $15\text{ }\mu\text{L}$ of $2\text{ }\mu\text{M}$ of the corresponding hairpin (or linear) capture probe

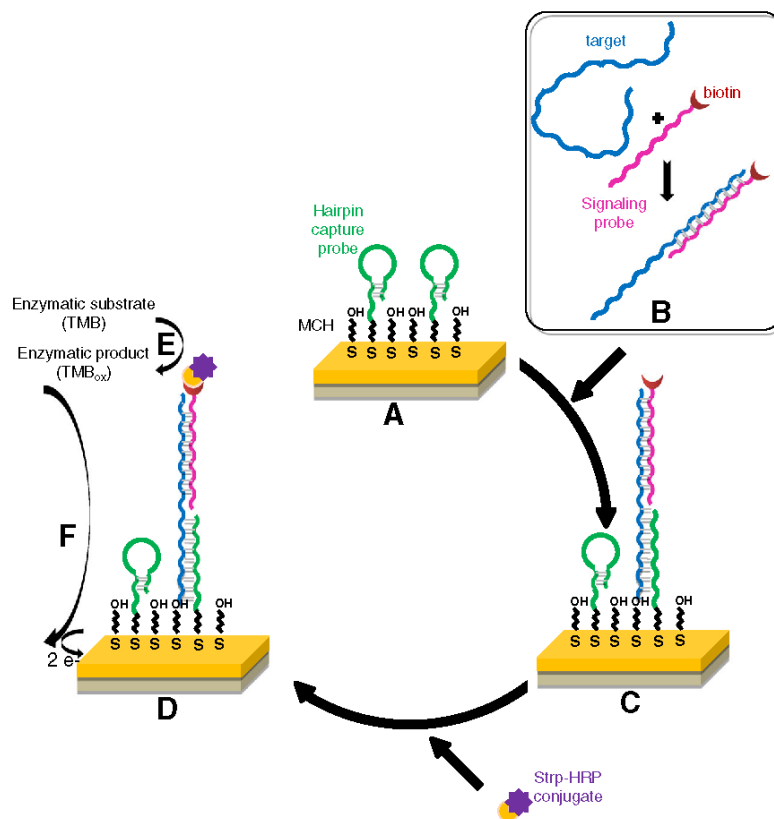
in $2\times\text{ SSPE pH }7.4$ solution (unless otherwise indicated), previously deprotected with DTT as indicated elsewhere [12]. Chemisorption proceeded overnight and after washing with $2\times\text{ SSPE}$ buffer in order to displace the nonspecifically adsorbed probe, $10\text{ }\mu\text{L}$ of 4.5 mM MCH in $2\times\text{ SSPE}$ was placed onto the probe modified surface for 30 min. The modified electrodes were then washed with buffer and dried with nitrogen before carrying out the sandwich assay (Fig. 1A).

Sandwich assay and measurement

Hybridization experiments were carried out in a sandwich-like format through two consecutive steps: homogeneous (Fig. 1B) and heterogeneous hybridization reactions (Fig. 1C).

For homogeneous hybridization, the target was added to a hybridization buffer ($2\times\text{ SSPE}$) solution containing $1.25\text{ }\mu\text{M}$ of the corresponding signaling probe labeled with biotin. DNA strands were thermally treated (5 min at $98\text{ }^{\circ}\text{C}$), and cooled down by placing in ice-water bath for 5 min . Then, the mixture was brought to room temperature over 30 min . Immediately after, $15\text{ }\mu\text{L}$ aliquot of this solution was placed onto the sensing phase for 2 h (heterogeneous hybridization) (unless otherwise indicated). Afterwards, the working electrode surface was washed and covered with $5\times\text{ SSPE}$ containing $2\text{ }\%$ BSA (w/v) and $0.1\text{ }\%$ Tween 20 (w/v) pH 7.4

Fig. 1 Schematic illustration of the sensing phase and the assay steps



(blocking buffer) for 2 min in order to prevent enzyme conjugate nonspecific adsorption (unless otherwise indicated). Fifteen microliters of a solution of 0.01 g/L Strp-HRP conjugate in the blocking buffer was added to the electrode surface. After 30 min, the sensing phase was washed, first with blocking buffer and then with 2× SSPE, and dried with nitrogen (Fig. 1D).

The last step was the chronoamperometric measurement of the bound enzymatic activity (Fig. 1E). 40 μ L of the enzymatic substrate (commercial TMB) was placed on the cell, covering the three electrodes, and after 60 s the product of the enzyme reaction was detected at 0 V sampling the current for 1 min (Fig. 1F). All measurements were carried out in three replicates.

Results and discussion

Instead of the 33 nt linear probe previously used [14], a capture probe with a stem-loop structure was designed. Generally, the target hybridizes only with the loop sequence, but for longer target sequences a very long stem would be required to maintain the hairpin closed, and some difficulties in the stem opening may occur in the presence of the target [12]. To avoid this, a shared-stem hairpin capture probe is desirable. The length of the stem for a fixed probe length is a major parameter of design to obtain stable hairpins against thermal fluctuations and low unspecific signal arisen from easy opening in the presence of partially complementary strands [11, 18]. So, a hairpin probe was designed extending the linear probe at both ends to achieve the formation of a 9-base pair stem (in italics in Table 1). The whole 25-base loop sequence and eight bases of the stem were complementary to the target at the 3' end (in bold in Table 1). The secondary structure of the tailored capture probe and the correct hybridization were checked using Mfold Web Server [19]. The hairpin with 9 bp in its stem has a free energy of -10.73 kcal/mol, which ensures a low background signal from mismatched sequences.

The successful formation of the binary self-assembled monolayer (SAM) was checked using 0.5 mM potassium ferrocyanide in 0.1 M KCl as redox probe in solution. The electron transfer from the probe to the bare Au electrode (Fig. 2, dashed line) is partially blocked on the electrode modified with the hairpin-MCH SAM (Fig. 2, solid line) due to the negative charges of the DNA (current intensity reduction along with increase in the peak potential separation).

In order to verify that the designed hairpin opens in the presence of the target, a preliminary experiment was carried out with 10 nM of target. A signal/blank ratio (S/B) of 2.0 was obtained, although the reproducibility was poor (20.9 %, $n=3$), with the concentration and reaction times of probes and MCH, previously studied [20]. This indicates that the target

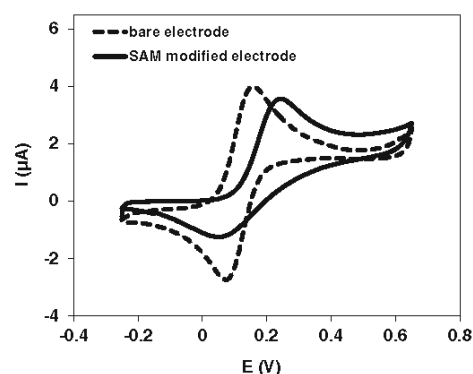


Fig. 2 Cyclic voltammetry of 0.5 mM potassium ferrocyanide in 0.1 M KCl on bare (dashed line) and binary SAM modified (solid line) electrodes

recognition takes place but an improvement of operating conditions is needed.

Improving the genosensor performance

Given the length and complexity of the specific sequence that codifies the 33-mer immunodominant peptide of gliadin, it is expected that the length of both the homogeneous and heterogeneous hybridization steps play a significant role in the genosensor performance.

The kinetics of hybridization on solid surfaces is notably slower than that occurring in solution, due to the lower accessibility to immobilized DNA probes [21], so we used longer reaction times for heterogeneous hybridization compared to homogeneous one. Accordingly, the homogeneous hybridization was assayed at 15 and 30 min and the heterogeneous one at 1 and 2 h using 10 nM of target and 1.25 μ M of biotinylated-signaling sequences in solution. For 15 min of homogeneous reaction time, an increase in the S/B was observed at the longest heterogeneous interaction time, S/B of 3.3 and 3.9, for 1 and 2 h, respectively. Also, a better performance was obtained for longer homogeneous reaction time (S/B of 5.8 for 30 min and 2 h, of homogeneous and heterogeneous interaction times, respectively). Therefore, the best S/B ratio was obtained for 30 min and 2 h for homogenous hybridization and heterogeneous hybridization, respectively, while similar blank currents were obtained in all the cases. The kinetics of the hybridization event is affected by the target secondary structure, which eventually would affect selectivity if short hybridization steps are performed, that is, equilibrium is not achieved [2]. In our case, the sandwich format disrupts the secondary structure of the target excluding this effect. However, the repetitive nature of the gliadin-specific target (Table 1, three repetitive domains of 21 bp in the target sequence, each one underlined), makes possible that the signaling probe can hybridize at several positions along the target sequence with very high stability (>70 kcal/mol). Some of

these hybrids block the further hybridization with the capture probe. Since several duplexes are in equilibrium after homogeneous hybridization, rearrangements have to occur in order to form the three-stranded perfect duplex. These unusual rearrangements might account for the extended heterogeneous hybridization in comparison with typical times ranging between 30 to 60 min [22–25]. Additionally, the length of the stem also influences negatively the hybridization rate because a larger energy barrier must be overcome [11]. In this sense, even the detection of microRNA needed 2 h of hybridization when using an 8-base pair stem hairpin probe [18].

Numerous studies have described the tendency of both the enzyme conjugate and the signaling probe to adsorb nonspecifically to the sensing surface [7]. Even in the case of using a small thiol such as mercaptohexanol as filling agent, some bare Au regions remain available for both signaling probe and enzyme conjugate adsorption [26]. In order to define a strategy to reduce this undesired contribution, the influence of both molecules on nonspecific signal was studied by adding commercial enzyme substrate TMB, in the absence of target on modified electrodes (SAM composed by capture probe and MCH). The electrochemical signal of TMB after incubation on the electrode with the signaling probe, the enzyme conjugate and both was registered. The adsorption of the biotinylated-signaling probe is minimal compared to the enzyme conjugate as indicated by the currents measured (1.88, 196, and 249 nA respectively). Therefore, we deduced that the unspecific adsorption of the enzyme conjugate was the main cause of the background noise, so the labeling step and the composition of the blocking buffer were considered to reduce this contribution.

First, the concentration of the enzyme and the length of the labeling reaction were studied. Using 0.01 g/L of enzyme conjugate, this step was lengthened up to 30 min where the highest difference between the specific signal at 10 nM and the blank responses was obtained (Fig. 3A). An increase in the conjugate concentration up to 0.1 g/L led to an unacceptable level of unspecific adsorption (data not shown), so this parameter remains at 0.01 g/L.

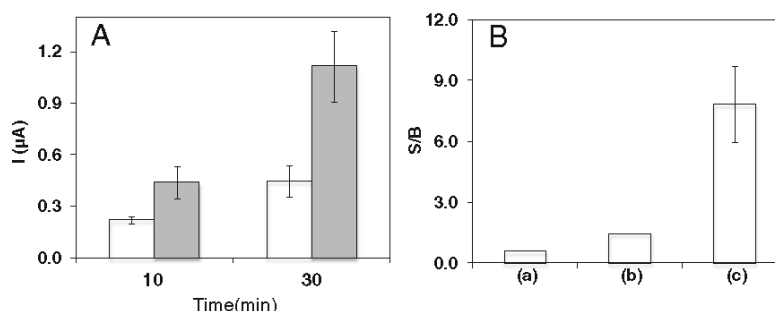


Fig. 3 **A** Variation of the current intensity obtained in the absence (*blank*, white bars) and in the presence of target 10 nM (*gray bars*) with the length of the labeling reaction ($n=3$, [HRP-Strp]=0.01 g/L). **B**

The composition of the blocking buffer also can help to reduce the unspecific binding. Detergents, such as Tween 20 or proteins such as BSA, have been widely employed with this aim [7, 27]. Nonetheless, some Tween preparations can contain peroxides [28, 29] which could interfere with the analysis if the enzyme label is HRP. So, the effect of suppressing Tween 20 was first evaluated at 10 nM of target. The absence of Tween in the blocking buffer resulted in large blank signals leading to very low S/B ratios (Fig. 3B (a)), probably because the surfactant reagent acts removing the excess of loosely bound biomolecules that it is expected to be higher when no target is present. Therefore, it is necessary to maintain Tween in the blocking buffer. The effect of the concentration of BSA was then varied in a blocking buffer containing 0.1 % Tween. As it can be seen in Fig. 3B ((b) and (c)), the S/B ratio was improved when 5 % of BSA was included and so, it was selected for further experiments.

Finally, the effect of buffer composition on the hybridization efficiency was also studied. In general, there are four strategies applicable when dealing with stable secondary structures (1) increasing the temperature during the contact of the DNA strands; (2) reducing the length of interacting DNA strands; (3) incorporating denaturing reagents; and (4) increasing the local concentration of the probes by the addition of volume-occupying reagents, to conduct the hybridization more efficiently [30, 7].

Regarding the former, the thermal denaturation carried out before the homogeneous hybridization ensures the disruption of secondary structures, so a complete hybridization between signaling probe and target is expected. The heterogeneous hybridization can be carried out at physiological or even higher temperatures on DNA thiolated monolayers [31–33]. However, increasing the temperature weakens the stability of the hairpin [11], which compromises the specificity, so it was discarded. The feasibility of reducing the length of the probes was also considered, because it might decrease the stability of secondary structures as that of the signaling probe used in this study (66 bases). Due to the repeating nature of the target model sequence: CTG CAG CTG CAA CCA TTT CCG

Influence of composition of blocking buffer on the S/B ratio for a target concentration of 10 nM: (a) 2 % BSA; (b) 2 % BSA + 0.1 % Tween; (c) 5 % BSA + 0.1 % Tween

CAG CCG CAA CTA CCA TAT]₂. CCG CAG CCG CAA CCA TTT shorter signaling probes can easily hybridize at multiple positions on the target, complicating the subsequent surface hybridization.

As a third alternative, the effect of the addition of binders and denaturing agents in the hybridization buffer solution was studied. Ku et al. [30] reported an improvement in the efficiency of hybridization of complementary DNA strands by using buffer solutions of phosphate containing 10 % dextran and 25 % formamide. Dextran sulfate, polyethylene glycol, and polyvinyl alcohol binders are “volume-occupying” reagents that increase the local concentration of interacting molecules [30, 34]. They are known to increase the interactions between biomolecules and to increase the contact between two complementary strands, improving the probability of collision between single-stranded DNA sequences, even without increasing the temperature and the background signal. Formamide acts as denaturing agent, which causes the opening of the secondary structure of the strands, and increases its contact and the probability of reaction between fully complementary strands. Discrimination with partially complementary sequences is also enhanced. It was also reported that SDS lowers the surface tension of aqueous solutions and improves the kinetics of nucleic acid interactions, and could reduce nonspecific signal [35].

So, dextran sulfate, formamide, and SDS were tested alone and in selected binary combinations in 6× SSPE buffer to investigate their effect on hybridization efficiency and then on the analytical response. The results were compared with those obtained in a buffer with lower ionic strength (2× SSPE) and displayed in Fig. 4. The use of 0.1 % SDS or 25 % formamide in 6× SSPE buffer (Fig. 4(e)), increases the *S/B* ratio with respect to the absence of both compounds (Fig. 4(a), (c), and (d)). The combined action of both components further increased the *S/B* ratio but the coefficients of variation (CV) were very large (up to 36 % for blank), so their use was

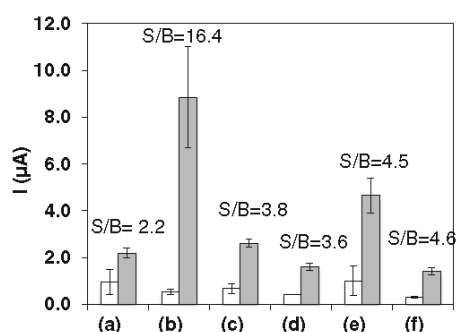


Fig. 4 Variation of the analytical signal obtained in the absence (*blank*) and in the presence of 10 nM of target and variation of the *S/B* ratio as a function of the composition of the hybridization buffer: (a) 6× SSPE; (b) 6× SSPE+10 % dextran + 25 % formamide; (c) 6× SSPE + 0.1 % SDS; (d) 6× SSPE + 25 % formamide; (e) 6× SSPE + 0.1 % SDS + 25 % formamide and (f) 2× SSPE

discarded. A dramatic increase in this ratio was obtained with a mixture of dextran sulfate and formamide (Fig. 4(b)) but the reproducibility was also negatively affected (~24 % for 10 nM). The irreproducibility was confirmed when assaying other higher (20 nM) and lower (5 nM) concentrations of target (data not shown). It is possible that the homogenization procedure was not as effective as expected. When the agglomerating agent is present, the buffer becomes much denser and very difficult to homogenize. So, despite having a positive effect on the hybridization reaction, as expected, it was decided to remove dextran from the buffer solution. The best *S/B* ratio with a reasonable reproducibility was obtained with 2× SSPE buffer (Fig. 4(f)) that was selected as the hybridization buffer.

Analytical characteristics

The analytical characteristics were evaluated in a range of the target concentrations between 0.5 and 200 nM (Fig. 5). The proposed genosensor exhibits a linear response from 5 to 50 nM of DNA, [I (µA) = 0.072 (±0.008) + 0.1 (±0.1) [DNA] (nM); $r=0.998$]. The limit of detection (LOD), calculated as three times the standard deviation divided by the slope of the regression equation, is estimated to be 1 nM ($n=6$). The CV was 21 and 4.3 %, for 10 and 50 nM, respectively ($n=3$).

Selectivity of the device

The selectivity of the assay is greatly influenced by the secondary structure of the capture probe immobilized to the electrode surface, so both the linear and the hairpin capture probes

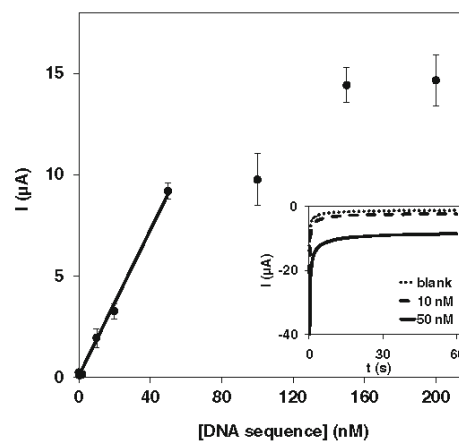


Fig. 5 Relationship between the analytical signal and the target concentration using HRP-Strp as enzymatic label. Experiences performed under the optimized conditions (see text for details). *Inset* chronoamperograms obtained at 0 V for blank (*dotted line*), 10 nM (*dashed line*) and 50 nM (*solid line*)

were challenged against related sequences that present mismatches.

Since the target model sequence encodes the immunodominant gliadin peptide from wheat, similar oligonucleotides are also present in closely related cereals that trigger celiac disease. The specific DNA sequences encoding barley and rye prolamins, previously described by Shan et al. [17], and a fully noncomplementary target, were tested. These sequences act as models of relative similar sequences that could interfere on the genosensor assay due to the cross-contamination of grains in origin or the frequent mixture of cereals to prepare food.

From Fig. 6, it is apparent that the linear capture probe gives rise to larger responses in terms of S/B than the hairpin probe in all cases (S/B of 17.1 and 4.6, respectively for wheat DNA). Although S/B values at 10 nM level of each DNA sequence are clearly lower for rye and barley, only the structured capture probe shows true specificity for the wheat DNA. This indicates a poor selectivity of the linear probe. In fact, the current intensities obtained for the related cereals with the hairpin probe were comparable to blank signals within the experimental error as S/B ratios close to 1 indicate. The response to a fully noncomplementary DNA sequence was negligible irrespective of the capture probe used.

The loss in sensitivity is a known consequence of using hairpin-DNA probes. In the present case, the penalty paid to overcome the energy barrier of the folded state of the hairpin is tolerable. While the lower limit of the linear range is 1 nM using the linear probe, 5 nM is achieved with the hairpin one. This is clearly compensated with the gain in selectivity.

Stability of the device

Self-assembled monolayers act as a two-dimensional liquid, and is influenced by numerous factors such as pre-cleaning of the surface, the orientation, the possible nonspecific adsorption and density of surface packaging [36], some of which are

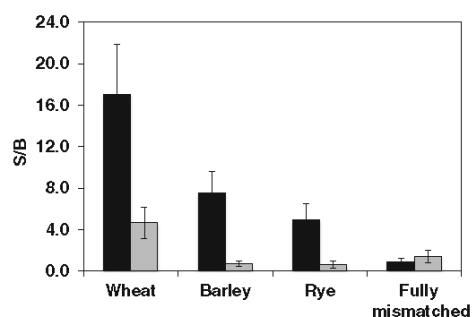


Fig. 6 S/B for linear capture probe (black) and hairpin capture probe (gray) for 10 nM of complementary and noncomplementary sequences

modified uncontrollably during storage of the sensor. A stability study of the sensing phase was also carried out. Modified electrodes prepared as described in the general protocol with the capture probe and the diluent were stored in a refrigerator at 4 °C in a closed Petri dish and with a small amount of water, to control the humidity. The long-term stability of the SAM-modified sensors was tested periodically by evaluating the response for 10 nM target DNA, during 12 days, using three electrodes each day. The S/B was gradually diminished, e.g., from 100 to 74.1 % and 72.8 %, for the 1st, 3rd, and 11th day, respectively, indicating relative instability of the sensing phase, justified by the complexity of the self-assembled monolayer. This is a well-known problem in genosensors based on self-assembled monolayers attributed to the capture probe displacement by MCH that leads to a rapid decrease in hybridization efficiency [25, 37]. However, in the present case, a slightly better stability was obtained, compared to the reported 62 % of signal loss after 10 days using a similar binary monolayer (1 mM MCH and 1 μ M capture probe) [37]. In contrast, a greatly improved S/B ratio when using ternary layers (capture probe, MCH and a third di-thiol spacer) for prolonged storage periods and slight decreasing of the sensitivity was reported [37]. This behavior was attributed to the limited MCH-induced displacement in the presence of the third thiol reagent.

Conclusions

In this work, we propose an improved method to selectively determine the presence of wheat DNA sequences, and discriminate between other related sequences. The strategy relied in the replacement of the linear capture probe by a 9-bp shared-stem hairpin capture probe. The genosensor performance was carefully tuned by selecting the best conditions. To approach the sensitivity of the linear probes with a repetitive DNA target sequence, the hybridization step was extended beyond the usual. The use of volume-occupying compounds as well as denaturing agents significantly enhanced the hybridization but led to a non-negligible irreproducibility, so their use was discarded. Under the optimum conditions, the superior selectivity of the designed hairpin capture probe was unambiguously demonstrated by comparing the responses towards the gliadin-encoding target and two phylogenetic related sequences. The proposed genosensor exhibits slightly lower sensitivity than that obtained with a linear probe but the specificity gained is essential for the identification of the gluten source required in some legislations. This study also shows that even the most complex target DNA sequences can be selectively detected with electrochemical genosensors, if an appropriated assessment of the genosensor design and the variables is performed.

Acknowledgments B.M.F. thanks to Universidad Complutense de Madrid for a predoctoral grant. This work has been cofinanced by Projects CTQ2008-02429/BQU granted to “Grupos Consolidados”, CTQ2012-31157 and the European Regional Development Fund.

References

- Bonanni A, Pumera M (2011) Graphene platform for hairpin-DNA-based impedimetric genosensing. *ACS Nano* 5(3):2356–2361
- Lubin AA, Vander Stoep Hunt B, White RJ, Plaxco KW (2009) Effects of probe length, probe geometry, and redox-tag placement on the performance of the electrochemical E-DNA sensor. *Anal Chem* 81(6):2150–2158
- Letowski J, Brousseau R, Masson L (2004) Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. *J Microbiol Methods* 57(2): 269–278
- Caruana DJ, Heller A (1999) Enzyme-amplified amperometric detection of hybridization and of a single base pair mutation in an 18-base oligonucleotide on a 7- μ m-diameter microelectrode. *J Am Chem Soc* 121(4):769–774
- Peterson AW, Wolf LK, Georgiadis RM (2002) Hybridization of mismatched or partially matched DNA at surfaces. *J Am Chem Soc* 124(49):14601–14607
- Kim K, Yang H, Park SH, Lee D-S, Kim S-J, Lim YT, Kim YT (2004) Washing-free electrochemical DNA detection using double-stranded probes and competitive hybridization reaction. *Chem Commun* 13:1466–1467
- Herzer S, Englert DF (2001) Nucleic acid hybridization. Molecular biology problem solver: a laboratory guide. Wiley-Liss, New York
- Ali MF, Kirby R, Goodey AP, Rodriguez MD, Ellington AD, Neikirk DP, McDevitt JT (2003) DNA hybridization and discrimination of single-nucleotide mismatches using chip-based microbead arrays. *Anal Chem* 75(18):4732–4739
- Miranda-Castro R, de-los-Santos-Álvarez N, Lobo-Castañón MJ, Miranda-Ordieres AJ, Tuñón-Blanco P (2009) A structured nucleic acid probes for electrochemical devices. *Electroanalysis* 21(19): 2077–2090
- Abi A, Ferapontova E (2013) Electroanalysis of single-nucleotide polymorphism by hairpin DNA architectures. *Anal Bioanal Chem* 405(11):3693–3703
- Tsourkas A, Behlke MA, Rose SD, Bao G (2003) Hybridization kinetics and thermodynamics of molecular beacons. *Nucleic Acids Res* 31(4):1319–1330
- Miranda-Castro R, de-Los-Santos-Alvarez P, Lobo-Castañón MJ, Miranda-Ordieres AJ, Tuñón-Blanco P (2007) Hairpin-DNA probe for enzyme-amplified electrochemical detection of *Legionella pneumophila*. *Anal Chem* 79(11):4050–4055
- Kjällman THM, Peng H, Soeller C, Travas-Sejdic J (2008) Effect of probe density and hybridization temperature on the response of an electrochemical hairpin-DNA sensor. *Anal Chem* 80(24):9460–9466
- Martín-Fernández B, Miranda-Ordieres AJ, Lobo-Castanón MJ, Frutos-Cabanillas G, de-los-Santos-Alvarez N, Lopez-Ruiz B (2014) Strongly structured DNA sequences as targets for genosensing: sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment. *Biosens Bioelectron* 60:244–251
- European Union (2009) Commission Regulation (EC) No.41/2009. *Off J Eur Union* L16
- Amigo CD, Popping B (2012) Labeling regulations, detection methods, and assay validation. *J AOAC Int* 95(2):337–348. doi:10.5740/jaoacint.SGE_Diaz-Amigo
- Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray G, Sollid L, Khosla C (2002) Structural basis for gluten intolerance in Celiac sprue. *Science* 297(5590):2275–2279. doi:10.1126/science.1074129
- Lin M, Wen Y, Li L, Pei H, Liu G, Song H, Zuo X, Fan C, Huang Q (2014) Target-responsive, DNA nanostructure-based E-DNA sensor for microRNA analysis. *Anal Chem* 86(5):2285–2288. doi:10.1021/ac500251t
- Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31(13):3406–3415
- Fernández BM, Palenzuela CLM, López MSP, Ruiz BL, Cabanillas GF (2012) Electrochemical genosensor for indirect wheat gluten detection in food. *An Real Acad Farm* 3:323–343
- Levicky R, Horgan A (2005) Physicochemical perspectives on DNA microarray and biosensor technologies. *Trends Biotechnol* 23(3): 143–149
- Liu Y, Li H, Chen W, Liu A, Lin X, Chen Y (2013) Bovine serum albumin-based probe carrier platform for electrochemical DNA biosensing. *Anal Chem* 85(1):273–277
- Gonzalez-Alvarez M, Perez-Ruiz E, Miranda-Castro R, de-los-Santos-Alvarez N, Miranda-Ordieres A, Lobo-Castanón M (2013) Effect of tags and labels on the performance of enzyme-amplified electrochemical genosensing assays. *Electroanalysis* 25(1):147–153
- Li Q, Cheng W, Zhang D, Yu T, Yin Y, Ju H, Ding S (2012) Rapid and sensitive strategy for salmonella detection using an InvA gene-based electrochemical DNA sensor. *Int J Electrochem Sci* 7(1):844–856
- Bettazzi F, Lucarelli F, Palchetti I, Berti F, Marrazza G, Mascini M (2008) Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Anal Chim Acta* 614 (1):93–102. doi:10.1016/j.aca.2008.03.027
- Campuzano S, Kuralay F, Wang J (2012) Ternary monolayer interfaces for ultrasensitive and direct bioelectronic detection of nucleic acids in complex matrices. *Electroanalysis* 24(3):483–493
- Raof M, Jans K, Bryce G, Ebrahim S, Lagae L, Witvrouw A (2013) Improving the selectivity by using different blocking agents in DNA hybridization assays for SiGe bio-molecular sensors. *Microelectron Eng* 111:421–424
- Wang W, Wang YJ, Wang DQ (2008) Dual effects of Tween 80 on protein stability. *Int J Pharm* 347(1–2):31–38
- Ha E, Wang W, Wang YJ (2002) Peroxide formation in polysorbate 80 and protein stability. *J Pharm Sci* 91(10):2252–2264
- Ku W, Lau W, Tseng Y, Tzeng C, Chiu S (2004) Dextran sulfate provides a quantitative and quick microarray hybridization reaction. *Biochem Biophys Res Commun* 315(1):30–37
- García-Mendiola T, Gamero M, Campuzano S, Revenga-Parra M, Alonso C, Pedrero M, Pariente F, Pingarrón J, Lorenzo E (2013) Nanostructured rough gold electrodes as platforms to enhance the sensitivity of electrochemical genosensors. *Anal Chim Acta* 788:141–147
- Flechsich G-U, Peter J, Hartwich G, Wang J, Gründler P (2005) DNA hybridization detection at heated electrodes. *Langmuir* 21(17):7848–7853
- Walter A, Surkus A-E, Flechsich G-U (2013) Hybridization detection of enzyme-labeled DNA at electrically heated electrodes. *Anal Bioanal Chem* 405(11):3907–3911
- Carpini G, Lucarelli F, Marrazza G, Mascini M (2004) Oligonucleotide-modified screen-printed gold electrodes for enzyme-amplified sensing of nucleic acids. *Biosens Bioelectron* 20 (2):167–175
- Rose K, Mason J, Lathe R (2002) Hybridization parameters revisited: solutions containing SDS. *Biotechniques* 33(1):54–58
- Chen D, Li J (2006) Interfacial design and functionalization on metal electrodes through self-assembled monolayers. *Surf Sci Rep* 61(11): 445–463
- Kuralay F, Campuzano S, Wang J (2012) Greatly extended storage stability of electrochemical DNA biosensors using ternary thiolated self-assembled monolayers. *Talanta* 99:155–160

4.4 Genosensor para la detección de Tri a 18

Del análisis de secuencias de ADN se dedujo que el método que más sensible es el que amplifica la secuencia que codifica la $\alpha 2$ -gliadina, secuencia multicopia. Entre las secuencias que se encuentran en una sola copia, la más adecuada debido a su sensibilidad y selectividad es la que amplifica la secuencia oligonucleotídica de ADN que codifica Tri a 18, esto es, una aglutinina presente en los cereales tóxicos.

Por este motivo se diseñó un genosensor electroquímico enzimático, con un formato idéntico a los descritos anteriormente, para la detección de la secuencia de ADN que codifica Tri a 18. La fase sensora se preparó mediante la adición de 15 μ L de 2 μ M de sonda CP-Tri a 18 tiolada (Tabla 3) para su inmovilización por quimisorción sobre la superficie de electrodos de oro serigrafiado (durante toda la noche), seguido del bloqueo de los sitios de la superficie no recubiertos por la sonda de captura con 10 μ L de 0,5 mM de MCH durante 30 min. Esta etapa proporciona además una reorganización de la CP. El ensayo se realizó en varias etapas. En una primera etapa, donde ocurre la hibridación homogénea, se ponen en contacto 1,25 μ M de sonda marcada con FITC, denominada SP-Tri a 18, con el analito (5 min en a 98 °C, 5 min en baño de hielo y 30 min a temperatura ambiente). El dúplex formado se puso en contacto con la CP inmovilizada sobre el electrodo (reacción heterogénea de 2 h). Posteriormente se adicionaron 15 μ L de 0,5 U/mL de enzima HRP-antiFITC que se dejaron reaccionar durante 30 min. Por último, se realizó la detección cronoamperométrica a -0.2 V tras la adición de 40 μ L de TMB que ha reaccionado durante 1 min para dar lugar al producto electroactivo.

Tabla 3. Secuencias de las sondas y analito del genosensor Tri a 18.

	Secuencias oligonucleotídicas 5' → 3'
Analito Tri a 18 (trigo, 125 nt)	CTG TTG TAG CAA GTG GGG ATC CTG TGG CAT CGG CCC GGG CTA TTG CGG TGC AGG CTG CCA GAG TGG CGG CTG CGA TGG TGT CTT CGC CGA GGC CAT CAC CGC CAA CTC CAC TCT TCT CCA AGA AT
SP- Tri a 18 (75 nt)	TCG CAG CCG CCA CTC TGG CAG CCT GCA CCG CAA TAG CCC GGG CCG ATG CCA CAG GAT CCC CAC TTG CTA CAA CAG -FITC
CP- Tri a 18 (50 nt)	HS-(CH ₂) ₆ - ATT CTT GGA GAA GAG TGG AGT TGG CGG TGA TGG CCT CGG CGA AGA CAC CA

Partiendo de las condiciones ya optimizadas en el genosensor de gliadina, se realizaron ensayos con el nuevo genosensor de Tri a 18. Las intensidades de corriente registradas fueron muy altas, tanto en presencia

como en ausencia de analito, lo que generaba una S/B muy baja, S/B~1 (Figura 12).

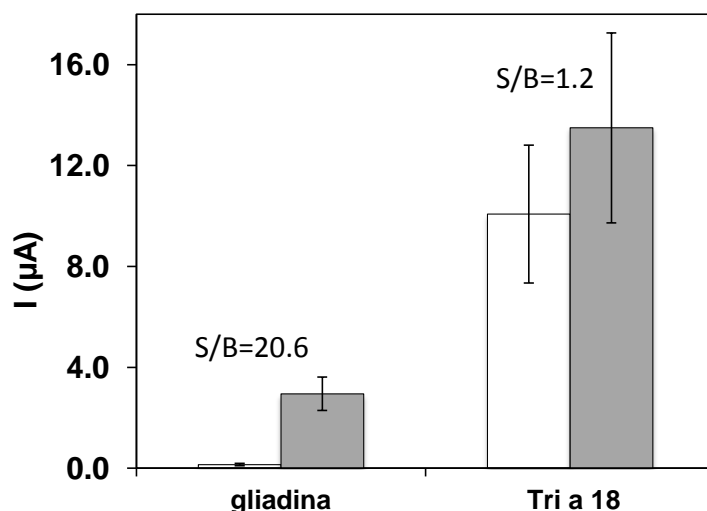


Figura 12. Comportamiento comparativo para los genosensores de gliadina y Tri a 18 (barra grises en presencia de 10 nM de analito, barras blancas en ausencia de analito). Cada ensayo repetido por triplicado.

Para intentar minimizar la señal del blanco, se probó a utilizar monocapas ternarias, que generalmente originan un mayor bloqueo de las adsorciones inespecíficas (Campuzano et al. 2011; Kuralay et al. 2012), debido a la disposición que adopta el tercer componente, un ditiol, sobre la superficie del electrodo (Figura 13). Se preparó el genosensor con una monocapa ternaria compuesta por 1 µM de hexanoditiol (HDT), 2 µM CP-Tri a 18, y 1 mM de MCH, pero se obtuvieron idénticos resultados.

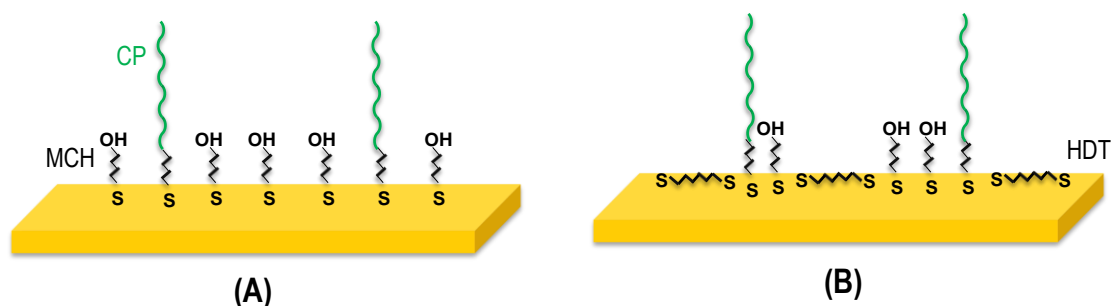


Figura 13. Representación esquemática de las monocapas del genosensor: (A) monocapa binaria con CP y MCH; (B) monocapa ternaria con CP, HDT y MCH.

Descartado el ineficiente bloqueo electródico, se pensó que la única causa posible de estos resultados podría ser la interacción de las sondas CP-

Tri a 18 y SP-Tri a 18 entre sí. Para verificar esta hipótesis se utilizó una herramienta informática disponible en la web, MFold Web Server (Zuker 2003) para las sondas que componen tanto el genosensor de gliadina como el de Tri a 18. Se obtuvo una energía de hibridación, ΔG , de -6,5 kcal/mol y -15,8 kcal/mol (Figura 14) para la reacción entre CP y SP, para gliadina y Tri a 18, respectivamente. Estudios previos realizados por nuestro grupo, para genosensores similares, han proporcionado buenos resultados para sondas CP y SP, que interactúan entre sí con hasta una ΔG de -9,83 kcal/mol. Aunque la diferencia entre estas energías libres de Gibbs no es muy grande, parece ser suficiente para que la hibridación entre las sondas CP-Tri a 18 y SP-Tri a 18 ocurra siempre que el blanco no esté presente, lo que impide el uso de estas sondas tal y como están diseñadas. Por lo tanto, parece que se puede establecer un límite práctico en el diseño de sondas en torno a $\Delta G_{\text{hibridación CP-SP}} = -10$ kcal/mol, a partir del cual la estabilidad del híbrido CP-SP es suficientemente alta para ser detectado mediante marcaje enzimático y detección electroquímica. El diseño de un genosensor para la secuencia específica de Tri a 18 previamente seleccionada fue finalmente descartado.

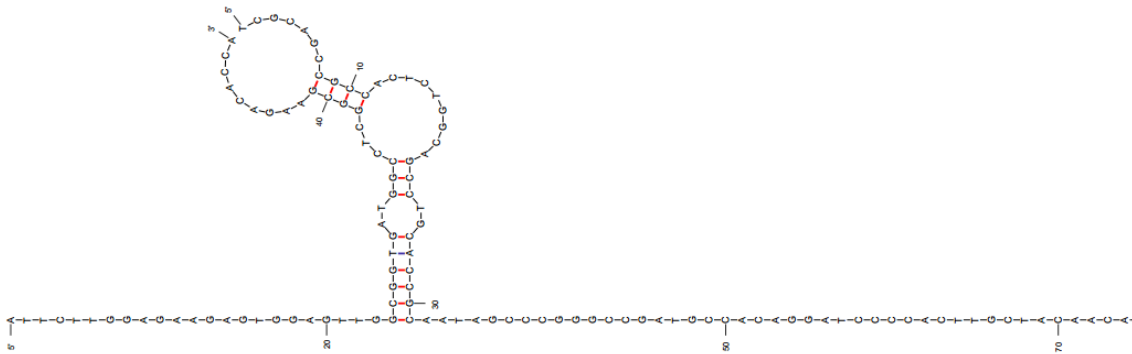


Figura 14. Estructura más estable de la hibridación entre CP-Tri a 18 + SP-Tri a 18.

Bibliografía

Campuzano, S., Kuralay, F., Lobo-Castañón, M.J., Bartošík, M., Vyavahare, K., Paleček, E., Haake, D.A., Wang, J., 2011. Ternary monolayers as DNA recognition interfaces for direct and sensitive electrochemical detection in untreated clinical samples. *Biosensors and Bioelectronics* 26(8), 3577-3583.

Kuralay, F., Campuzano, S., Wang, J., 2012. Greatly extended storage stability of electrochemical DNA biosensors using ternary thiolated self-assembled monolayers. *Talanta* 99, 155-160.

5 ANÁLISIS DE CURVAS DE DISOCIACIÓN DE ALTA RESOLUCIÓN (HRM)

High resolution melting analysis as a new approach to
discriminate gluten-containing cereals

ANÁLISIS DE CURVAS DE DISOCIACIÓN DE ALTA RESOLUCIÓN (HRM)

La detección de secuencias de ADN que codifican tanto gliadina como aglutinina (Tri a 18) es de gran utilidad para detectar gluten en alimentos, tanto por PCR en tiempo real como con el genosensor de gliadina, que aporta un nivel adicional de selectividad a los métodos de PCR ya descritos, ya que es capaz de discriminar el origen del gluten. El genosensor permitió determinar selectivamente secuencias de ADN de trigo respecto a secuencias homólogas de otros cereales tóxicos para los celíacos.

Sería deseable, además el desarrollo de un método capaz de discriminar entre todas las diferentes especies nocivas para los pacientes celíacos, esto es, diferenciar si el alimento en cuestión contiene trigo, cebada, centeno o avena. Esto requeriría la puesta a punto de genosensores específicos de secuencias para cada uno de estos cereales y el análisis en paralelo de las muestras con todos ellos, lo que resultaría relativamente caro y laborioso. Por este motivo, se exploró otra técnica: el análisis de curvas de disociación de alta resolución, HRM análisis. Esta técnica es no destructiva, y relativamente rápida para genotipado de secuencias de ADN similares sin necesidad de secuenciación. El análisis HRM se basa en la monitorización de la disociación o deshibridación del amplicón de PCR mediante la adición de una molécula intercalante fluorescente, cuando se la somete a un aumento gradual de la temperatura (Wittwer 2009). La curva de disociación es característica de cada secuencia, por lo que mediante el uso de un instrumento que permita el control preciso de la temperatura y un fluoróforo adecuado, se pueden detectar pequeñas variaciones de la secuencia, que conducen a perfiles no distinguibles en curvas de disociación convencionales. Este tipo de análisis permite la identificación del fragmento amplificado mediante la caracterización del perfil de disociación o fusión; la pureza de la muestra mediante la ausencia de distorsiones de la masa disociada de control de la curva (y la ausencia de picos de disociación adicionales), y el rendimiento de la amplificación mediante la intensidad de la señal de fluorescencia (Vossen et al. 2009).

Este tipo de análisis ya ha demostrado la alta capacidad de discriminación entre especies vegetales estrechamente relacionadas (Costa et al. 2012). Sin embargo, a pesar de que es capaz de diferenciar entre secuencias que varían sólo unos pocos nucleótidos, no permite el análisis cuantitativo de las muestras, por lo que constituye un complemento a otros métodos cuantitativos como los desarrollados previamente.

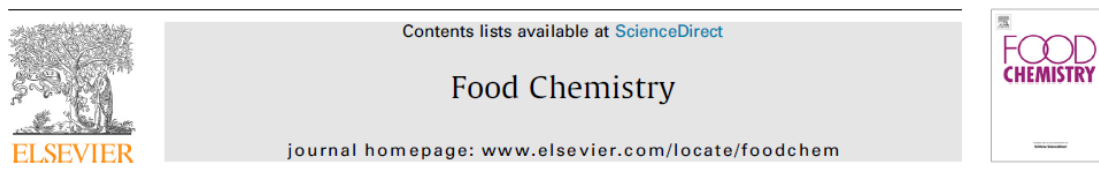
Bibliografía

Costa, J., Mafra, I., Oliveira, M.B.P.P., 2012. High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry* 133(3), 1062-1069.

Vossen, R., Aten, E., Roos, A., den Dunnen, J.T., 2009. High-Resolution Melting Analysis (HRMA)-More Than Just Sequence Variant Screening. *Human Mutation* 30(6), 860-866.

Wittwer, C.T., 2009. High-Resolution DNA Melting Analysis: Advancements and Limitations. *Human Mutation* 30(6), 857-859

(submitted)



High resolution melting analysis as a new approach to discriminate gluten-containing cereals

Begoña Martín-Fernández^{a,b}, Joana Costa^a, Noemí de-los-Santos-Álvarez^c, Beatriz López-Ruiz^b, M. Beatriz P.P. Oliveira^a and Isabel Mafrá^{a,*}

^aREQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

^bSección Departamental de Química Analítica, Facultad de Farmacia, Universidad Complutense de Madrid, Pz. Ramón y Cajal s/n, 28040 Madrid, Spain

^cDepartamento Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain

* Corresponding author. Tel.: +351 220428640; fax: +351 226093390.

E-mail address: isabel.mafrá@ff.up.pt (I. Mafrá)

ABSTRACT

Gluten-containing cereals (wheat, barley, rye) are responsible for triggering allergic responses and celiac pathologies in predisposed individuals as a result of an abnormal immunological reaction to them. As a consequence, it is essential to have easy and reliable methodologies to verify the safety of food products for these patients. Providing the close relationship and high homologies among these cereals, their discrimination is difficult to achieve. Here we developed a novel, simple, fast and highly specific approach based on the high resolution melting (HRM) analysis to detect wheat and discriminate it from other related cereals, such as barley, rye and oat through the comparison of DNA melting profiles. The method consists of a real-time PCR assay targeting the gene encoding for the Tri a 18 allergen, using the fluorescent EvaGreen[®] dye combined with HRM analysis. Results demonstrated that this analysis can act as a cost effective tool for the efficient detection of cereals in foods, discriminating their origin at trace levels.

Keywords

Real-time PCR, gluten-containing cereals, wheat, celiac disease, wheat allergy, agglutinin isolectin.

INTRODUCTION

Food allergy is defined as an adverse immunological response in sensitised individuals that occurs reproducibly upon exposure to a given food(s) (Boyce et al., 2010). Among foods classified as allergenic, wheat and other gluten-containing cereals are part of one of the eight groups responsible for triggering almost 90% of the reported food allergic reactions. Allergies related to the consumption of wheat (or other gluten-containing cereals) are mainly classified as IgE-mediated (baker's asthma and wheat-dependent exercise-induced anaphylaxis) or as non-IgE mediated (celiac disease), depending on the activation mechanism (Tatham & Shewry, 2008). In IgE-mediated allergy, wheat allergens are the main cause for most of clinical symptoms, while in celiac disease the immunological inflammatory response is triggered by the gluten fraction (glutenins and gliadins) that is common to several cereals: wheat, rye, barley, kamut, spelt, and, in some cases, oat (Denham & Hill, 2013; Haraszi, Chassaing, Maquet, & Ulberth, 2011; Tatham & Shewry, 2008).

To protect the health of patients with celiac disease or wheat allergy, the avoidance of gluten-containing cereals is required. However, their total elimination from most diets is extremely difficult. In the specific case of celiac disease, the concept of "gluten-free", referring to a level of gluten that is harmless when ingested indefinitely, is currently widely accepted (Hischenhuber et al., 2006). Presently, food products containing a maximum of 20 mg/kg of gluten are labelled as "gluten-free" (Regulation (EU) No. 41/2009), being this limit considered as a safe threshold level for most celiac individuals. Nevertheless, the definition of an upper safe limit is still not universally accepted in food allergy since the sensitivity of celiac/wheat-allergic patients to gluten varies on an individual basis (Hischenhuber et al., 2006). Therefore, the referred threshold level (20 mg/kg of gluten) represents a reference value for both celiac and wheat-allergic individuals, contributing to a better management of their personal allergies.

In spite of the recent legislation that establishes limits of 20 mg/kg or 100 mg/kg, respectively for "gluten-free" or "very low gluten" products, no specific analytical methodology has yet been recommended for gluten monitoring. Due to the high stability of DNA molecules, methods relying on them have attained an increasing role in food safety control. In the specific case of allergen analysis, DNA-based techniques have been pointed out as highly reliable, sensitive and specific tools for the detection, identification and quantification of gluten-containing cereals in foods. Presently, methods based on polymerase chain reaction (PCR) are widely used for gluten analysis, being available either commercially (e.g. SureFood[®] Allergen Quant Gluten, R-Biopharm AG, Darmstadt, Germany) or reported in the literature (Martin-Fernandez, Miranda-Ordieres, Lobo-Castanon, Frutos-Cabanillas, de-los-Santos-Alvarez, & Lopez-

Ruiz, 2014; Martin-Fernandez, Costa, Oliveira, Lopez-Ruiz, & Mafra, 2015; Mujico, Lombardia, Mena, Méndez, & Albar, 2011; Pinto, Polo, Henry, Redondo, Svodova, & O'Sullivan, 2014).

With the recent advances on high resolution instrumentation and with the development of specialised and more efficient fluorescent DNA-binding dyes, the novel approach of high-resolution melting (HRM) analysis has been recently applied to food analysis. HRM measures the rate of dissociation of double stranded DNA to single stranded DNA through small increments of temperature (0.01-0.2 °C/s) (Druml & Cichna-Markl, 2014; Reed, Kent, & Wittwer, 2007), generating DNA melt curve profiles that are both specific and sensitive to distinguish nucleic acid species based on minor nucleic acid differences. In theory, a single substitution of a nucleotide (e.g. adenine by guanine) is sufficient to discriminate two fragments of similar size and composition (Reed et al., 2007). With respect to food analysis, HRM has been applied for the identification/differentiation of varieties and closely related species, genotyping and serotyping of pathogenic microorganisms, screening of genetically modified organisms and detection of food allergens (see review of Druml & Cichna-Markl, 2014). Reports in the literature describe the successful discrimination of almond allergens from other related species namely peach, nectarine and apricot (Costa, Mafra, & Oliveira, 2012a) and of hazelnut from other nuts (Madesis, Ganopoulos, Bosmali, & Tsaftaris, 2013) by HRM analysis. Following the referred achievements, this work intends to propose, to the best of our knowledge, a first attempt of using HRM analysis to differentiate and detect gluten-containing cereals targeting the gene encoding for the germ agglutinin isolectin A (Tri a 18) allergen.

MATERIALS AND METHODS

Sample preparation

Flours of different plants from the Poaceae family (wheat, rye, barley, oat, maize, soybean and rice) were obtained at local markets. In the absence of certified or testing reference standards for wheat, binary model mixtures containing: 1 mg/kg, 5 mg/kg, 10 mg/kg, 20 mg/kg, 50 mg/kg, 100 mg/kg, 500 mg/kg, 1,000 mg/kg and 5,000 mg/kg, 10,000 mg/kg, 50,000 mg/kg, 100,000 mg/kg and 500,000 mg/kg of wheat flour in soybean material were prepared. The first sample containing 500,000 mg/kg (50%) of wheat was prepared by adding 50 g of wheat flour to 50 g of soybean flour. All the other model mixtures were prepared by successive additions of soybean flour until the spiked level of 1 mg/kg (0.0001%) in the equivalent proportion to a final weight of 100 g in a non-target plant matrix (soybean flour). All the mixtures were thoroughly homogenised separately in a laboratory knife mill Grindomix GM200 (Retsch, Haan, Germany), using different containers and material, previously treated with

a DNA decontamination solution (DNA-ExitusPlus™, AppliChem, Darmstadt, Germany). To avoid accidental cross-contaminations among samples, cereals and reference mixtures were prepared on different days. After preparation, all samples and reference mixtures were immediately stored at -20°C until further DNA extraction.

DNA extraction

DNA was extracted from 100 mg of each sample or model mixture using the commercial Nucleospin Food kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions with minor alterations, as previously described (Costa, Mafra, Kuchta, & Oliveira, 2012b). A negative control sample was included in every DNA extraction. Yield and purity of extracts were assessed by agarose gel electrophoresis and by UV spectrophotometric DNA quantification on a SynergyHT multi-mode microplate reader (BioTek Instruments, Inc., Vermont, USA), using a Take3 micro-volume plate accessory. DNA content was determined using the nucleic acid quantification protocol with sample type defined for double-strand DNA in the Gen5 data analysis software version 2.01 (BioTek Instruments, Inc., Vermont, USA).

Target gene selection and oligonucleotide primers

In the official list of allergens of WHO/IUIS (World Health Organization/International Union of Immunological Societies) there are 21 wheat allergenic proteins, from which 9 are classified as food allergens, namely Tri a 12, Tri a 14, Tri a 18, Tri a 19, Tri a 20, Tri a 25, Tri a 26, Tri a 36 and Tri a 37. As a potential DNA marker for wheat and other gluten-containing cereals detection, the nucleotide sequence encoding the allergenic protein agglutinin isolectin A was retrieved from the NCBI database (<http://www.ncbi.nlm.nih.gov/>). This allergen belongs to the pathogenesis-related (PR)-3 family of proteins or class I chitinases, which is an important family of allergenic proteins (Breiteneder & Radauer, 2004). Primers, presented in Table 1, were specifically designed on the Tri a 18 encoding sequence (GenBank accession no. M25536.1), using the software Primer-BLAST designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

The basic local alignment search tool BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was also used to identify regions of local similarity between the chosen nucleotide and homologue sequences of different species. *In silico* analysis estimated the statistical significance of the matches and confirmed the specificity of the designed primers with 100% and 93% of identity for wheat species (*Triticum aestivum* and *Triticum durum*) and 91% identity for barley (*Hordeum vulgare*) considering 100% of query cover. As a positive amplification control for end-point PCR and real-time PCR assays,

universal primers (18SRG-F/18SRG-R) targeting a conserved DNA region (nuclear 18S rRNA gene) were also used (Table 1).

Table 1. Key data of primers designed to specifically target agglutinin isolectin A (Tri a 18) encoding sequence of *Triticum aestivum* and a conserved eukaryotic region (Nuclear 18S rRNA).

Nucleotide sequence (GenBank accession no.)*	Primer sequences (5' → 3')	Amplicon (bp)	References	
Agglutinin isolectin A (Tri a 18) (*M25536.1)	Tri18-F Tri18-R	CTGTTGTAGCAAGTGGGGATCCT ATTCTTGGAGAAGAGTGGAGTTGG	125	Martin-Fernandez et al. (2015)
	Tri18-FS Tri18-RS	TACGGGTACTGCGGCTTCGG CCATGCATGCATCCTGACAACAG	649	This work
Nuclear 18S rRNA	18SRG-F 18SRG-R	TCTGCCCTATCAACTTTCGATGG TAATTTGCGCGCCTGCTG	113	Costa et al. (2013)

Primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

Sequencing

In order to verify the identity of the amplified fragments, a second set of primers (Tri18-FS/ Tri18-RS) was specifically designed to produce a longer fragment for wheat and related species (Table 1), covering the target region amplified by the primers (Tri18-F/Tri18-R). PCR products were purified with Jetquick PCR purification kit (Genomed, Löhne, Germany) to remove any possible interfering components and sent to a specialised research facility (STABVIDA, Lisbon, Portugal) for sequencing. Each target fragment was sequenced twice, performing the direct sequencing of both strands in opposite directions, which allowed the production of two complementary sequences of high quality.

End-point PCR

PCR amplifications were carried out in 25 µL of total reaction volume containing 2 µL of DNA extract (200 ng), 67 mM of Tris–HCl (pH 8.8), 16 mM of (NH₄)₂SO₄, 0.01% of Tween 20, 200 µM of each dNTP, 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience, Ulm, Germany), 3.0 mM of MgCl₂ and 120 nM of each primer (Tri18-F/Tri18-R) (Table 1). For sequencing, the PCR components were the same, but using the set of primers Tri18-FS/Tri18-RS (Table 1). The reactions were performed in a MJ Mini thermal cycler (BioRad, Hercules, CA, USA), using the following program: initial denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 30 s, 63 °C for 30 s or 60°C for 45s and 72°C

for 30 s or 1 min, respectively for primers Tri18/Tri18R or Tri18-FS/Tri18-RS; and a final extension at 72°C for 5 min. PCR amplifications targeting the universal region (18S rRNA) were performed as described by Costa, Oliveira, & Mafra (2013).

The amplified fragments were analysed by electrophoresis in a 1.5% agarose gel containing 1× Gel Red (Biotium, CA, USA) for staining and carried out in 1× SGTB buffer (GRISP, Porto, Portugal) for 20 min at 200 V. The agarose gel was visualised under a UV light tray Gel Doc™ EZ System (BioRad Laboratories, Hercules, CA, USA) and a digital image was obtained with Image Lab software version 5.1 (BioRad Laboratories, Hercules, CA, USA).

Real-time PCR assays and HRM analysis

Real-time PCR assays were performed in 20 µL of total reaction volume containing 2 µL of DNA extract (100 ng), 1× of SsoFast Evagreen Supermix (BioRad, Hercules, CA, USA) and 200 nM of each primer Tri18-F/Tri18-R (Table 1). The reactions were performed in a fluorometric thermal cycler CFX96 real-time PCR detection system (BioRad, Hercules, CA, USA), following the program: 95 °C for 5 min, 40 cycles at 95 °C for 15 s and 63 °C for 45 s, with the fluorescence signal acquisition at the end of each cycle. Data were collected and processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA).

For HRM analysis, PCR products were denatured at 95°C for 1 min and then annealed at 63 °C with temperature increments of 0.02°C/s and acquisition of fluorescence data at the end of each melting temperature. The collected data were further processed using the Precision Melt Analysis Software 1.2 (Bio-Rad Laboratories, Hercules, CA, USA) to generate melting curves as a function of temperature and difference curves for easier visualisation of the clusters. Melting curve shape sensitivity determines the stringency used to classify melting curves into different clusters, while temperature of melting (T_m) difference threshold is a parameter that determines the lowest amount of T_m difference between samples. In both cases, cluster detection parameters were set to high sensitivity and threshold yields aiming at providing more heterozygote clusters. Therefore, melting curve shape sensitivity was adjusted to a percentage value of 70% and T_m difference threshold was set as 0.20. Samples were analysed in triplicates in two independent assays.

RESULTS AND DISCUSSION

Tri a 18 is a wheat germ agglutinin that was firstly identified as allergen for bakers by Sutton, Skerritt, Baldo, & Wrigley (1984). This small disulphide-rich lectin protein has a homodimeric structure (Breiteneder & Radauer, 2004) and appears in multiple molecular forms due to the polyploidy nature of wheat.

In hexaploid wheat (*T. aestivum*), agglutinin presents three isolectins, namely A, B and D, which are encoded by homologous genes, presenting 95-97% and 91-94% sequence identity at the amino acid (Wright & Raikhel, 1989) and at nucleotide levels (Raikhel & Wilkins, 1987; Smith & Raikhel, 1989), respectively. Based on a previous study concerning the selection of new DNA markers for gluten detection (Martin-Fernandez et al., 2015), the Tri a 18 (wheat germ agglutinin isolectin A) (GenBank accession no. M25536.1) was the selected gene for the development of the novel HRM approach for the discrimination of gluten-containing cereals.

End-point PCR

Prior to the target gene specific amplification, to eliminate any false negative results, all DNA extracts were evaluated for their amplifiability by end-point PCR targeting a conserved nuclear 18S rRNA gene, as a universal eukaryotic marker sequence with primers 18SRG-F/18SRG-R (Costa et al., 2013). All samples amplified positively the expected fragment of 113 bp, confirming that the DNA extracts had the adequate quality, yield and purity for PCR amplification. The specificity of the primers targeting Tri a 18 encoding sequence was previously assayed using a total of 27 plant species, from which only wheat, barley, rye and oat amplified positively the target gene. Therefore, there was no cross-reactivity with other plant species rather than gluten-containing cereals (barley, wheat, rye and oat).

Real-time PCR and melting analysis

Wheat, barley, rye and oat DNA extracts were amplified with Tri18-F/Tri18-R primers by real-time PCR using EvaGreen as fluorescence dye. All cereals amplified positively as expected based on the end-point PCR results, but with different cycle threshold (Ct) values. For the same quantity of DNA (200 ng), wheat was the first cereal to be amplified, being followed by rye, barley and oat with differences of 1.4, 4.5 and 7.9 cycles, respectively (Table 2). From the amplification results, products from wheat seem to present higher similarity to rye and more distantly related to oat. However, by conventional melting analysis, the fragments from the four cereals presented two distinct melting profiles at ~87.5 °C for wheat and oat peaks and at ~89 °C for barley and rye (Figure 1, Table 2). As a result, from the melting temperatures, fragments from wheat/oat and from barley/rye seemed to have similar composition and/or size, disabling the unequivocal identification of each pair of cereals.

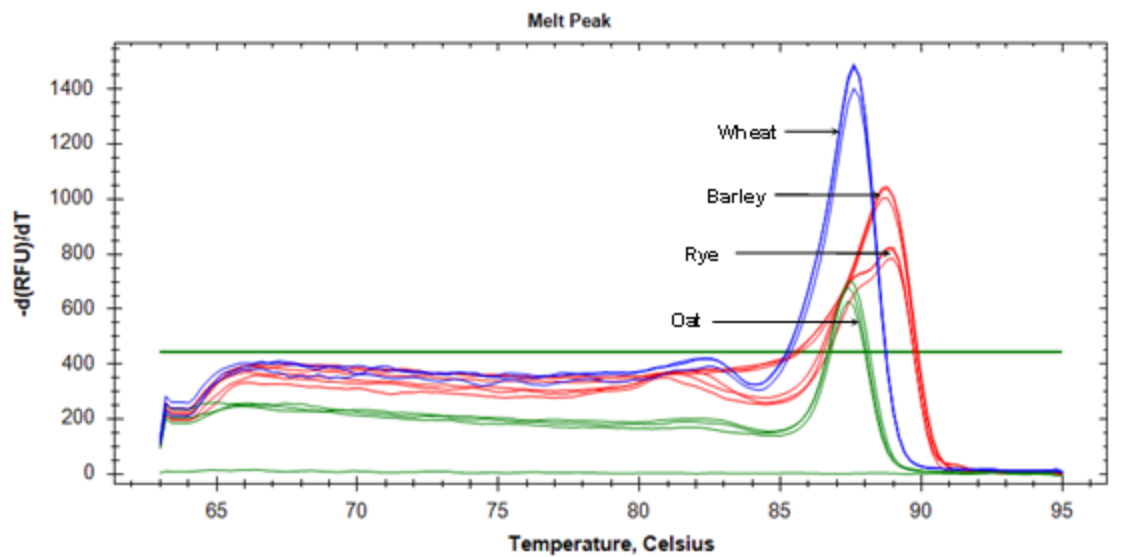


Figure 1. Melting curves obtained by real-time PCR with EvaGreen® dye targeting the Tri a 18 gene of wheat and applied to gluten-containing cereals.

HRM analysis

HRM analysis is reported as a fast, simple and cost-effective approach for scanning and genotyping similar DNA sequences, being based on the transition of the double-stranded DNA. Using an instrument with precise temperature control, slight sequence variations can be detected by the denaturation of DNA fragments as a result of decreasing fluorescence due to dissociation of the double-stranded DNA-specific dye (Costa et al, 2012a). Therefore, it is a very useful tool to discriminate sequences from highly phylogenetically related species, such as the gluten-containing cereals. As such, HRM analysis was applied to differentiate the four cereal species that conventional melting curve analysis was not capable to do.

Figure 2 shows the resultant application of HRM analysis to discriminate wheat, rye, barley and oat. The small differences among melting curves presented in the normalised plot (Figure 2A) can be better visualised in the difference curve plot (Figure 2B). In Figure 2B, it is possible to observe that wheat curves have similar shape and were included in the same group (cluster 1), being defined as the reference cluster with percentages of confidence over 99.5%. Rye and barley, which formerly produced fragments with similar melting temperatures (Figure 1B), were distinguished from wheat and included in a distinct group (cluster 3) with a percentage of confidence above 98.7% (Table 2). Oat amplicons were included in cluster 2, with high level of confidence (>98.5%) (Figure 2, Table 2), being now differentiated from wheat. As result, HRM analysis allowed discriminating the wheat Tri a 18 allergen encoding sequence from other closely related species, which is in good agreement with other reports (Costa et al., 2012a; Madesis et al., 2013).

Table 2. Results of two independent real-time PCR assays (n=6) with melting and high resolution melting (HRM) analysis applied to gluten-containing cereals (wheat, rye, barley and oat)

Gluten-containing cereal	Ct \pm SD ^a	Tm \pm SD ^b (°C)	Cluster (% of confidence \pm SD)
Wheat	28.00 \pm 0.27	87.60 \pm 0.00	Cluster 1 (99.5 \pm 0.1)
Rye	29.36 \pm 0.17	88.80 \pm 0.00	Cluster 3 (99.2 \pm 0.5)
Barley	32.56 \pm 0.25	88.93 \pm 0.10	Cluster 3 (99.2 \pm 0.5)
Oat	35.92 \pm 0.20	87.40 \pm 0.00	Cluster 2 (98.6 \pm 0.1)

^a Ct \pm SD – mean cycle threshold (Ct) values \pm standard deviation; ^b Tm \pm SD – mean melting temperature values \pm standard deviation; Cluster 1 considered as the reference cluster.

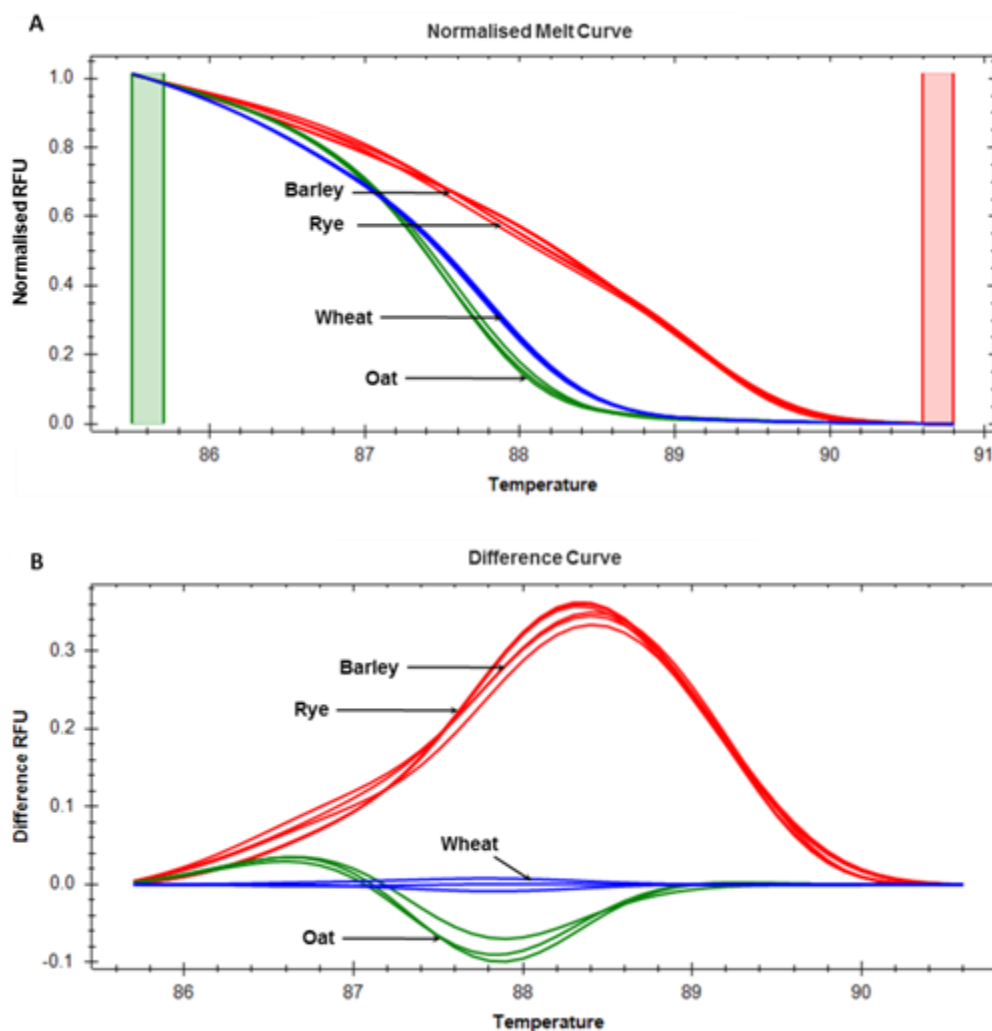


Figure 2. HRM analysis of real-time PCR products with EvaGreen® dye targeting the Tri a 18 gene of wheat and applied to different cereal species. (A) Normalised melting curves and (B) difference curves.

Despite the fact that oat is not clearly related to celiac or wheat allergy, its consumption is commonly not recommended due to the possibility of cross-reactivity with allergenic proteins from wheat (Tatham & Shewry, 2008). However, close genetic proximity can be attributed to all these cereals (wheat, rye, barley and oat), which is herein highlighted by both end-point PCR and real-time PCR results, suggesting potential cross-reactivity phenomena. These species produced fragments with similar size and melting temperatures, mostly due to their phylogenetic proximity and the conserved nature of the targeted sequence, a wheat germ agglutinin (Chapot, Peumans, & Strisberg, 1986). With the application of HRM analysis, it was possible to distinguish small variations among these related species, contributing to discriminate wheat from other gluten-containing cereals.

Sequencing results

The results presented by HRM analysis were further investigated and confirmed by sequencing the PCR products from wheat and the other tested cereals of the Poaceae family. For this purpose, sequencing primers (Tri18-FS/Tri18-RS) to produce PCR products of 649 bp were specifically designed to encompass the target DNA sequence of 125 bp flanked by Tri18-F/Tri18-R primers. Although oat DNA could be amplified positively for the target fragment of 125 bp, no fragments were obtained using Tri18-FS/Tri18-RS primers, thus precluding its sequencing.

Sequencing results from wheat, rye and barley were aligned and compared with an available wheat sequence from the Genbank database of NCBI (M25536.1). Since each analysed cereal was amplified twice, with direct sequencing of both strands in opposite directions, a total of $n=4$ strands were obtained. All sequencing results evidenced high resolution electropherograms, being consistent among replicates of each target. Considering that the platform often does not allow perfect resolution for the reading of the first ~50 bp at the 5'-end of the sequence, data on Figure 3 refers to sequencing of 532 bp regions with double coverage. Marked differences could be observed among wheat, rye and barley sequences, as well as with the sequence from the Genbank database. Within the region of interest (125 bp) used for HRM analysis, some differences could also be observed among the same sequences. In relation to the available Genbank sequence, a total of 5 nucleotide differences were evidenced in positions 121, 160, and 181, where sequenced products present residues of cytosine (C) instead of thymine (T) and in positions 236-237 guanine (G) and cytosine (C) are present instead of cytosine (C) and adenine (A), respectively. These consistent differences could be attributed to potential sequencing mistakes on the available Genbank sequence or to the genetic variability of multiploid species such as wheat (Eilam, Anikster, Millet, Manisterski, & Feldman, 2008; Wright, & Raikhel, 1989).

In the case of wheat the differences were the five referred mismatches, while in rye and barley sequences two and five additional mismatches were identified, respectively. Regarding the alignment of rye with wheat, the two additional differences were located in positions 166 and 195, with 2 C residues instead of T and G, respectively (Figure 3). These two nucleotide differences could be responsible for the shift in temperature of melting verified between rye and wheat (Table 2). The 5 additional nucleotide differences present in barley were located in positions 124 and 166, corresponding to C→T, in position 172 (T→C), in position 215 (G→A) and in position 233 (G→C). Since two of the additional differences were located in the region of primer annealing, in both forward and reverse, this might be responsible for the much delayed amplification of barley (4.6 cycles) in relation to wheat (Table 2).

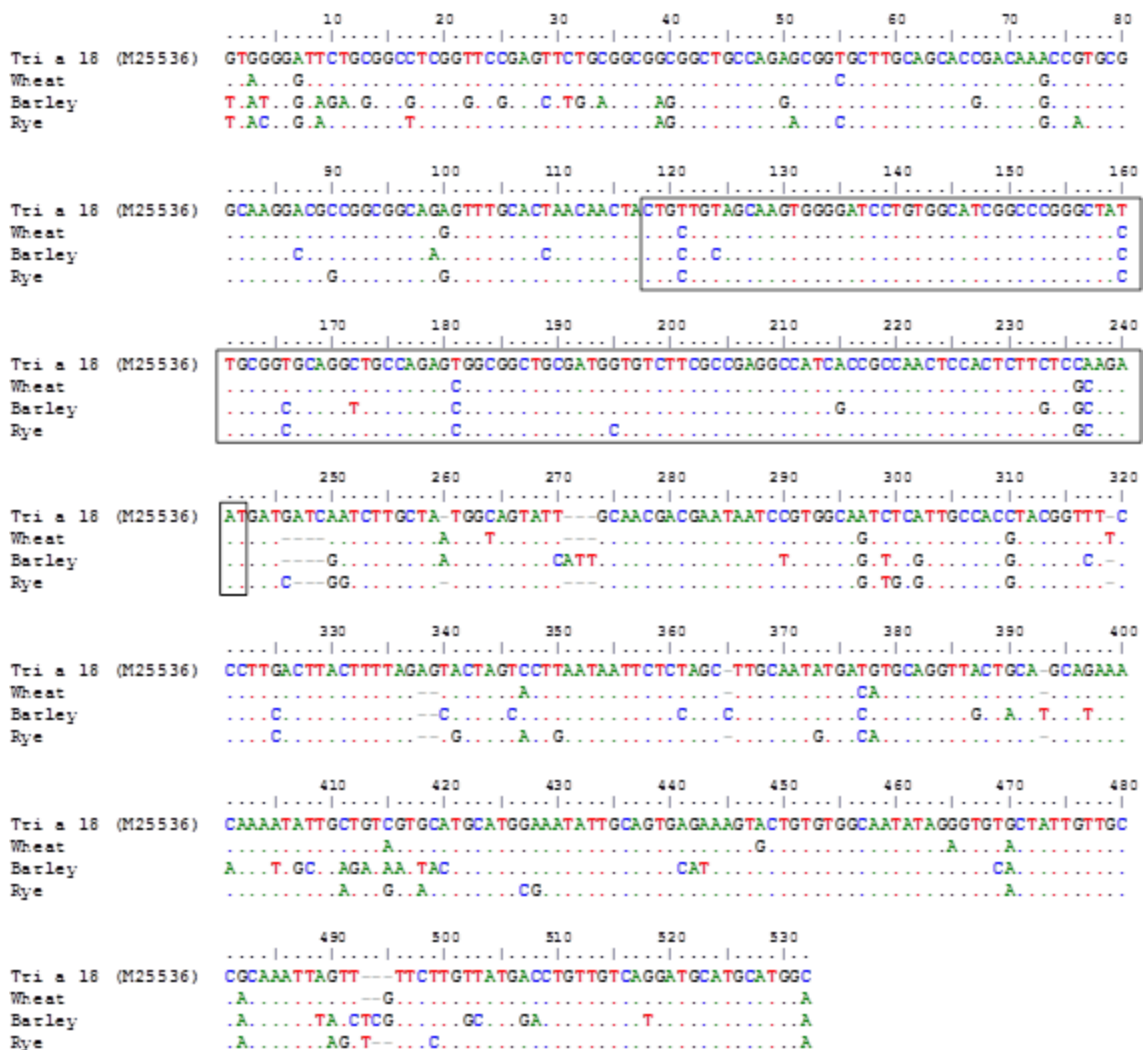


Figure 3. Sequence analysis of wheat, barley and rye, evidencing the differences in DNA alignments. The region inside the boxes refers to the amplified sequences with primers (Tri18-F/Tri18-R).

According to studies on immunological and biochemical properties, similar lectins and wheat agglutinin isolectin can be found in rye and barley (Peumans, Stinissen, & Carlier, 1982). Barley and wheat B-isolectin share 95% of sequence identity at protein level. Also, cDNA clones encoding barley lectin are homologous to the wheat agglutinin (Lerner & Raikhel, 1989). Despite the small nucleotide differences between wheat and the other cereals from the Poaceae family (rye and barley), this can justify the exclusion of rye, barley and oat, with high level of confidence (>98.5%), from the same cluster of wheat by HRM analysis. Therefore, sequencing enabled to confirm and reinforce the results of the HRM analysis. These findings are in good agreement with previous reports. In the work described by Costa et al. (2012a), the development of a HRM system allowed the discrimination of almond (*P. dulcis*) from other *Prunus* species such as apricot (*P. armeniaca*) and peach (*P. persica*) based on two nucleotide differences in Pru du 5 allergen. Likewise, Madesis et al. (2013) used an universal chloroplast *trnL* region (barcode), which amplifies polymorphic products in eleven nut species, being further discriminated by HRM analysis.

CONCLUSIONS

Herein, we report the application of HRM for genotyping of wheat and the detection/discrimination of other phylogenetically related cereals, such as barley, rye and oat, based on a DNA region that encodes the wheat germ agglutinin isolectin A (Tri a 18) allergen. These species of Poaceae family are responsible for inducing allergic reactions both in celiac and wheat-sensitised individuals, presenting highly conserved primary sequences, which difficult their analysis and discrimination. Among other plant species, a specific amplification was obtained for wheat, barley, rye and oat samples by both end-point PCR and real-time PCR targeting Tri a 18 encoding sequence. Providing the close relationship among wheat, oat, barley and rye cereal species, similar melting temperatures of real-time PCR amplicons were observed. Nevertheless, we have developed a novel and simple approach to detect very similar allergenic cereals and to discriminate them by means of HRM analysis. To the best of our knowledge, this is the first time that HRM analysis was applied as a tool for simple, fast and high-throughput identification of gluten-containing cereals in foods.

ACKNOWLEDGMENTS

This work was supported by Fundação para a Ciência e Tecnologia (FCT) through grant no. Pest-C/EQB/LA0006/2013 financed by POPH-QREN (subsidised by FSE and MCTES), the bilateral project Acções Integradas Luso-Espanholas/2012 between University of Porto (E-38/12) and University of Oviedo (Ministerio de Ciencia e Innovación, PRI-AIBPT-2011-0769). B. Martin-Fernandez and J. Costa are grateful to Complutense University PhD grant

(BE45/10) and to FCT post-doctoral grant SFRH/BPD/102404/2014, respectively.

REFERENCES

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., Plaut, M., Cooper, S. F., & Fenton, M. J. (2010). Guidelines for the Diagnosis and Management of Food Allergy in the United States: Report of the NIAID-Sponsored Expert Panel. *Journal of Allergy and Clinical Immunology*, 126(6, Supplement), S1-S58.

Breiteneder, H., & Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, 113, 821-830.

Chapot, M., Peumans, W., & Strisberg, A. (1986). Extensive homologies between lectins from non-leguminous plants. *Febs Letters*, 195, 231-234.

Costa, J., Mafra, I., & Oliveira, M. B. P. P. (2012a). High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. *Food Chemistry*, 133, 1062-1069.

Costa, J., Mafra, I., Kuchta, T., & Oliveira, M. B. P. P. (2012b). Single-tube nested real-time PCR as a new highly sensitive approach to trace hazelnut. *Journal of Agricultural and Food Chemistry*, 60, 8103-8110.

Costa, J., Oliveira, M. B. P. P., & Mafra, I. (2013). Effect of thermal processing on the performance of the novel single-tube nested real-time PCR for the detection of walnut allergens in sponge cakes. *Food Research International*, 54, 1722-1729.

Denham, J. M., & Hill, I. D. (2013). Celiac disease and autoimmunity: review and controversies. *Current Allergy and Asthma Reports*, 13, 347-353.

Druml, B., & Cichna-Markl, M. (2014). High resolution melting (HRM) analysis of DNA – Its role and potential in food analysis. *Food Chemistry*, 158, 245-254.

Eilam, T., Anikster, Y., Millet, E., Manisterski, J., Feldman, M. (2008). Nuclear DNA amount and genome downsizing in natural and synthetic allopolyploids of the genera *Aegilops* and *Triticum*. *Genome*, 51, 616-627.

Haraszi, R., Chassaing, H., Maquet, A., & Ulberth, F. (2011). Analytical methods for detection of gluten in food--method developments in support of food labeling legislation. *Journal of AOAC International*, 94, 1006-1025.

Hischenhuber, C., Crevel, R., Jarry, B., Mäki, M., Moneret-Vautrin, D.A., Romano, A., Troncone, R., & Ward, R., (2006). Review article: safe amounts of gluten for patients with wheat allergy or coeliac disease. *Alimentary Pharmacology & Therapeutics*, 23, 559-575.

Lerner, D. R., & Raikhel, N. V. (1989). Cloning and characterization of root-specific barley lectin. *Plant Physiology*, 91, 124-129.

Martin-Fernandez, B., Costa, J., Oliveira, M. B. P. P., Lopez-Ruiz, B., & Mafra, I. (2015). Screening new gene markers for gluten detection in foods. *Food Control*, 56, 57-63.

Martin-Fernandez, B., Miranda-Ordieres, A. J., Lobo-Castanon, M. J., Frutos-Cabanillas, G., de-los-Santos-Alvarez, N., & Lopez-Ruiz, B. (2014). Strongly structured DNA sequences as targets for genosensing: Sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment. *Biosensors & Bioelectronics*, 60, 244-251.

Madesis, P., Ganopoulos, I., Bosmali, I., & Tsaftaris, A., (2013). Barcode High Resolution Melting analysis for forensic uses in nuts: a case study on allergenic hazelnuts (*Corylus avellana*). *Food Research International*, 50, 351-360.

Mujico, J. R., Lombardía, M., Mena, M. C., Méndez, E., Albar, J. P., 2011. A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chemistry*, 128, 795-801.

Peumans, W. J., Stinissen, H. M., & Carlier, A. R. (1982). Isolation and partial characterization of wheat-germ-agglutinin-like lectins from rye (*Secale cereale*) and barley (*Hordeum vulgare*) embryos. *Biochemical Journal*, 203, 239-243.

Pinto, A., Polo, P., Henry, O., Redondo, M.C., Svobodova, M., O'Sullivan, C., 2014. Label-free detection of gliadin food allergen mediated by real-time apta-PCR. *Analytical and Bioanalytical Chemistry*, 406, 515-524.

Raikhel, N., & Wilkins, T. (1987). Isolation and characterization of a cDNA clone encoding wheat-germ-agglutinin. *Proceedings of the National Academy of Sciences of the United States of America*, 84(19), 6745-6749.

Reed, G. H., Kent, J. O., & Wittwer, C. T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics*, 8, 597-608.

Regulation (EU) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. *Official Journal of European Union*, L16, 3-5.

Smith, J., & Raikhel, N. (1989). Nucleotide-sequences of cDNA clones encoding wheat-germ agglutinin isolectin-A and isolectin-D. *Plant Molecular Biology*, 13(5), 601-603.

Sutton, R., Skerritt, J. H., Baldo, B. A., & Wrigley, C. W. (1984). The diversity of allergens involved in bakers' asthma. *Clinical Allergy*, 14, 93-107.

Tatham, A.S., & Shewry, P.R., 2008. Allergens to wheat and related cereals. *Clinical & Experimental Allergy*, 38, 1712-1726.

Wright, C., & Raikhel, N. (1989). Sequence variability in three wheat germ agglutinin isolectins: Products of multiple genes in polyploid wheat. *Journal of Molecular Evolution*, 28(4), 327-336.

6 CONCLUSIONES Y CONSIDERACIONES FINALES

CONCLUSIONES Y CONSIDERACIONES FINALES

Dada la complejidad del problema de la cuantificación del gluten en alimentos, durante las últimas décadas se han probado numerosas estrategias. Falsos negativos, sobre y subestimaciones del contenido en gluten así como tratamientos térmicos y químicos sufridos por el alimento limitan la aplicación universal de los métodos basados en proteínas como los ELISA. Por ello es importante contar con métodos alternativos y confirmatorios que detecten otro tipo de analitos para verificar la seguridad del consumo de los alimentos por los enfermos celíacos. Esto es especialmente interesante teniendo en cuenta que el consumo de bajas cantidades de gluten por estos enfermos durante largos períodos de tiempo se ha asociado con un aumento del riesgo de sufrir enfermedades autoinmunes. Los métodos basados en la detección de ADN, presentan el problema de que la relación entre el gluten y el ADN es variable entre diversas especies y variedades. Sin embargo, teniendo en cuenta que esta variabilidad no excede de un orden de magnitud, los métodos basados en ADN han sido considerados como excelentes alternativas a los métodos inmunoanalíticos, como ELISA, en el análisis de gluten.

Los métodos de ADN descritos hasta el momento se basan en la reacción en cadena de la polimerasa, PCR, y PCR en tiempo real, pero en general no han proporcionado sensibilidades que permiten controlar el cumplimiento de la legislación vigente. Esto es llamativo, dado que la sensibilidad que proporcionan estos métodos para analitos similares, como los alérgenos, es muy elevada. Esto puede ser debido a la gran complejidad del trigo. Por un lado, el gran tamaño del genoma del trigo (17,33 copias de ADN/genoma haploide), que es probablemente el más complicado de las especies de plantas domesticadas, y la enorme variabilidad genética debida a la gran capacidad de hibridación entre diferentes variedades y especies, dificultan enormemente su análisis a través del ADN.

Sin embargo, en la Tesis Doctoral se exploraron varios métodos de análisis de ADN para la detección de gluten en alimentos. Los tres métodos de amplificación aquí desarrollados por PCR convencional y PCR en tiempo real (gliadina, Tri a 18 y Tri a 25) constituyen los más sensibles descritos hasta el momento. Además se puso de manifiesto un efecto de interferencia de matriz, del que no se había informado hasta el momento. Esto supone un gran avance y una gran controversia al mismo tiempo, dado que pone de manifiesto la necesidad de cuantificación una muestra alimentaria teniendo en cuenta los componentes principales de su matriz para poder proporcionar datos cuantitativos fiables.

En cuanto al genosensor que aquí se propone, es el primer genosensor electroquímico para la detección de gluten en alimentos, y su diseño ha requerido superar una dificultad doble. Por un lado, la complejidad del genoma

del trigo y por el otro las características del analito, repetitivo y con fuerte estructura secundaria. Una vez superadas estos retos, se consiguió desarrollar un sensor de ADN capaz de detectar la presencia de trigo en muestras alimentarias de forma altamente selectiva.

A continuación se exponen las principales conclusiones extraídas de este trabajo:

1. El análisis *in-silico* del genoma del trigo suministró tres secuencias cortas (menos de 150 pb) que codifican proteínas alergénicas del trigo (α 2-gliadina, aglutinina isolectina Tri a 18 y tiorredoxina h Tri a 25). Su especificidad se verificó experimentalmente mediante amplificación por PCR convencional de ADN extraído de casi 30 especies vegetales.
2. Se desarrollaron métodos de PCR a tiempo real empleando sondas de hidrólisis, especialmente diseñadas para cada secuencia, que permitieron detectar y amplificar secuencias de ADN que codifican Tri a 18 y Tri a 25 en cantidades muy bajas, de hasta 20 pg de ADN de trigo y 500 mg/kg de trigo en matriz de soja, y de α 2-gliadina hasta 2 pg y 50 mg/kg. La secuencia que codifica para gliadina presentó el mejor límite de detección debido a que es una secuencia multicopia, frente a secuencias de Tri a 18 y Tri a 25, que se encuentran en una sola copia en el genoma del trigo. Todos los métodos fueron selectivos para la detección de cereales que contienen gluten (trigo, cebada, centeno y la controvertida avena) con una excelente reproducibilidad (CV <1%).
3. Se observó un acusado efecto de la matriz para los 3 métodos de amplificación propuestos, manifestado en una mejora del límite de detección de cómo mínimo 5 veces en el caso de las matrices de arroz y maíz, al compararlas con soja. Este efecto, nunca antes descrito, es consecuencia del mayor rendimiento de extracción del ADN de la soja respecto a otros cereales, esto es, debido a una competición del ADN de soja con el de trigo durante la extracción, así como al elevado contenido de lípidos de la soja que pueden actuar como inhibidores de la PCR y puede ocasionar importantes desviaciones positivas o negativas del contenido real en gluten. Los límites de detección obtenidos en matrices de arroz y maíz son de 5 ó 10 mg/kg para α 2-gliadina, respectivamente y 100 mg/kg para Tri a 25. Para Tri a 18 los límites encontrados fueron de 50 mg/kg en matriz de maíz y 20 mg/kg en arroz. La mayor sensibilidad se obtuvo de nuevo para la secuencia multicopia α 2-gliadina, muy superior a las conseguidas hasta el momento.
4. Asumiendo que el contenido promedio de gluten en harinas es del 9% en peso, y asumiendo que se mantiene tal proporción en el ADN, se obtiene un límite de detección de 0,9 mg/kg de gluten, lo que permitiría usar este método para evaluar el cumplimiento de la normativa vigente en Europa y

las recomendaciones del Codex Alimentarius de 20 mg/kg gluten para alimentos etiquetados como “sin gluten”.

5. La secuencia que codifica la α 2-gliadina presentó las mejores características de respuesta. Se seleccionó un fragmento de 99 pb de esta secuencia, que se empleó como analito para el diseño de un genosensor con detección electroquímica. Debido a la naturaleza repetitiva de la secuencia y a su fuerte estructura secundaria, favorecida por la presencia de cuartetos de guanina, se adoptó un formato de ensayo tipo sándwich, especialmente ventajoso en este tipo de analitos aportando una gran selectividad.
6. Se optimizó la composición de la fase sensora, usando una monocapa autoensamblada formada por ADN de captura y un agente bloqueante, mercaptohexanol. El dispositivo resultante dio una respuesta lineal para la concentración de analito sintético hasta 50 nM, con un límite de detección de 0,3 nM y una reproducibilidad del 3,5 %, para 5 nM, n=5.
7. El análisis de muestras reales requirió una etapa de amplificación previa por PCR convencional, que generó 3 bandas de distinto tamaño. La secuenciación dio como resultado que la de mayor tamaño era el fragmento deseado. Al llevar a cabo la detección del amplicón con el genosensor se observó una disminución de la respuesta en comparación con la obtenida para el ADN sintético, que se explica por la mayor longitud del amplicón (134 pb frente a 99 pb del sintético) que deja una larga cadena monocatenaria en las cercanías del electrodo. Se rediseñaron las sondas, para que formaran un dúplex completo. El límite de detección del nuevo diseño fue de 0,2 nM.
8. La aplicación del genosensor a muestras reales requirió una optimización de las condiciones de la PCR previa para maximizar la amplificación selectiva de la secuencia de interés. El producto de PCR obtenido solo requirió de un mínimo pretratamiento de dilución (1:5) para su detección con el genosensor. En estas condiciones se logró detectar hasta 15 pg de ADN de trigo y 0,001% de trigo en matriz de arroz, lo que indica que la combinación de PCR/genosensor tiene un límite de detección equivalente o incluso superior a la técnica considerada de elección, la PCR a tiempo real, pero a un menor coste.
9. Se observó una relación sigmoidea entre el logaritmo del porcentaje en peso de trigo y la intensidad de corriente medida en un amplio intervalo (0,001-100%). El dispositivo permitió cuantificar tanto muestras con alto contenido en gluten, como muestras etiquetadas como “sin gluten” obteniendo resultados concordantes con los obtenidos con el inmunoensayo oficial.

10. El genosensor propuesto es selectivo para cereales que contienen gluten no dando respuesta a secuencias sintéticas totalmente no complementarias. Tampoco se obtuvieron señales significativamente distintas del blanco para muestras de arroz y soja. Sí se obtuvo respuesta para hordeínas de cebada, secalinas de centeno y aveninas de avena, con diferente sensibilidad debido a que las secuencias amplificadas no son 100% homólogas a la de trigo. Para discriminar entre el cereal del que procede el gluten, tal y como exigen algunas legislaciones, se utilizó una sonda de captura estructurada con formato horquilla que mejoró radicalmente la selectividad del genosensor. En estas condiciones de ensayo, las señales de ADN de cebada y centeno fueron similares a las del blanco. Se obtuvo una respuesta lineal entre 5 y 50 nM, con LOD de 1 nM y una reproducibilidad del 4,3% para 50 nM (n=3). Además el sensor es estable a 4 °C durante más de 11 días (mantiene aproximadamente un 73% de la S/B), por encima de otros genosensores previamente publicados basados en el mismo tipo de inmovilización.
11. Se intentó utilizar la secuencia que codifica Tri a 18 para el diseño de un genosensor pero la elevada estabilidad del híbrido entre la sonda de captura y la indicadora ocasionaron blancos demasiado elevados para detectar de forma sensible ADN de trigo.
12. Como estrategia alternativa al diseño de genosensores específicos de cada tipo de cereal, se desarrolló un método cualitativo: el análisis de curvas de disociación de alta resolución (HRM). Esta técnica permitió un análisis más específico que la PCR para discriminar entre cereales con un grado muy elevado de homología (2-5 bases), diferenciando claramente muestras de trigo de muestras de avena y de muestras que contienen cebada o centeno. Estos dos últimos no pudieron ser diferenciados entre sí.

7 SUMMARY

7.1 Introduction

7.2 Objectives

7.3 Conclusions and final considerations

Indirect determination of gluten using DNA-based methods: PCR and electrochemical genosensors

7.1 Introduction

Celiac disease is a common autoimmune enteropathy (which affects 1% of the population) that occurs in genetically predisposed individuals as a result of an exacerbated immune response to a well-defined environmental factor, gluten. Thus, it is important for celiac patients avoid consuming foods that contain gluten. Furthermore, it seems that the ingestion of relatively small amounts of gluten for long periods of time is correlated with an increased risk of developing autoimmune complications.

Therefore, identification of the presence of gluten and its quantification in foodstuffs are a cornerstone issue, and a challenge to scientists due to the difficulty of analysis. Nowadays, either the analytical verification of compliance with the labeling requirements and the detection of wheat in food are mainly carried out by methods that detect proteins, like Enzyme-Linked ImmunoSorbent Assay (ELISA). However, despite the enormous advances in the detection of gluten proteins, some of the limitations of existing methods have encouraged the development of alternative technologies. Thus, DNA-based methods such as polymerase chain reaction (PCR), real-time PCR or DNA sensors (also called genosensors) represent attractive options. They all share a significant advantage: the target itself can be amplified so extremely low amounts of target can be detected. Also the increased stability of DNA molecules, compared to proteins, makes DNA-based methods very convenient for this kind of analysis, especially when dealing with processed food. However, the enormous complexity of the wheat genome (probably one of the most complex of domesticated plant species), and its high genetic variability make it a challenging but highly interesting task. In general, methods based on DNA detection are mainly used as screening tools to detect the presence of food allergens, but PCR and genosensors can also provide high sensitivity and specificity, compared to ELISA, even when a quantitative analysis is needed. Furthermore, taking into account that ELISA and PCR methods detect different types of targets, a positive result in both assays represents almost an undeniable evidence that allergen is present.

Due to the advantages of detecting DNA, in the last decade various methods of PCR and real-time PCR have been reported to detect wheat in food, though, most of them did not reach the necessary sensitivity. A simpler and cheaper alternative to PCR-based methods are the genosensors or DNA biosensors. These devices appeared as an alternative for the detection of DNA and their main advantages, especially regarding electrochemical genosensors, are relative their simplicity, low cost of implementation, possibility of on-line analysis, and their compatibility with miniaturization techniques. However their application for the detection of wheat in food is unexplored.

This PhD Thesis focuses on the indirect detection of gluten using specific DNA sequences belonging to harmful cereals for celiac patients, as an alternative to protein

detection methods. Appropriate DNA sequences from toxic cereals were rationally selected to use them as a target.

The behavior of three specific DNA sequences was studied using real-time PCR. A remarkable higher sensitivity was obtained with all of them, compared to previous works comply the requirements imposed by the legislation (<20 ppm). A strong influence of the food matrix was also found, showing that rice and maize matrices offered the best analytical performance. This finding points out to the need for adapting food standards for each matrix in order to avoid negative or positive deviations from the true value. The best results were obtained for the oligonucleotide sequence encoding the immunodominant gliadin peptide, so an electrochemical genosensor was designed to detect this target sequence, being the first that specifically detects gluten. Unlike other electrochemical genosensors, the detection of PCR-amplified fragments coming from food samples could be performed without prior purification of amplicons, and allowed the quantification of 15 pg of wheat DNA and 0.001% of wheat in an inert rice matrix. This sensitivity competes with the most sensitive real-time PCR methods. The genosensor allowed the determination of DNA of all toxic cereals including oats, and it did not show cross reactivity with other safe species, such as rice and soy. The selectivity of the sensor was modified by using structured probes in order to obtain a highly specific method for the detection of wheat, over other phylogenetically related cereals (barley, rye, oats), without compromising the good analytical performance of the genosensor. High resolution melting analysis (HRM) was also used for the identification of toxic cereals, discriminating the origin of gluten, i.e. the-cereal that is present in a food sample.

In sum, the methods proposed in this work represent an advance in the detection of gluten through specific wheat DNA sequences and are pioneers in a field that is in its infancy, but have a bright future ahead.

7.2 Objetives

The high incidence of celiac disease (1 in 100 people), together with the serious consequences that gluten consumption could cause in celiac patients, has forced the development of sensitive, selective and reliable methods for detection and quantification of gluten in food samples. ELISA methods are traditionally the most used, and also recommended by international organizations. However, cross-reactivity and false negative results due to denaturation of proteins during food processing have led to the development of alternative methods to confirm those immunoanalytical results, and to check the food safety for celiac patients.

The emergence of DNA-based methods, such as PCR or genosensors, has made a breakthrough in the identification and quantification of specific sequences from several targets: pathogens, allergens and genetically modified organisms. PCR-based methods provide a high sensitivity, while genosensors are portable, simple and inexpensive analysis methods. Despite its versatility, to date, there are no genosensors for gluten detection in food matrices.

In this sense, the general objective of this work is the development of alternative analytical methods for the detection and quantification of gluten in foods using a

different target: DNA. For this purpose the development PCR and genosensors for gluten detection is intended.

The specific objectives of this Thesis are:

1. *In silico* analysis for the selection of gluten specific DNA sequences encoding allergenic wheat protein. Verification of the suitability of these sequences for its use as targets of PCR amplification reactions.
2. Design and development of methods for gluten quantification using real-time PCR of the selected sequences and hydrolysis probes employing a fluorescent detection. Evaluation of selectivity towards nontoxic cereals, sensitivity and matrix effects of the method.
3. The sequence or sequences that provide the best analytical performance in real-time PCR will be used to design an electrochemical genosensor on gold electrodes. For this purpose, different sensing phases based on self-assembled monolayers will be explored. A sandwich assay format with enzyme labeling will be employed in order to maximize the selectivity. Different capture probes, linear and structured, will be compared. The design of genosensors and the performance will be assessed.
4. The resulting electrochemical genosensor will be applied to the analysis of real samples. To this purpose a previous extraction of the DNA and an amplification steps will be needed, taking into account the length of the genome and the presence of the complementary strand, that precludes the direct analysis of extracted DNA. Conventional PCR method will be optimized in order to detect both flours and processed food samples with the electrochemical genosensor.
5. Considering that certain legislations impose the identification of the cereal or gluten origin, the high resolution melting analysis (HRM) will be employed. It is a more specific method compared to PCR, which allows the discrimination between homologous sequences.

7.3 Conclusions and final considerations

Given the complexity of the gluten quantification in foods, numerous strategies have been reported during decades. False negatives and the underestimation of gluten content, as well as its thermal and chemical instability have limited the universal application of protein-based methods such as ELISA. It is important to have alternative and confirmatory methods to detect other targets to verify the food safety for celiac patients, such as DNA-based methods. DNA methods described so far are based on the polymerase chain reaction (PCR) and real-time PCR, but they have not provided the sensitivity for controlling the compliance of foodstuff with the legislation. This may be due to the complexity of wheat. On the one hand, genome of wheat has a large size (17.33 DNA copies/haploid genome), and on the other the enormous genetic variability, due to the great capacity of hybridization between different varieties and species, result in the high difficulty of wheat detection through its DNA analysis.

However, in this PhD Thesis various DNA analytical methods for the detection of gluten in food were explored. The three developed conventional PCR and real-time PCR amplification methods (gliadin, Tri a 18 and 25) were the most sensitive described so far. Additionally an interference matrix effect was observed, which had not been

reported before. This implies an important finding and a great controversy at the same time, because it highlights the need for quantify a food sample in function of the main components of the sample to provide reliable quantitative data. Regarding the genosensor herein proposed, it is the first electrochemical genosensor for detecting gluten in food, and its design overcome two difficulties, the complexity of the wheat genome and the repetitive and strong secondary nature of the target DNA sequence. Once these challenges have been surpassed, it was possible to develop a DNA sensor capable of detecting the presence of wheat in food samples in a highly selective manner.

The main conclusions of this work are as follows:

1. The *in-silico* analysis of the wheat genome provided three short sequences (less than 150 bp) encoding protein allergens from wheat (α 2-gliadin, Tri a 18 agglutinin isolectin and Tri a 25 thioredoxin h). Their specificity was experimentally verified by standard PCR amplification of DNA extracted from almost 30 plants.
2. Real-time PCR methods using hydrolysis probes were specially designed for each sequence, which enabled the detection and amplification of DNA sequences encoding Tri a 18 and Tri a 25 in very low amounts, 20 pg of wheat DNA and 500 mg/kg of wheat in a soybean inert matrix, and a limit of detection of 2 pg and 50 mg/kg for α 2-gliadin. The sequence encoding gliadin presented the best detection limit because of its multicopy nature compared to Tri a 18 and Tri a 25 sequences that are single copy genes. All methods selectively detected gluten-containing cereals (wheat, barley, rye and controversial oats) with an excellent reproducibility (CV <1%).
3. A strong matrix effect were noticed for the 3 proposed amplification methods, which was manifested in an improved detection limit of at least 5 times in the case of rice and maize matrices, when compared to soybean. This effect, never described before, was a consequence of the greater efficiency of extraction of soybean DNA compared to other cereals, namely, due to a competition of soybean DNA and wheat DNA during the extraction procedure, as well as the high lipid content of soy that can act as a PCR inhibitor, which could cause significant positive or negative deviations from the actual gluten content. Detection limits of rice and maize matrices were 5 or 10 mg/kg for α 2-gliadin, respectively; and 100 mg/kg for Tri a 25. Tri a 18 presented a limit of detection of 50 mg/kg in a maize matrix and 20 mg/kg in rice. The highest sensitivity was also obtained for the α 2-gliadin multicopy sequence, higher than those reported before.
4. Assuming that the gluten content in flour is around 9%, and assuming that such ratio is maintained in the DNA, a detection limit of 0.9 mg/kg gluten was obtained. So this method could be employed to assess the compliance with current European labeling regulations and the Codex Alimentarius recommendations, which imposes a maximum of 20 mg/kg gluten content to label foods as "gluten-free".
5. The sequence encoding the α 2-gliadin presented the best response performance. A 99 bp fragment was used as a target for designing a genosensor with electrochemical detection. Due to the repetitive nature of the sequence and strongly secondary structure, favored by the presence of guanine quartets, a sandwich format assay was adopted, which is especially advantageous in this type of targets providing a improved selectivity.

6. The sensing phase composition was optimized using a self-assembled monolayer formed by capture DNA and blocking agent, mercaptohexanol. The resultant device gave a linear response between intensity and synthetic target concentration up to 50 nM, with a detection limit of 0.3 nM and a reproducibility of 3.5% for 5 nM (n=5).
7. Analysis of real samples required a prior stage of conventional PCR amplification, which generated three bands of different sizes in gel electrophoresis. Sequencing revealed that only the larger was the desired fragment. A decrease in genosensor response was observed when comparing the amplicon analysis to that obtained for synthetic DNA, which was explained by the longer length of the amplicon sequence (134 bp compared to the synthetic sequence of 99 bp), leaving a highly flexible single stranded chain in the vicinity of the electrode. Probes were redesigned so to form a full duplex. The detection limit of the new design was 0.2 nM.
8. The analysis of real samples with the designed genosensor required the optimization of PCR conditions to maximize the selective amplification of the sequence of interest. The obtained PCR product required only a minimal pretreatment dilution (1: 5) prior the detection with genosensor. Under these conditions it was possible to detect as low as 15 pg of DNA from wheat and 0.001% wheat in a rice inert matrix, indicating that the combination of PCR/genosensor had an equivalent, or even higher, detection limit when comparing with the preferred method, real-time PCR, but a lower cost.
9. A sigmoid relationship between the logarithm of wheat percentage (% w/w) and the current intensity was observed, between a wide range of concentrations (0.001 to 100%). The device allowed the quantification of samples with both high and low (labeled as gluten free) gluten content achieving consistent results with those obtained with the official immunoassay.
10. The proposed genosensor was selective for cereals containing gluten, and it did not respond to not-complementary synthetic sequences. Rice and soybean samples provided negligible signals, similar to blank signals. The device responded to hordeins from barley, secalins from rye and avenins from oat with different sensitivity, because they are not 100% homologous to wheat. The discrimination of the origin of gluten is required by some legislations, so a hairpin-structured capture probe was designed in order to dramatically improve the genosensor selectivity. Under these conditions, signals from barley and rye DNA were similar to blank. A linear response between 5 and 50 nM, a LOD of 1 nM, and a reproducibility of 4.3% to 50 nM (n = 3) were obtained. In addition, the sensor was stable at 4 °C more than 11 days (keeping about 73% of the S/B), above other published genosensors based on the same kind of immobilization protocol.
11. The sequence encoding Tri a 18 was also employed to design a genosensor, but the high stability of the hybrid between capture probe and the signaling probe caused too high blank signals and prevented the wheat DNA detection.
12. As an alternative to the design of specific genosensors for each toxic cereal, a qualitative method was used: the high resolution melting analysis (HRM). This technique allowed a more discriminative detection between closely-related cereals with a high degree of sequence homology (2-5 bases), compared to PCR. It was able to clearly differentiate between wheat and oat samples, and between samples containing barley or rye. The two latter could not be discriminated each other.

8 RESUMO

7.1 Introdução

7.2 Objetivos

7.3 Conclusões e considerações finais

Determinação indireta de glúten usando métodos baseados em ADN: PCR e genosensores eletroquímicos

8.1 Introdução

A doença celíaca é uma enteropatia auto-imune comum (que afeta ao 1% da população), que ocorre em indivíduos geneticamente predispostos como um resultado de uma resposta imune exacerbada a um factor ambiental bem definido, o glúten. Assim, é importante que os pacientes com doença celíaca evitem consumir alimentos que contêm glúten. Além disso, parece que a ingestão de quantidades relativamente pequenas de glúten por longos períodos de tempo é correlacionado com um aumento do risco de desenvolver complicações autoimunes.

Portanto, a identificação da presença de glúten e sua quantificação nos produtos alimentícios são questões fundamentais e um desafio para os cientistas devido à dificuldade da sua análise. Hoje em dia, tanto a verificação analítica de conformidade com os requisitos de rotulagem como a detecção de trigo em alimentos são realizadas, principalmente, através de métodos que detectam proteínas como os enzimo-imunoensaios (ELISA). No entanto, apesar dos enormes avanços na detecção de proteínas de glúten, algumas das limitações dos métodos existentes têm estimulado o desenvolvimento de tecnologias alternativas. Assim, os métodos baseados em DNA tais como: a reação em cadeia da polimerase (PCR), a PCR em tempo real e os sensores de DNA (também chamados de genosensores) representam opções atrativas. Todas elas compartilham uma vantagem significativa: o próprio alvo pode ser amplificado e, assim, quantidades extremadamente baixas de alvo podem ser detectadas. Além disso, a maior estabilidade de moléculas de DNA, em comparação com proteínas, faz com que os métodos baseados em DNA sejam muito convenientes para este tipo de análise, especialmente, quando se precisa analisar alimentos processados. No entanto, a enorme complexidade do genoma do trigo (provavelmente um dos mais complexos dentre as espécies de plantas domesticadas), e a sua alta variabilidade genética torna a análise deste genoma uma tarefa difícil, porém muito interessante. Em geral, os métodos baseados na detecção de DNA são utilizados principalmente como ferramentas de rastreio para detectar a presença de alérgenos alimentares, porém a PCR e os genosensors também podem proporcionar uma elevada sensibilidade e especificidade em comparação com o ELISA, mesmo quando seja necessária uma análise quantitativa. Além disso, tendo em conta que os métodos ELISA e PCR detectam diferentes tipos de alvos, um resultado positivo nos dois ensaios, quase representa uma evidência inegável de que o alérgeno está presente.

Devido às vantagens da detecção de DNA, vários métodos de PCR e PCR em tempo real têm surgido na última década para a detecção de trigo em alimentos, no entanto, a maioria deles não alcança a sensibilidade necessária. Uma alternativa mais simples e mais barata que os métodos baseados em PCR são os genosensores ou biosensores de DNA. Estes dispositivos apareceram como uma alternativa para a detecção de DNA e as suas principais vantagens, especialmente, em relação

genosensors eletroquímicos são a sua simplicidade, baixo custo de implementação, possibilidade de análise on-line e a sua compatibilidade com as técnicas de miniaturização. No entanto, a sua aplicação para a detecção de trigo na alimentação está ainda inexplorada.

Esta Tese de Doutorado tem como principal objetivo a detecção indireta de glúten utilizando sequências de DNA específicas como alvo que pertencem a cereais prejudiciais para os pacientes com doença celíaca, como uma alternativa aos métodos de detecção de proteínas.

O comportamento das três sequências de DNA específicas selecionadas foi estudado utilizando PCR em tempo real. Uma notável maior sensibilidade foi obtida com todos eles, em comparação com os trabalhos anteriores e em conformidade com as exigências impostas pela legislação (<20 ppm). Uma forte influência da matriz nos alimentos foi encontrada também, mostrando que as matrizes do arroz e do milho oferecem um melhor comportamento analítico. Esta constatação aponta para a necessidade de adaptar os padrões alimentares para cada matriz, a fim de evitar desvios positivos ou negativos em relação ao valor verdadeiro. Os melhores resultados foram obtidos para a sequência de oligonucleótido que codifica para o péptido imunodominante da gliadina e, portanto, o genosensor eletroquímico foi concebido para detectar esta sequência alvo, sendo o primeiro genosensor que detecta especificamente glúten. Ao contrário dos outros genosensors eletroquímicos, a detecção de fragmentos de PCR amplificados a partir de amostras provenientes de alimentos pode ser realizada sem a purificação prévia dos fragmentos amplificados, e permitiu a quantificação de 15 pg de DNA de trigo e 0,001% de trigo em uma matriz inerte do arroz. Esta sensibilidade compete com os métodos mais sensíveis de PCR em tempo real. O genosensor permitiu a determinação de DNA de todos os cereais tóxicos, incluindo a aveia e não mostraram reatividade cruzada com outras espécies seguras, tais como o arroz e a soja. A seletividade do sensor foi modificada pela utilização de sondas estruturadas para obter um método altamente específico para a detecção de trigo frente a outros cereais filogeneticamente relacionados (cevada, centeio, aveia), sem comprometer o bom comportamento analítico do genosensor. A análise de fusão de alta resolução ou High Resolution Melting (HRM) também foi utilizada para a identificação de cereais tóxicos discriminando a origem do glúten, ou seja, identificando o tipo de cereal que está presente numa amostra de alimento.

Em suma, os métodos propostos neste trabalho representam um importante avanço na detecção de glúten de trigo através de sequências de DNA específicas e são pioneiros em um campo crescente, mas que tem um futuro promissor.

8.2 Objetivos

A elevada incidência da doença celíaca (1 em cada 100 pessoas) e as graves consequências que o consumo de glúten causa em pacientes com doença celíaca empurram ao desenvolvimento de métodos sensíveis, confiáveis e seletivos para a detecção e quantificação do glúten em amostras de alimentos. Os métodos ELISA são, tradicionalmente, os mais utilizados e também os recomendados pelas organizações internacionais. No entanto, a reatividade cruzada e os falsos negativos,

devido à desnaturação de proteínas durante o processamento dos alimentos, têm levado ao desenvolvimento de métodos alternativos para confirmar esses resultados imunanalíticos e com o objetivo da verificação da segurança alimentar para pacientes celíacos.

O surgimento de métodos baseados em DNA, tal como a PCR ou os genosensores, supõe um grande avanço na identificação e quantificação de sequências específicas a partir de vários alvos: agentes patogénicos, alérgenos e organismos geneticamente modificados. Os métodos baseados em PCR proporcionam uma elevada sensibilidade, mas também os genosensores são portáteis, simples e baratos. Apesar da sua versatilidade, até o momento não há genosensores para detecção do glúten em matrizes de alimentos.

Neste sentido, o objectivo geral do presente trabalho consiste no desenvolvimento de métodos analíticos alternativos para a detecção e quantificação de glúten em produtos alimentares usando outro alvo, o DNA. Para este objetivo, desenvolvem-se a PCR e os genosensores para a detecção glúten.

Os objetivos específicos desta tese são:

1. A análise *in silico* para a seleção das sequências de DNA específicas que codificam proteínas do glúten de trigo alérgicas. A verificação da aptidão destas sequências para a sua utilização como alvos de reações de amplificação por PCR.
2. Desenvolvimento de métodos para a quantificação de glúten usando PCR em tempo real das sequências selecionadas e sondas de hidrólise que usam uma detecção fluorescente. A avaliação da seletividade para cereais, da sensibilidade e dos efeitos da matriz do método.
3. A sequência ou sequências que proporcionem o melhor desempenho analítico na PCR em tempo real serão utilizadas para conceber um genosensor eletroquímico com eletrodos de ouro. Para este efeito, diferentes fases de detecção usando monocamadas autoestruturadas serão exploradas. Um formato de ensaio em sanduíche com a marca enzimática será utilizado, a fim de maximizar a seletividade. As sondas de captura linear e estruturada também serão comparadas. Os genosensores serão avaliados.
4. O genosensor eletroquímico resultante será aplicado à análise de amostras reais. Para esse efeito será necessário uma extração prévia do DNA e de uma etapa de amplificação. O método de PCR convencional será otimizado, a fim de detectar as amostras de farinhas e as amostras de alimentos processados com o genosensor eletroquímico.
5. Será empregada a análise de fusão de alta resolução (HRM) para a identificação do cereal de origem do glúten tendo em vista as determinações impostas pela legislação alimentar. É um método mais específico em relação à PCR que permite a discriminação entre sequências homólogas.

8.3 Conclusões e considerações finais

Dada a complexidade da quantificação do glúten em alimentos, numerosas estratégias têm sido descritas durante as últimas décadas. Os falsos negativos e a subestimação do conteúdo em glúten, a sua instabilidade térmica e química têm limitado a aplicação universal dos métodos que detectam proteínas, tais como, ELISA. É importante ter métodos alternativos e confirmatórios para verificar a segurança alimentar dos pacientes com doença celíaca, tais como, métodos baseados em DNA. Os métodos de DNA descritos até agora baseiam-se na reação em cadeia da polimerase (PCR) e PCR em tempo real, mas não têm proporcionado a sensibilidade para controlar a conformidade de produtos alimentares como a legislação requer. Isto pode ser devido à complexidade do trigo. Por um lado, o genoma de trigo tem um tamanho grande (17,33 cópias de DNA/genoma haplóide), e por outro, a enorme variabilidade genética, devido à grande capacidade de hibridação entre diferentes variedades e espécies, resultam em uma elevada dificuldade da detecção do trigo através da análise do seu DNA.

Nesta Tese de Doutorado foram explorados diversos métodos de análise de DNA para a detecção do glúten nos alimentos. Os três métodos de amplificação por PCR convencional e em tempo real desenvolvidos (gliadina, Tri a 18 e Tri a 25) foram os mais sensíveis até agora descritos. Além disso, um efeito de interferência da matriz foi observado, que não tinha sido relatado antes. Isto implica uma descoberta importante e uma grande controvérsia ao mesmo tempo, e realça a necessidade de quantificar as amostras de alimentos em função dos principais componentes da amostra para fornecer dados quantitativos confiáveis. O genosensor proposto nesta tese, o primeiro genosensor eletroquímico utilizado para detectar glúten nos alimentos, teve que superar duas dificuldades: a complexidade do genoma do trigo e a de capacidade de formar uma estrutura secundária forte e a natureza repetitiva da sequência de DNA alvo. Uma vez que estes desafios foram ultrapassados, foi possível desenvolver um sensor de DNA capaz de detectar a presença de trigo em amostras de alimentos de uma maneira altamente seletiva.

As principais conclusões deste trabalho são os seguintes:

1. A análise *in silico* do genoma do trigo mostrou três sequências curtas (menos de 150 pb) que codificam proteínas alergênicas do trigo (α 2-gliadina, agglutinin isolectin Tri a 18 e thioredoxin h Tri a 25) e sua especificidade foi experimentalmente verificada por amplificação de PCR dos DNA extraídos de quase 30 plantas diferentes.
2. As PCR em tempo real utilizando sondas de hidrólise foram especialmente concebidos para cada sequência, o que permitiu a detecção e a amplificação de sequências de DNA que codificam Tri a 18 e Tri a 25 em quantidades muito baixas, de 20 pg de DNA de trigo e 500 mg/kg de trigo numa matriz inerte de soja, e um limite de detecção de 2 pg e 50 mg/kg para α 2-gliadina. A sequência que codifica gliadina apresentou o melhor limite de detecção devido à sua natureza multicópia em comparação com as sequências de Tri a 18 e Tri a 25 que são genes de cópia única. Todos os métodos detectaram seletivamente cereais que contêm glúten (trigo, cevada, centeio e aveia), com uma excelente reprodutibilidade (CV <1%).

3. Observou-se um forte efeito da matriz para os três métodos de amplificação propostos, que se manifestou em um limite de detecção melhorado pelo menos 5 vezes, no caso das matrizes do arroz e do milho em comparação à matriz da soja. Este efeito nunca tinha sido descrito antes e corresponde a uma consequência da maior eficiência de extração de DNA da soja em comparação com os cereais, ou seja, devido à competição do DNA da soja e do DNA do trigo durante o processo de extração, assim como o elevado conteúdo de lípidos na soja que podem atuar como inibidores da PCR, o que poderia causar desvios positivos ou negativos do conteúdo real de glúten. Os limites de detecção das matrizes do arroz e do milho foram de 5 e 10 mg/kg para α 2-gliadina, respectivamente, e ambos apresentaram 100 mg/kg para Tri a 25. A sequência Tri a 18 apresentou um limite de detecção de 50 mg/kg na matriz do milho e 20 mg/kg na matriz do arroz. A maior sensibilidade foi também obtida para a sequência multicópia da α 2-gliadina e foi superior aos dados relatados anteriormente.
4. Partindo do princípio de que o conteúdo de glúten na farinha é por volta de 9%, e assumindo que a mencionada relação é mantida no DNA, foi obtido um limite de detecção de 0,9 mg/kg de glúten. Portanto, este método pode ser utilizado para avaliar a conformidade com as regulamentações europeias em vigor em matéria de rotulagem e as recomendações do Codex Alimentarius que impõe um máximo de 20 mg/kg de conteúdo de glúten em alimentos rotulados como "sem glúten".
5. A sequência que codifica a α 2-gliadina apresentou o melhor comportamento. Um fragmento de 99 pb foi utilizado como o alvo para a preparação de um genosensor com detecção eletroquímica. Devido à natureza repetitiva da sequência e a estrutura secundária forte, favorecida pela presença de quartetos de guanina, adotou-se um ensaio em formato de sanduíche, que é especialmente vantajoso para este tipo de objetivo, pois proporciona uma seletividade melhorada.
6. A composição da fase de detecção foi otimizada usando uma monocapa autoestruturada formada por uma sonda de captura de DNA e um agente bloqueador, mercaptohexanol. O dispositivo resultante apresentou uma resposta linear entre a intensidade e a concentração alvo sintético até 50 nM com um limite de detecção de 0,3 nM e uma reprodutibilidade de 3,5% para 5 nM ($n = 5$).
7. A análise de amostras reais requereu uma etapa prévia de amplificação por PCR convencional que gerou três bandas de tamanhos diferentes em eletroforese em gel. A sequenciação revelou que apenas o fragmento de maior tamanho era o desejado. Uma diminuição na resposta do genosensor foi observada ao comparar a análise do amplicon com o DNA sintético. Isso foi explicado pela maior extensão da sequência de amplificação (134 pb em comparação com a sequência sintética de 99 pb), deixando uma cadeia simples de DNA altamente flexível na proximidade do eletrodo. As sondas foram redesenhadas de modo a formar um duplex completo e o limite de detecção do novo genosensor foi de 0,2 nM.
8. Para a análise de amostras reais com o genosensor concebido foi necessário otimizar as condições da PCR para maximizar a amplificação seletiva da sequência de interesse. O produto de PCR obtido somente foi necessário uma diluição mínima (1:5) como etapa de pré-tratamento para detecção com este genosensor. Nestas condições foi possível detectar quantidades muito baixas do DNA de trigo, 15 pg e 0,001% de trigo numa matriz inerte de arroz, indicando que

a combinação de PCR/genosensor produz um limite de detecção igual, ou superior aos métodos da PCR em tempo real, porém com um menor custo.

9. Observou-se uma relação sigmóide entre o logaritmo da percentagem de trigo (% w/w) e a intensidade de corrente em uma grande faixa de concentrações (0,001 a 100%). O dispositivo permitiu a quantificação de amostras (rotulados como sem glúten) com alto e baixo conteúdo de glúten e alcançou resultados consistentes com aqueles obtidos com o imunoensaio oficial.
10. O genosensor proposto foi seletivo para os cereais que contêm glúten e não respondeu a sequências de não complementares. As amostras do arroz e da soja forneceram sinais insignificantes semelhantes aos sinais do branco. O dispositivo respondeu às hordeínas da cevada, às secalinas do centeio e às aveninas da aveia com sensibilidade diferente porque elas não são 100% homólogas ao trigo. A discriminação do cereal da origem de glúten é requerida por algumas legislações, por isso desenhou-se uma sonda de captura estruturada com forma de forquilha a fim de melhorar a seletividade genosensor. Sob estas condições, os sinais de DNA da cevada e do centeio foram semelhantes ao branco. Foram obtidos uma resposta linear entre 5 e 50 nM, uma LOD de 1 nM e uma reprodutibilidade de 4,3% a 50 nM (n = 3). Além disso, o sensor foi estável a 4 °C por mais de 11 dias (mantendo cerca de 73% do S/B) mostrando-se mais estável que outros genosensors publicados e baseados no mesmo tipo de protocolo de imobilização.
11. A sequência que codifica Tri a 18 foi também utilizada para conceber um genosensor, porém a elevada estabilidade do híbrido entre a sonda de captura e a sonda de sinalização apresentou sinais de branco muito elevados e impediu a detecção de DNA de trigo.
12. Como uma alternativa dos genosensors específicos para cada cereal tóxico, um método qualitativo foi utilizado: a análise de fusão de alta resolução (HRM). Esta técnica permitiu uma detecção mais discriminativa entre cereais estreitamente relacionados com um elevado grau de homologia nas suas sequências (2-5 bases) em comparação com a PCR. O HRM foi capaz de diferenciar claramente as amostras de trigo, aveia, cevada e centeio. A cevada e o centeio não puderam ser discriminados entre si.

9 ANEXO I: PUBLICACIONES DERIVADAS DE LA TESIS DOCTORAL

ANEXO I: Publicaciones derivadas de esta Tesis Doctoral

Los estudios realizados en la presente Tesis Doctoral, han dado lugar a los siguientes trabajos científicos, aceptados para su publicación en revistas científicas internacionales y que conforman la tesis:

- Begoña Martín-Fernández, Noemí de-los-Santos-Álvarez, María Jesús Lobo-Castañón, Beatriz López-Ruiz. “Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a Gliadin fragment”. *Analytical and Bioanalytical Chemistry*, 407: 12 (2015) 3481-3488. DOI: <http://dx.doi.org/10.1007/s00216-015-8560-2>.
- Begoña Martín-Fernández, Joana Costa, M. Beatriz P.P. Oliveira, Beatriz López-Ruiz, Isabel Mafra. “Screening new gene markers for gluten detection in foods”. *Food Control*, 56 (2015) 57–63. DOI: <http://dx.doi.org/10.1016/j.foodcont.2015.02.047>.
- Begoña Martín-Fernández, Arturo J Miranda-Ordieres, María Jesús Lobo-Castañón, Gloria Frutos Cabanillas, Noemí de-los-Santos-Álvarez, Beatriz López-Ruiz. “Strongly structured DNA sequences as targets for genosensing: sensing phase design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment”. *Biosensors and Bioelectronics*, 60 (2014) 244-251. DOI: <http://dx.doi.org/10.1016/j.bios.2014.04.033>.
- Begoña Martín-Fernández, C.Lorena Manzanares Palenzuela, Marta Sánchez-Paniagua López, Beatriz López-Ruiz, Gloria Frutos Cabanillas. “Diseño de un genosensor electroquímico para la detección indirecta de gluten en alimentos”. *Anales de la Real Academia Nacional de Farmacia*, 78 (2012) 323-343.
- Begoña Martín-Fernández, Joana Costa, Noemí de-los-Santos-Álvarez, Beatriz López-Ruiz, M. Beatriz P.P. Oliveira, Isabel Mafra. “High resolution melting analysis as a new approach to discriminate gluten-containing cereals”. *Food Chemistry* (submitted).
- Begoña Martín-Fernández, Joana Costa, M. Beatriz P.P. Oliveira, Beatriz López-Ruiz, Isabel Mafra. “Effect of matrix on the performance of quantitative real-time PCR of gluten-containing cereals”. *Food and Chemical Toxicology* (submitted).

- Begoña Martín-Fernández, Noemí de-los-Santos-Álvarez, Juan Pedro Martín-Clemente, María Jesús Lobo-Castañón, Beatriz López-Ruiz “Challenging genosensors in food samples: the case of gluten determination”. *Talanta* (submitted).

Además, durante la realización de la Tesis Doctoral también se han publicado artículos en revistas internacionales relacionados con el tema desarrollado:

- C.Lorena Manzanares-Palenzuela; Begoña Martín-Fernández; Marta Sánchez-Paniagua, Beatriz López-Ruiz. “Electrochemical genosensors as innovative tools for detection of genetically modified organisms”. *Trends in Analytical Chemistry*, 66 (2015) 19-31. DOI: <http://dx.doi.org/10.1016/j.trac.2014.10.006>.
- Begoña Martín-Fernández, C. Lorena Manzanares-Palenzuela, Marta Sánchez-Paniagua López, Noemí de-los-Santos-Álvarez, Beatriz López-Ruiz. “Electrochemical Methods based on DNA in Food Safety Assessment”. *Critical Reviews in Food Science* (accepted).

(accepted)

Critical Reviews in Food Science and Nutrition
Copyright © Taylor and Francis Group, LLC



Electrochemical Methods based on DNA in Food Safety Assessment

Begoña Martín-Fernández^{a,b}, C. Lorena Manzanares-Palenzuela^a, Marta Sánchez-Paniagua López^a, Noemí de-los-Santos-Álvarez^b, Beatriz López-Ruiz^{*a}

^aSección Departamental de Química Analítica, Facultad de Farmacia. Universidad Complutense de Madrid, Pz. Ramon y Cajal s/n, 28040 Madrid, Spain.

^bDepartamento de Química Física y Analítica, Universidad de Oviedo, Av. Julián Clavería 8, 33006 Oviedo, Spain.

*Corresponding autor e-mail: bealopru@ucm.es (B. López-Ruiz).

ABSTRACT

The main goal of food safety assessment is to provide reliable information on the identity and composition of food and reduce the presence of harmful components. Nowadays, there are many countries where rather than the presence of pathogens, common public concerns are focused on the presence of hidden allergens, fraudulent practices and genetic modifications in food. Accordingly, food regulations attempt to offer a high level of protection and to guarantee transparent information to the consumers. The availability of analytical methods is essential to comply these requirements. Protein-based strategies are usually employed for this purpose, but present some limitations. Because DNA is a more stable molecule, present in most tissues, and can be amplified, there has been an increasing interest in developing DNA-based approaches (polymerase chain reaction, microarrays and genosensors). In this regard, electrochemical genosensors may play a major role in fulfilling the needs of food industry, such as reliable, portable and affordable devices. This work review the achievements of this technology applied to allergen detection, species identification and genetically modified organisms testing. We summarized the legislative framework, current design strategies in sensor development, their analytical characteristics and future prospects.

Keywords

Allergen; Food authenticity; Species identification; Genetically modified organisms; Electrochemical DNA sensor; Food Safety

1. INTRODUCTION

During the past few decades, food quality and food safety have become a topic of public awareness. Guaranteeing food safety is nowadays the main focus of authorities in the food supply chain. The importance of food safety has grown, not only due to foodborne diseases, but also to the concern raised by allergic consumers inadvertently exposed to allergen containing foods (Monaci and Visconti, 2010), as well as various food-related crises undermining public confidence, such as species adulteration, e.g. undeclared horsemeat. Additionally, there has been an increased alarm regarding the introduction of GMOs into the food and feed market due to their controversial safety for human consumption and loss of biodiversity.

In this sense, the main objective is to provide safe food that consumers can trust. Public opinion, along with scientific research and the opinion of experts are continually engaged into promoting careful evaluation of all aspects related to food (Figure 1). Authorities are pushing the food and the feed industry to restructure the food inspection system and try to enhance consumer information to regain consumers trust in food (Röhr et al., 2005). For health, economical and religious reasons, of the promotion of the consumer protection has fuelled the need of legal regulations enforcing the labeling of all food products containing potentially harmful (allergens) or controversial ingredients (GMOs). Species identification, also known as food authenticity, allows the consumer to prevent fraud by substitution of products by others of lesser value for economic purposes. One of the key challenges to ensure food safety is the availability of fast, sensitive and reliable analytical methods to identify specific hazards associated to food before they become a health problem (Amaya-González et al., 2013).

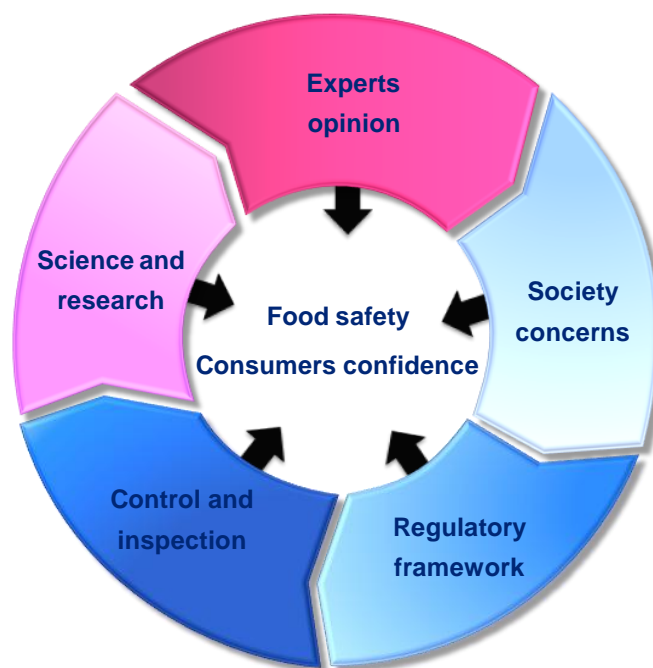


Figure 1. Representation of the main agents that improve food safety and quality and increasing consumer confidence.

The methodology for testing allergens, authenticity and GMOs are well-known topics in food science since many years. Food products can be characterized through classical approaches such as fatty acid and protein composition profiling. However, for safety-related purposes, proteins and nucleic acids are highly discriminating targets. ELISA methods are the common approaches to detect proteins, though their low thermal stability and the availability of specific antibodies are the main obstacles for the general application of protein-based methods. This is especially true for transgenic proteins, where the absence of antibodies is notorious and also for allergenic proteins, whose sequence is conserved among different species complicating the discrimination ability of the antibodies. Consequently, since the introduction of the PCR technique, DNA has been the target of choice due to its relatively high stability in processed products (Mafrá et al., 2008a). Also this target presents a remarkable advantage, it is self-amplifying and, thus, extremely low amounts of DNA can be detected. Specific recognition of unique sequences of DNA from samples can provide the basis of highly specific assays for allergen-coding sequences, species identification and GMO testing.

The limitations of existing methods for DNA detection have encouraged the development of new alternative technologies, especially to comply with current desirable analytical features, such as low-cost equipment, portability, automation and robustness. Electrochemical genosensors can play a major role in addressing these needs. Genosensors are DNA biosensors that rely on a

hybridization recognition reaction between two complementary strands, the target and a recognition element, called probe. These devices use an electrode-based platform as transducer and, in order to convert this highly specific hybridization reaction into a measurable signal, a reporter molecule (electroactive molecules or enzymes) is usually incorporated. In the past decades, many of these electrochemical sensors have been developed for food safety assessment. In Figure 2, we have summarized the quantity of publications in the last 15 years regarding DNA sensors for general food safety. An increased interest on these devices is clearly observed especially in the last years, with a minimum of 20 papers reported per year. The main focus has been pathogen detection in food, which has been a widely reviewed topic (Alonso-Lomillo et al., 2010; Arora et al., 2011; Pividori and Alegret, 2010; Poltronieri et al., 2014; Sforza et al., 2011; Van Dorst et al., 2010). When the literature analysis was refined to another type of food control, namely “non-pathogenic”, including the terms “genetically modified OR allergen OR gluten OR authenticity”, the number of papers were considerably less, yet the chart reflects an increasing number of publications in the past year. This rise may be in response to the enforcement of food regulations and the growing interest of consumers in being more informed about food composition. Moreover, when the search included the keyword “electrochemical”, the chart indicates that most “non-pathogenic” devices were based on electroanalytical techniques. As expected, electrochemical devices have gained more notice as an alternative to conventional optical or mass approaches for DNA detection, keeping in thought that electrochemical instrumentation bears great advantages; portability, automation and low cost being the most important ones.

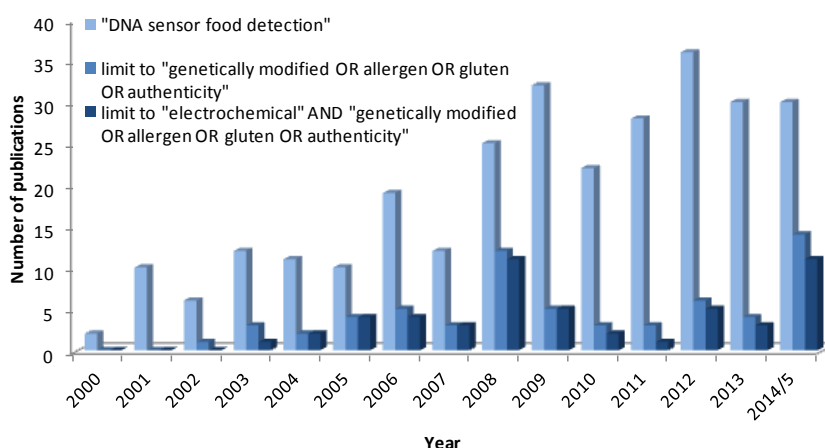


Figure 2. Number of publications reporting DNA sensors since 2000 till 2014/5. The publications were retrieved from ISI Web of Knowledge and Scopus and analyzed.

The aim of this review is to collect and discuss the electrochemical DNA sensors reported so far for food authentication (meat and fish), allergens

(hazelnut, peanut and gluten) and recent advances achieved in GMO assessment. We will focus on these specifics not only because they are paramount to the industry and to the consumers, but also because there has been a growing trend in pursuing this type of food assessment by researchers in the biosensor field. Our interest was driven into carefully reviewing this topic of up-to-date prominence, considering that it has not been thoroughly addressed yet. This work summarizes the requirements imposed by the legislative framework regarding food, the current knowledge about electrochemical genosensors development, the possible gaps and how they should be addressed. Thus, the main progresses to date are also presented, highlighting potential prospects for the future and emphasizing aspects that deserve special attention for improvement of these devices.

2. LEGISLATION

The increasing use of packaged foods has forced governments to subject the food industry to more and more stringent labeling of their products. Labeling helps consumers to choose the product and identify its contents, allowing the consumer to exercise freedom of choice (Premanandh, 2011). Accordingly, one of the main objectives of the governments is to achieve a high level of protection and to guarantee their right to information, ensuring that consumers are appropriately informed in regards to the food they consume (Regulation (EU) 1169/2011).

Legal framework related to GMOs, authenticity and allergen in foodstuffs is wide and comprises different goals. It covers issues ranging from personal choice (GMOs), the prevention of food fraud (food authentication), and to certify the safety of food to allergic or celiac individuals (allergen issue).

2.1. Allergen legislation

Labeling rules generally only cover deliberately added ingredients in pre-packed foods, but supply chain and manufacturing processes are extremely complex, resulting in the adventitious presence of small amounts of allergens (traces) in many products and restricted variety of food products for allergenic people (Crevel et al., 2008). Besides, recently the use of a precautionary label "may contain" in food products has risen to become overused, thus leading to unnecessary limitation of consumer choice and devaluation of the allergen labeling information (van Hengel, 2007). Because of that, allergic people can find avoidance of specific food very difficult.

To date, most regulatory authorities have focused on ingredient labeling irrespective of the amount in the food. Presently, the European Union (EU) ruled through the Regulation (EU) 1169/2011 the mandatory labeling of fourteen groups of certain substances or products that are known that cause allergies or

intolerances (annex II) (Regulation (EU) 1169/2011). It is required to highlight these ingredients from the rest of the ingredients enumerated in processed food, regardless of their quantity. The list includes crustaceans, eggs, fish, peanuts, soybean, milk, nuts, gluten containing cereals, celery, mustard, sesame, sulphur dioxide, lupin and molluscs. The United States (US) also implemented legislation to improve the protection for allergic consumers, with the Food Allergen Labeling and Consumer Protection Act (FALCPA). The US lay down 8 groups of allergenic ingredients, while the legal basis for food allergies imposed by the EU includes a list of 14 groups, thus being the most complete legislation framework so far. Other countries such as Canada (11 groups), Australia and New Zealand (10 groups), and Japan (6 groups) also establish restrictive legislation to protect the allergic patients. In spite of the advances achieved in labeling requirements throughout the world (Gendel, 2012), there is a lack of consensus regarding the establishment of threshold values for allergen labeling (Taylor et al., 2002).

Unlike most allergens, gluten is the only one identified with a clear and explicit threshold at least in the EU. Although universal safe levels for consumption of gluten has not yet been defined, literature suggests that small amounts of gluten could be sufficient to cause damage to the celiac intestinal mucosa over time (Catassi et al., 2007) and an increased risk of autoimmune disorders (Ventura et al., 1999). Therefore, mandatory labeling of celiac harmful food ingredients has been established to inform the consumer about the presence of gluten in food products. The Codex Alimentarius and the Regulation (EC) 41/2009 endorse a maximum gluten contamination of 20 mg/kg in gluten free labeled products as a safe threshold (CODEX-STAN118, 1979; Regulation (EC) 41/2009). However, these regulations do not specify an analytical methodology for gluten analysis.

2.2. Authenticity legislation

One of the risks gaining attention from industry, governments, and standards-setting organizations is fraud conducted for economic gain by food producers, manufacturers, processors, distributors, or retailers (Moore et al., 2012). It is essential to establish that species of high commercial value declared are not replaced, partially or entirely, with other lower value species (Mafra et al., 2008a). Religious reasons also play an important role in this subject. Labeling legislation has to ensure that food is properly described, and it has to protect consumers from being sold a product with a fake description (Dennis, 1998). According to Regulation (EC) 178/2002 and 1169/2011, it is a general principle of food law to provide a basis for consumers to make informed choices in relation to food they consume and to prevent any practices that may mislead the consumer (Regulation (EC) 1169/2011). In agreement with these rules it is

necessary to provide comprehensible and trustworthy information on the identity and composition of the food.

2.3. GMOs legislation

According to the European legislation, the presence of genetically modified (GM) material in food and feed is governed by Regulation (EC) 1829/2003, which insists on the mandatory labeling of all products consisting of, produced or containing authorized GMO above 0.9%. Below this limit, GMO labeling is not required when its presence is considered adventitious or technically unavoidable. For non-authorized GM ingredients, the threshold is set at 0.5% (Regulation (EC) 1829/2003). Unlike the European legislation, the labeling of transgenic products is voluntary in the United States.

Since the correct labeling of foods is the only effective way of gain the consumers' confidence and protecting the allergic patients, legislation in terms of food assurance has undoubtedly been improved in the last decades. However, the issue of adventitious contamination and cross-contact during manufacturing is not addressed yet, even though substantial incorporation of undeclared constituents can occur (Crevel et al., 2008). Differences not only in food regulation but also in the different threshold levels set for labeling in many countries, as well as the complexity and variety of food matrices, make extremely difficult the development of only one analytical method universally applicable to assess food safety. This is why there is a compendium of existing methods, some of which are implemented on a routine basis and others that are more difficult to apply in every research or industry facility. The number and diversity of available methods to detect species, allergen-coding sequences and GM DNA, keeps growing. The common goal is to develop robust and reliable analytical methods, cost-effective and easy to implement in food control practices.

3. ALLERGEN DETECTION

Food allergy is an adverse, abnormal immune-mediated reaction to a certain food or food ingredient that occurs in susceptible individuals who often require a strict avoidance of the offended component. Food sensitivity also includes food intolerance, which is clinically undistinguishable from allergy but it is a non-immune mediated reaction (Koppelman and Hefle, 2006). Up to 1-2% of the total human population suffers from clinically proven food allergies (Sicherer and Sampson, 2006). Prevalence among children is even higher, about 5-8% (Branum and Lukacs, 2009; Gupta et al., 2011). Food allergy has thus evolved from being a restricted problem for the food-allergic population to an emerging public health issue.

Allergens determination in foodstuffs is a major concern for both the food industry and food-allergic consumers. Ultrasensitive analytical approaches are demanded to detect even traces of allergens. Thus biosensors have been applied to numerous kinds of allergen targets as reviewed recently (Alves et al., 2015). Even though the target itself is the allergenic protein, there is a very important limitation regarding protein-targeting methods, such as ELISA, i.e., food thermal processing can promote degradation of food constituents, modifications, or interactions with other components of the food matrix and therefore thermal processing has a potential to modify allergenic properties of proteins (Sathe et al., 2005; van Boekel et al., 2010). Given the difficulty of detecting allergenic proteins under certain circumstances (e.g. with ELISA assays), DNA-based methods are also used for screening or confirmation, relying on the high stability of DNA upon food processing when compared with proteins (Mafra et al., 2008a). Since DNA detection methods are completely orthogonal to ELISA, a positive result with both assays gives almost irrefutable proof that the allergen is present (Janssen, 2006).

Usually, DNA target sequences are selected among genes coding for allergenic proteins or simply on a taxon-specific region of the allergenic species genome. Most of electrochemical genosensors rely on the determination of DNA sequences that encode allergenic proteins (Table 1). Various types of designs have been reported, using different sorts of immobilization strategies and electrochemical methods. Most methods were applied to real sample analysis.

Table 1. Summary of electrochemical genosensors applied to allergen detection

Target	Electrode type	DNA immobilization strategy	Detection method	Sample type	LOD (M)	Reference
Hazelnut (Cor 1.04 and Cor 1.03)	Array 8 SPEAu (homemade)	SAMs	DPV with enzymatic amplification	Synthetic DNA and commercial food samples with PCR amplification. Amplicon purification	3×10^{-10} (synthetic) 1×10^{-9} (amplicons)	(Bettazzi et al., 2008)
Hazelnut (Cor 1.04)	Microfluidic device (8 parallel microchannels SPEC) and magnetic microparticles	Biot-Strp	DPV with enzymatic amplification	Synthetic DNA and commercial food samples with PCR amplification. Amplicon purification	10×10^{-9} (synthetic) 0.2×10^{-9} (amplicons)	(Berti et al., 2009)
Peanut (Ara h1)	Au electrode	SAMs	EIS	Synthetic DNA and one peanut milk beverage with PCR amplification.	3.5×10^{-16} (synthetic)	(Sun et al., 2012)
Peanut (Ara h1)	GCE	Multilayered Au /graphene	DPV	Synthetic DNA and one peanut milk beverage with PCR amplification	4.1×10^{-17} (synthetic)	(Sun et al., 2015a)

Peanut (Ara h1)	GCE	Au film/chitosan-MWCNT	CA with enzymatic amplification	Synthetic DNA and one peanut milk beverage with PCR amplification	1.3×10^{-17} (synthetic)	(Sun et al., 2015b)
Peanut (Ara h2)	SPEAu	SAMs	DPV with enzymatic amplification	Synthetic DNA	10×10^{-12}	(Sánchez-Paniagua López et al., 2014)
Gluten ($\alpha 2$ -gliadin)	SPEAu	SAMs	CA with enzymatic amplification	Synthetic DNA and commercial food samples with PCR amplification. Without amplicon purification	3×10^{-10} (synthetic) 2×10^{-10} (amplicons)	(Martín-Fernández et al., 2014)
Gluten ($\alpha 2$ -gliadin)	SPEAu	SAMs	CA with enzymatic amplification	Synthetic DNA	1×10^{-9} (synthetic)	(Martín-Fernández et al., 2015)

3.1. Hazelnut

Hazelnut, *Corylus avellana*, is one of the most commonly consumed tree nuts being virtually found in a wide range of processed foods (Costa et al., 2012). It has been regarded as a food with potential heart-protective benefits (FDA, 2003), but also as a source of allergens capable of inducing mild to severe allergic reactions in sensitized individuals. Hazelnut allergy is one of the most global incidence, with an estimated prevalence in Europe of 0.1–4% of the population (Zuidmeer et al., 2008). Several allergenic proteins have been identified and characterized: Cor a 1, Cor a 2, Cor a 8, Cor a 9, Cor a 11, Cor a 12 and Cor a 13 and Cor a 14 (Akkerdaas et al., 2006; Breiteneder et al., 1993; Garino et al., 2010; Iniesto et al., 2013; Lüttkopf et al., 2002; Pastorello et al., 2002)

Bettazzi et al. (2008) reported an electrochemical genosensor for the detection Cor a 1.04 and of the isoform Cor a 1.03. To achieve the desired sensitivity, genosensors generally require previous DNA extraction and subsequent amplification; being PCR the most frequent amplification procedure coupled to biosensing protocols. In this work, the amplification of these two hazelnut sequences by PCR rendered long amplicons. Because these long strands present a peculiar hybridization behavior with immobilized probes, compared to the predictable hybridization kinetics of synthetic oligonucleotides, this work also included a careful evaluation of the hybridization efficiency with PCR products. The high steric hindrance of long amplicons and the presence of the sister strand in solution competing with the immobilized short capture probe, make difficult the hybridization on the surface. To enhance hybridization efficiency and sensor performance, sandwich hybridization was implemented using a biotinylated signaling probe. The sandwich hybridization is often recommended with long target sequences and involves two probes: a capture probe partially complementary to the target and anchored to the electrode, and a labeled signaling probe that hybridizes with the rest of the target. The relative hybridization position of probes in a sandwich assay is of main importance, as it has been previously reported (Del Giallo et al., 2005; Holzhauser et al., 2000),

since higher hybridization efficiencies are obtained when one of the termini of the amplicon was involved. In this work, Bettazzi et al. demonstrated that the signaling probe should be designed in order to be contiguous to the capture probe sequence to get the optimal hybridization yield and therefore the highest electrochemical response. On the contrary, when fringe single-stranded regions between the probes exist, intramolecular folding may occur reducing the availability of the target strand, and lowering the hybridization efficiency at the electrode surface. Enzymatic signal amplification using streptavidin–alkaline phosphatase with DPV was performed. After optimizing the device, an extensive analysis of real samples without precedent was conducted. DNA was extracted from several hazelnut-containing commercial samples (including chocolates and lecithin-based products) with a commercial kit. PCR amplicons were analyzed by gel electrophoresis, purified and then quantified with PicoGreen. These pretreated samples were diluted and analyzed with the genosensor. The obtained data correlated well with those obtained with an ELISA method. Despite all efforts, only qualitative data was reported in nanomolar detection levels.

Another work reported the detection of the same hazelnut DNA sequence Cor 1.04, coupled to a microfluidic system (Berti et al., 2009). Capture probes were immobilized to streptavidin-coated paramagnetic micro-beads. The complementary sequence was then recognized via sandwich hybridization with a capture probe and a biotinylated signaling probe. After labeling the biotinylated hybrid with a streptavidin–alkaline phosphatase conjugate, the particles were introduced in a disposable cartridge composed of eight parallel microchannels etched in a polyimide substrate. The modified particles were trapped with a magnet addressing each microchannel individually. The presence of one microelectrode in each channel allowed direct electrochemical detection of the enzymatic product within the microchannel. The main advantage of these systems is their portability and the multi-target assessment, which together with the lower propensity to nonspecific signals of the magnetic particles, resulted in improved detection limits for diluted PCR amplicons.

3.2. Peanut

Another highly frequent food allergy is caused by peanut consumption. Their symptoms range from mild oral allergy syndrome to anaphylactic reactions and even death (Bock et al., 2001). Up to now, there are 13 listed allergens that account for peanut allergy, including 11 allergens named Ara h 1 (*Arachis hypogaea* allergy 1) to Ara h 11, and two recently identified allergens, agglutinin and 18 kDa oleosin (Mari et al., 2006). The major peanut allergens are Ara h 1–3 that are recognized by over 90% of peanut-allergic adults (Koppelman and Hefle, 2006).

Ara h 1 is a 7S vicillin-like globulin also known as cupin, which affects 35-95% of peanut-allergic patients. The group of Li reported different sensors for Ara h 1 detection, with a variety of immobilization and detection strategies (Sun et al., 2012; Sun et al., 2015a; Sun et al., 2015b).

Initially, they reported the detection of a 125 bp sequence of Ara h 1 peanut DNA (Sun et al., 2012). The device relied on the formation of a SAM of a thiolated hairpin capture probe and MCH onto gold electrodes. A signal-off detection was employed, based on the decrease of the electron transfer of ferrocyanide/ferricyanide ($[\text{Fe}(\text{CN})_6]^{3-/4}$) redox probe after the hybridization with the target measured by EIS. They obtained femtomolar sensitivity for synthetic samples, and detected a concentration level of 0.32 pM of peanut DNA extracted from one sample of peanut milk.

Later, the same group reported another device based on the previous design, but using a multilayer graphene-gold nanocomposite onto a GCE electrode for the immobilization of the hairpin capture probe. Monitoring of $[\text{Fe}(\text{CN})_6]^{3-/4}$ by DPV provided a lower detection limit for synthetic oligonucleotides (sub-femtomolar) (Sun et al., 2015a). They also reported the detection of DNA extracted from a peanut milk beverage sample. When known amounts of target DNA were added to the extract, adequate recoveries were obtained.

Another genosensor for Ara h 1 detection involved a mixture of chitosan and multiwalled carbon nanotubes and a spongy gold film, employed to coat a glassy carbon electrode (Sun et al., 2015b). The adsorption of a dually-tethered hairpin capture probe was carried out. Its 5' end was functionalized with a thiol group and its 3' end with a biotin tag. Streptavidin-horseradish peroxidase was used as enzymatic label with CA detection. They also reached a femtomolar quantification level for synthetic samples. In addition, quantification of one real sample of DNA extracted from peanut milk was reported, showing a good correlation with the results obtained with real-time PCR.

All of the previous sensors detected Ara h 1, which represents about 20% of the total peanut proteins. However, Ara h 2 (conglutin family, 2S albumin) constitutes the most frequently recognized allergen in children (Flinterman et al., 2007; Nicolaou et al., 2010). An electrochemical sensor for detection of an 86-mer DNA sequence encoding part of the allergenic protein Ara h 2 was developed (Sánchez-Paniagua López et al., 2014). The detection system of synthetic oligonucleotides was based on a sandwich hybridization assay and enzymatic amplification. The study was focused on the optimization of the sensing phase, a mixed SAM of thiolated capture probe and MCH, using the Design of Experiments approach, to improve the performance of the device. The use of Response Surface for desirability function generated the overlay contour plots for each response, signal and blank, which allowed finding the

optimum experimental area with maximum signal for target and minimal for blank. As a result, picomolar sensitivity was achieved.

3.3. Gluten

Celiac disease is defined as a common autoimmune inflammatory disease with both genetic and environmental components. The celiac disease is one of the most frequent chronic health illness, being estimated to affect as much as 1% of the general population, although this number could be underestimated owing to difficulties in diagnosing this autoimmune disorder (Green and Cellier, 2007; Kagnoff, 2007). Clinical manifestations of celiac disease are highly variable, including both gastrointestinal and non-gastrointestinal features, which may result in systemic manifestations (Briani et al., 2008). The major environmental trigger is the consumption of gluten, which corresponds to a significant portion of protein fraction in cereals, namely wheat, barley and rye (Fasano and Catassi, 2012). In the celiac disease, one of the most well studied peptides is the so-called 33-mer peptide, which is the immunodominant fragment of the alpha2-gliadin playing a major role in the immunogenicity (Shan et al., 2002). Mutant peptides lacking this fragment lose their immunotoxicity. The immunogenicity of the referred peptide was attributed to its resistance to breakdown in the human intestine, mainly due to its high content of proline residues as well as high susceptibility to transglutaminase deamidation (Qiao et al., 2004). Nowadays, the only effective treatment consists on the avoidance of gluten- containing food.

So far, most of the methods employed in the detection of gluten rely on protein-based approaches, namely ELISA method (Diaz-Amigo and Popping, 2013; Janssen, 2006). However, false negative results, poor reproducibility and cross-reactions are frequently considered as the major drawbacks of these methods (Haraszi et al., 2011; Mujico et al., 2011). Hence, DNA-based technologies have arisen as an alternative strategy for gluten detection. The enormous complexity of the wheat genome (probably one of the most complicated genomes among domesticated plant species), along with its high genetic variability, make the detection of wheat-related DNA sequences both a challenging but stimulating task. For these reasons, only two electrochemical sensors have been reported to detect wheat DNA in food samples (Martín-Fernández et al., 2015; Martín-Fernández et al., 2014). In both works, a 99 nucleotide DNA sequence from wheat was selected as target to develop a sandwich type DNA genosensor. This sequence is a highly structured, long and repetitive target fragment that encodes the alpha2-gliadin 33-mer allergenic protein. The high content of guanine in this fragment and in its complementary probes (capture and signaling probes) might favor the formation of G-quartets that provide additional stable 3D conformations that further prevent the strands hybridization. In this work it was shown that a rational design of the capture and

tagged-signaling probes along with a two-hour hybridization time coupled to a heating shock can be considered a general strategy to overcome these problems. PCR was also optimized to be applied to DNA extracted from cereal flour samples. The genosensor developed allowed the selective detection of this fragment in the presence of both DNA spurious products and PCR reagents without the need of amplicon purification. The successful detection of gliadin DNA verifies the suitability of the described PCR-coupled genosensor to complement the immunochemical methods to detect gluten for celiac disease patients' safety.

Although the EU Regulations establishes gluten labeling regardless of the cereal species, in other countries the identification of the specific gluten-containing cereal origin is compulsory (Diaz-Amigo and Popping, 2012). In this sense, it is important to have highly discriminatory methods for selective detection of wheat, to meet these standards. In order to improve the selectivity of the previously described genosensor against wheat DNA sequences, and to provide the highest discrimination ability, the former design was modified by incorporating a structured capture probe instead of a linear one (Figure 3) (Martín-Fernández et al., 2015). Usually, the selectivity of the hybridization reaction is based on mismatches that cause a destabilization of double-stranded DNA. Therefore, structured oligonucleotides, hairpin or stem-loop structures, provide a competition reaction with probe-target hybridization that increases probe specificity and prevents the hybridization of undesired sequences (with a few mismatches). The hairpin-based genosensor provided slightly inferior sensitivity than that obtained with linear probes due to energetic penalty associated to the need for hairpin opening, but exhibited complete specificity against wheat with respect to closely-related species (barley and rye).

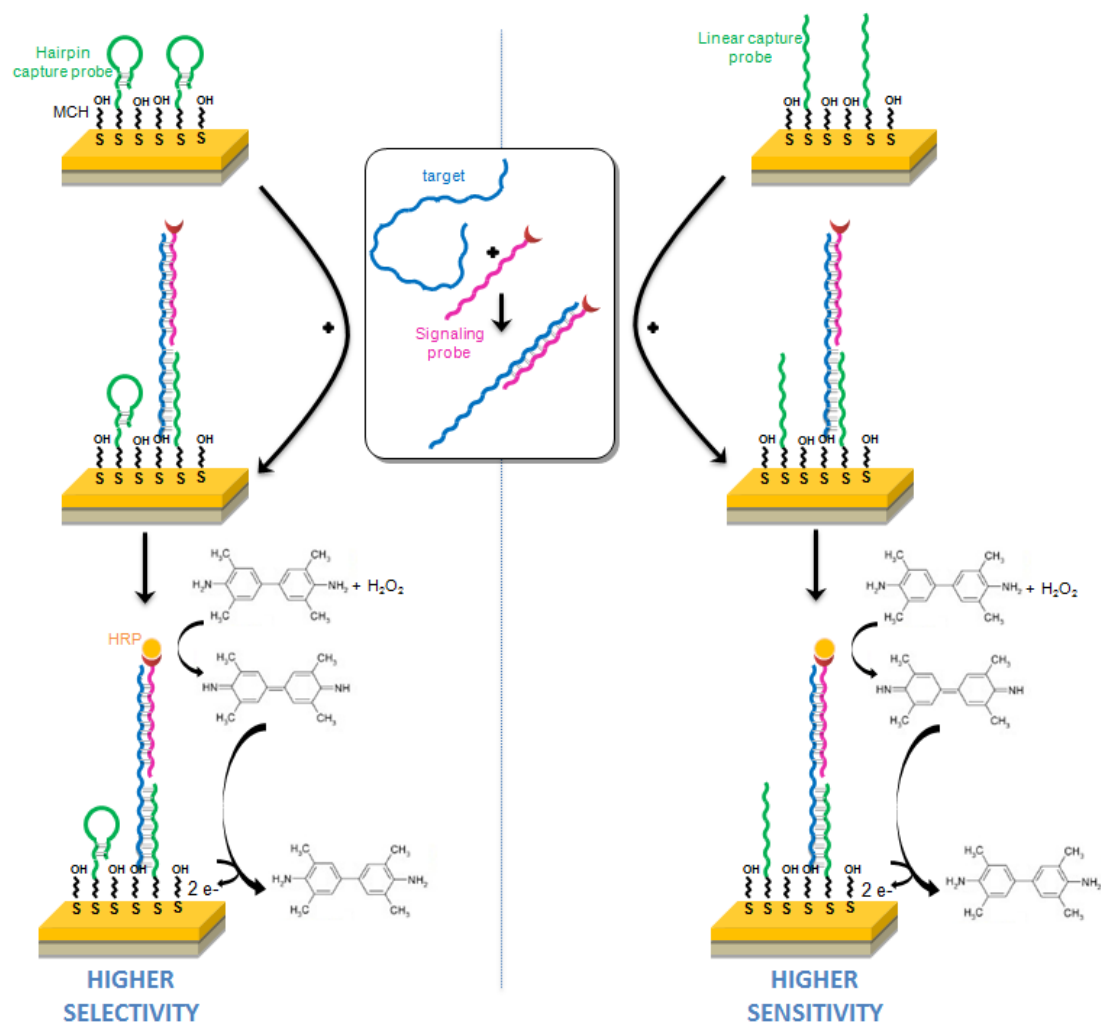


Figure 3. Schematic illustration of the sensing phase and the assay steps of the genosensors reported for gluten detection.

4. SPECIES IDENTIFICATION

Food and feedstuffs are generally of plant or animal origin. The identity of ingredients in processed or composite mixtures is not always readily apparent and verification that the components are authentic and belong to sources acceptable for human consumption is needed.

The reliable identification of the species is a key issue for food authenticity and should preferably be based on parameters that do not undergo too many alterations during food processing, e.g., the taxonomic identification of fish species based on morphological characteristics is a difficult task because most species are available commercially after the removal of external features (viscera, skin, head, etc.) (Ward et al., 2009). Routinely traditional analysis involves detection of species-specific proteins by means of electrophoretic and immunological methods (Gallardo et al., 1995; Piñeiro et al., 1999). As

mentioned before, heating and processing treatments are prone to alter proteins and modify the results (Lockley and Bardsley, 2000). DNA is more thermostable, and is present in the majority of the cells of an organism, enabling the identification regardless of the tissue of origin. For these reasons, in recent years mitochondrial DNA has been used as target for fish and meat species identification.

Fish species can only be visually identified while the fish remains in its whole state. The matter becomes further complicated as fish is processed where mincing, battering, crumbing and frying operations take place. Consequently, in this type of food, substitution for toxic or low valued fish species represents a risk (Asensio Gil, 2007). One example of the need of ensuring authenticity of fish species comes from the dangerous consumption of members of the *Tetraodontidae* family (pufferfish). Pufferfish poisoning is one of the most dangerous intoxications from marine species. In Japan and in some parts of the world, pufferfish are prepared by highly trained cooks, and are considered a delicacy, regardless of the fact that they contain a deadly toxin (Lau et al., 1995). Del Giallo et al. (2005) developed a genosensor for differentiation of pufferfish (*Takifugu niphobles*, *Tetraodontidae* family) from anglerfish (*Lophius budegassa*, *Lophiidae* family), considering that the latter lacks from toxicity. Long (~600 bp) sequences of mitochondrial DNA of both were amplified using primers that are common to both species. Purification and quantification (with Picogreen) steps of the amplified target were required prior to analysis. The amplified sequences were hybridized with a thiol-thethered capture probe, immobilized to SPEAu, and different biotinylated signaling probes, forming a dendritic-like assay (Figure 4). Afterwards, labeling and detection were done by means of an enzymatic reaction using ALP-streptavidin. They demonstrated that both the surface coverage by the probe and its relative position on the target strand are important to control the hybridization efficiency. Providing the long target sequence, they proposed a multiple signal amplification design, anchoring several enzymes to multiple sites on the target strand. The high steric hindrance limited the use of more than three closely located enzymes. An unusual bell-shaped relationship between the amplicon concentration and the electrochemical response was found. It was attributed to the renaturation of the two strands of the extremely long target sequence, favored over the formation of the surface-immobilized capture probe amplicon complex. This work is worthy of mention because it was the only genosensor for fish identification, and one of the first works involving authenticity assessment.

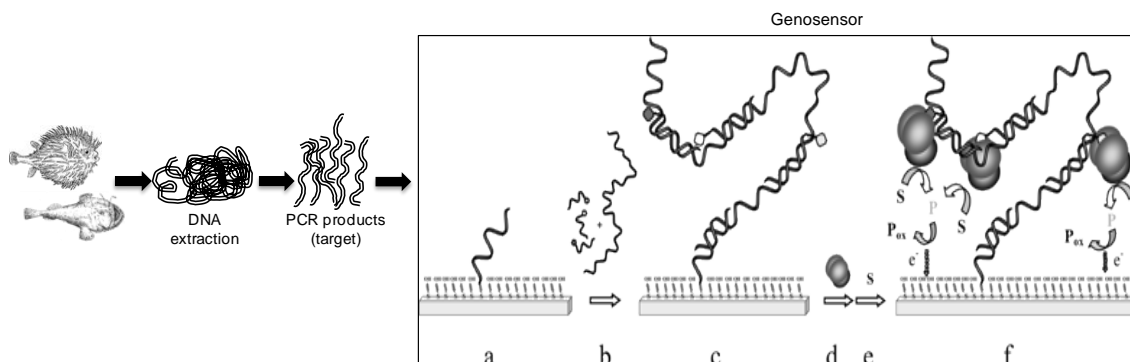


Figure 4. Schematic representation of the dendritic-like signal amplification path. Unmodified PCR products (b) were captured at the sensor interface (a) via sandwich hybridization with the surface-tethered probe and up to three biotinylated signaling probes. The polybiotinylated hybrid (c) was then coupled with streptavidin-alkaline phosphatase conjugates (d) and exposed to the enzyme substrate solution (e). DPV was finally used to detect the oxidation signal of the product of enzymatic hydrolysis (f). Reprinted with permission from (Del Giallo et al., 2005) Copyright (2005) American Chemical Society.

On the other hand, meat species adulteration represents a relatively frequent issue in the food industry, as it is the case of the addition of pork to beef products for economic gain. This type of adulteration is especially important in some religions, where pork meat consumption is forbidden (Soares et al., 2010). There is also an increased concern over undeclared meat species due to the relatively recent outbreaks of bovine spongiform encephalopathy, commonly known as Mad-Cow Disease, a fatal, neurodegenerative disease in cattle. The potential for transferring this disease to humans in the form of variant Cruetzfeldt Jacobs Disease has increased the need for traceability of meat, meat products and feedstuffs (Mafra et al., 2008b). Similarly, consumers may be unwilling to ingest meat because of the risk of avian influenza virus and foot and mouth disease, which are associated with the consumption of chicken and swine meats, respectively.

One of the first reports in regards of meat species identification with genosensors was carried out by Mascini et al. (2005). They designed two different genosensors for the detection of bovine and sheep samples targeting satellites of DNA (~250 bp and 430 bp, respectively), which are highly repetitive oligonucleotide sequences. A label-free approach was employed, based on the square-wave voltammetry detection of guanine oxidation when the target is present. To avoid background signals given by the probe itself, inosine-modified (guanine-free) DNA probes of ~25 bases were designed. The probe was designed to hybridize with the peripheral zone of the DNA satellite fragment to avoid steric hindrance effects (Figure 5). Bovine genomic DNA from extracted samples was analyzed with the genosensors without PCR, after performing restriction enzyme digestion to reduce the size of the genomic fragments,

consequently reducing the steric hindrance. The bovine genomic DNA was applied to both bovine and sheep genosensors. As expected, it only provided a positive response in the former, confirming the selectivity of the designed device. So, they reported the detection of 3 $\mu\text{g/mL}$ of synthetic target, and a clear discrimination of the mammalian species, bovine and sheep, in real samples with a detection limit lower than 30 $\mu\text{g/ml}$ of total genomic bovine DNA.

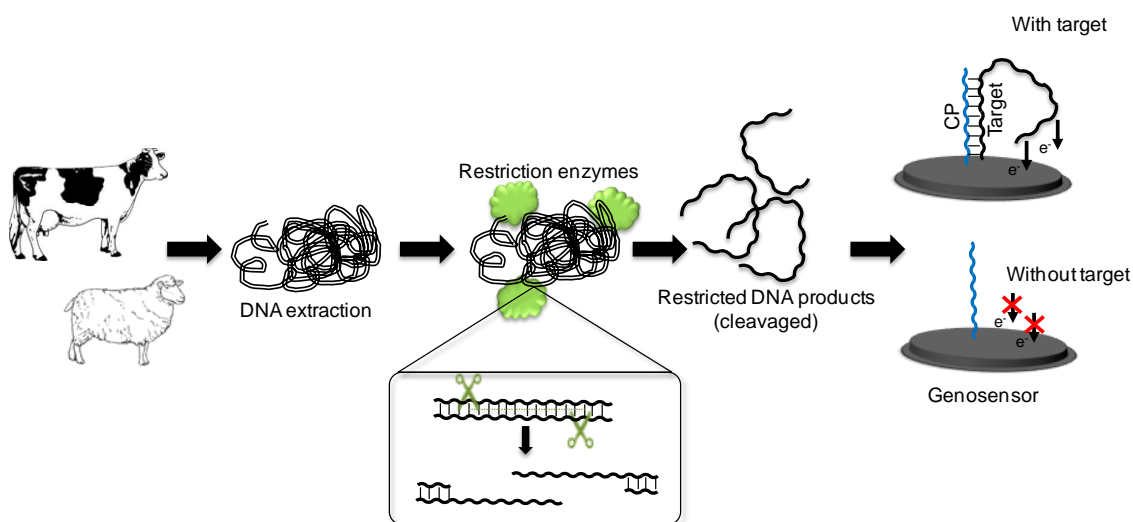


Figure 5. Meat species identification with genosensors with previous genomic DNA cleavage by restriction enzyme digestion

Chaumpluk et al. (2006) reported an electrochemical platform for bovine DNA detection and semi-quantification in feedstuff using amplification by PCR and LSV detection. This work used Hoeschst 33258, H33258, a DNA minor groove binder used as a fluorescent dye to stain DNA. Its electrochemical activity was exploited by mixing it with the PCR-amplified double-stranded DNA and measuring the change in the anodic peak current of free and DNA-bound H33258. In the absence of DNA, the highest diffusion rate of the free H33258 resulted in a higher signal on screen-printed carbon electrodes. On the contrary, lower oxidation currents were measured in the presence of amplicons due to binding of dye to DNA.

The PCR amplification was designed to target two sequences, one bovine-specific (bovine parathyroid, Pth) and the other for an eukaryote common gene (12S rRNA). Qualitative (discrimination of bovine-containing products) and quantitative determination of the bovine content as percentage were reported. For the quantitative analysis, the authors introduced a plasmid reference system to build standard calibration curves with both genes. Cloned portions of Pth and 12S rRNA were used as known DNA copies and serial dilutions were performed to obtain a wide concentration interval. Animal blood and bone meals for feedstuffs production and commercial pet foods were

extracted and purified with commercial kits. By performing 30 cycles of PCR amplification (before saturation), semi-logarithmic relationships between the anodic current and copy number of the Pth gene and the 12S rRNA gene were found. Bovine composition was expressed as a ratio between the Pth gene copy number and total copy number of 12S rRNA. Only semi-quantitative data was obtained because 12S rRNA could not be estimated accurately in mixed animal and plant ingredients, making it impossible to achieve a full quantitative analysis.

Later, Ahmed et al. (2010) developed a faster and simpler method for bovine, chicken and swine meat identification, using a combination of isothermal amplification of DNA and electrochemical detection on disposable carbon-based electrochemical printed chips (Figure 6). This work also used H33258 to measure DNA products by LSV. Mitochondrial sequences were amplified in DNA extracted from raw meats and processed food, by means of LAMP, instead of the most widely employed temperature-dependent PCR amplification. In this type of isothermal amplification, four loop primers for species-specific detection were used, where six distinct regions are targeted and amplified without the use of a thermal cycler. It is considered to be more specific than PCR as it uses a greater number of primers to delimit the region to be amplified, which may diminish the occurrence of false-positive results. The amplified products were diluted and then mixed with H33258. The mixture was placed onto the chip surface for each measurement. The limits of detection were 20.33 ng/ μ L, 78.68 pg/ μ L and 23.63 pg/ μ L for pork, chicken and bovine species, respectively. The results from this method were compared to a multiplex-PCR and capillary gel electrophoresis system. The authors claimed that their LAMP-based electrochemical genosensor was more specific, reduced the cross-reactivity and avoided unspecific amplicon formation in raw and processed meats, compared to the PCR-based method.

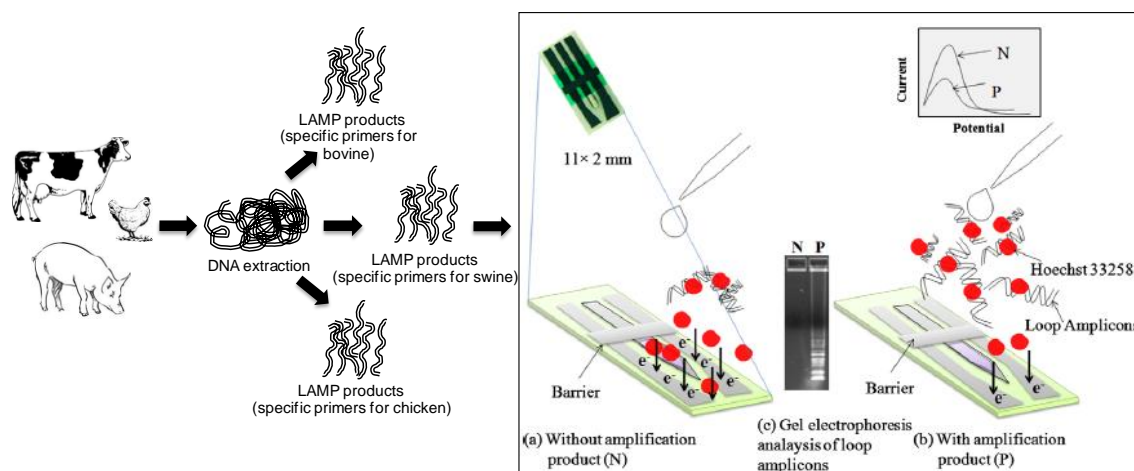


Figure 6. Species-specific identification and detection of loop amplicons based on DNA–H33258 interaction using the disposable carbon-based electrochemical printed chip. A carbon barrier is used to prevent the reaction mixture to adhere

with the chip connector. (a) Higher rate of diffusion and oxidation of H33258 molecules on the electrode surface in the absence of DNA. (b) Lower oxidation observed due to DNA–H33258 interaction and for the low amount of unbound/free H33258 molecule. (c) Gel electrophoresis analysis of negative sample, N and positive sample, P are shown. On the right top inset, the illustration is showing the electrochemical response. Adapted with permission from Elsevier (Ahmed et al., 2010).

5. GMO TESTING

Genetically modified foods are those produced from organisms that have undergone specific changes into their DNA using genetic engineering methods in order to introduce new traits (Figure 7) to improve crop production (herbicide or insect resistance) or to enhance nutritional properties. Shortly after their release to the food and feed market, many genosensors have been reported as alternative methods towards their detection (Arugula et al., 2014) Target selection is relatively easy to undertake because the transgenic inserts and specific sequences are fully described in open databases and in validated PCR methods when they have been authorized. Some of these sequences serve as “generic” targets for all GMO-containing products, such as 35S or PEP promoters and NOS terminator (van den Eede et al., 2004), so the methods that target such sequences are called “screening methods” and they assist in preliminary analysis. As it has been recently reviewed by Manzanares-Palenzuela et al. (2015b), most electrochemical devices have been reported for screening purposes, followed by the group of genosensors that target gene-specific sequences, i.e. part of the gene that encodes the protein conferring herbicide resistance or insect resistance in crops. However, the highest level of specificity is given by event-specific methods, in which the targets are the unique junction, characteristic of each transgenic event, found at the integration locus between the inserted DNA and the recipient genome. Only few works have been developed for detecting event-specific sequences. Taxon-specific detection is required for GMO quantification and for species identification in preliminary screening analysis but this is not usually accomplished when developing electrochemical methods. This fact seriously limits the applicability of the methods so far reported that remain restricted to simple identification or quantitation of transgenic events in an absolute fashion that is not acceptable to establish compliance with legislation, which requires relative quantitation (content expressed in percentage).

In this section we review the most recent and relevant electrochemical genosensors reported for GMO detection.

5.1. Screening methods

As it is shown in Figure 7, many genosensors have been reported as screening methods (Carpini et al., 2004; Kerman et al., 2006; Lien et al., 2010; Ligaj et al., 2003; Lucarelli et al., 2005; Meric et al., 2004; Sun et al., 2008; Sun et al., 2007; Tichoniuk et al., 2008; Ulianas et al., 2014; Wang et al., 2009; Wang et al., 2008; Xie et al., 2008; Xu et al., 2006; Yang et al., 2012b; Zhu et al., 2008). The group of Mascini (Lucarelli et al., 2005; Meric et al., 2004) pioneered the development of electrochemical sensing platforms for GMO screening. Their contribution is worthy of mention due to the high sensitivity achieved by their proposed device and the application to reference materials (see Figure 7, screening methods). They reported an EIS-based sensor using mixed SAMs as immobilization method and sandwich hybridization with enzymatic labeling. The amplification of the signal was mediated by the addition of ALP-streptavidin conjugate that binds to biotin-signaling probe (Lucarelli et al., 2005). The enzyme transforms 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium mixture in an insoluble and insulating product which precipitates on the sensing interface. Electrochemical transduction of the hybridization process was performed by EIS in presence of $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The signal increases after hybridization due to the precipitation of the enzymatic product onto the surface. A remarkable sensitivity was achieved, with a LOD of 1.2 pM. However, this impedimetric genosensor was not able to differentiate between two different concentrations of both GM soy and maize powders reference materials, amplified by PCR. Therefore, the authors concluded that this method could only work as a “yes or no” system, until an adaptation for quantitative determination of GMOs could be done. Lucarelli’s work is one of the most sensitive sensors reported up to this date.

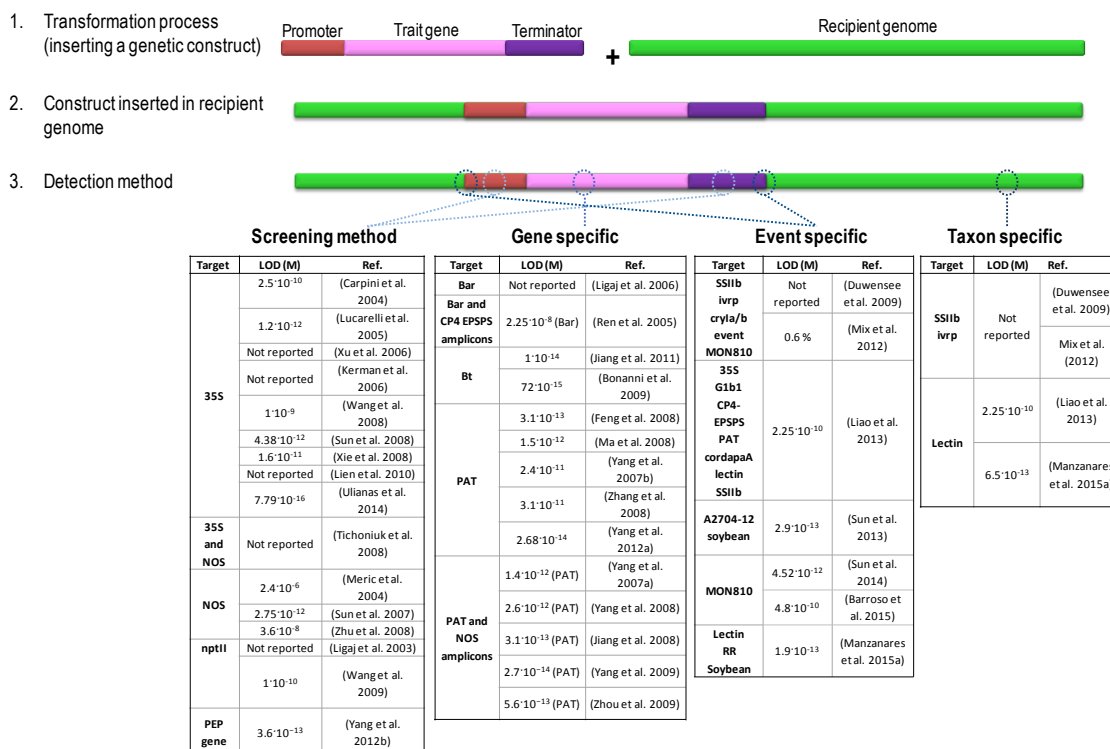


Figure 7. Scheme of the transformation process in GMOs. Levels of specificity of analytical methods targeting DNA sequences and electrochemical genosensors described for each level, highlighting their sensitivity and the detected target. LOD obtained with synthetic oligonucleotides.

Recently, Ulianas et al. (2014) reported the use of a composite based on acrylic microspheres and gold nanoparticles onto screen-printed carbon electrodes, achieving sub-femtomolar levels of detection, which is considered to be a remarkable contribution to the field. The nanoparticles, used as electron transfer material, were suspended in ethanol and placed onto the electrode surface. Subsequently, acrylic microspheres were deposited onto the modified electrode for DNA probes immobilization via covalent bonding. The hybridization process was monitored by DPV of anthraquinone-2-sulfonic acid monohydrate sodium salt as hybridization redox indicator, because of its intercalation capacity in the double helix, increasing the signal when hybridization occurs. The biosensor was used for the determination of GMO extracted from GM soybean samples. They reported a wide linear range of six orders of magnitude with a sub-femtomolar detection limit (0.779 fM) with synthetic oligonucleotides. Such sensitivity was attributed to a robust covalent bond of the DNA probe onto the enhanced large area given by the acrylic microspheres/gold nanoparticles composite.

5.2. Gene-specific methods

Many genosensors have been reported as gene-specific methods (Bonanni et al., 2009; Feng et al., 2008; Jiang et al., 2008; Jiang et al., 2011; Ligaj et al., 2006; Ma et al., 2008; Ren et al., 2005; Yang et al., 2007a; Yang et al., 2012a; Yang et al., 2007b; Yang et al., 2008; Yang et al., 2009; Zhang et al., 2008; Zhou et al., 2009), accounting for more than 30% of the reported GMO sensors so far (Manzanares-Palenzuela et al., 2015b). One of the commonly inserted gene in genetically engineered plants is the Bt gene obtained from *Bacillus thuringiensis*, which encodes an insecticidal protein in plants. Jiang et al. (2011) presented an electrochemical DNA biosensor for a 30-mer Bt fragment. A MCH-mixed SAM was used to immobilize a 15-mer capture probe in a gold electrode (Figure 8). The authors proposed a sandwich hybridization format, with a signaling probe modified with biotinylated Ag nanoparticles. The modified electrode was incubated with streptavidin and subsequently more biotinylated Ag nanoparticles were added in order to form aggregates that amplified the response. Detection of Ag nanoparticles was carried out using solid-state Ag/AgCl voltammetric process in which the Ag was oxidized to Ag^+ and the electrogenerated Ag^+ formed insoluble AgCl on the electrode surface in the presence of Cl^- . The solid AgCl on the electrode was then reduced to Ag. By measuring this reduction current, a detection limit of 10 fM was obtained, three orders of magnitude less than single Ag nanoparticle label detection.

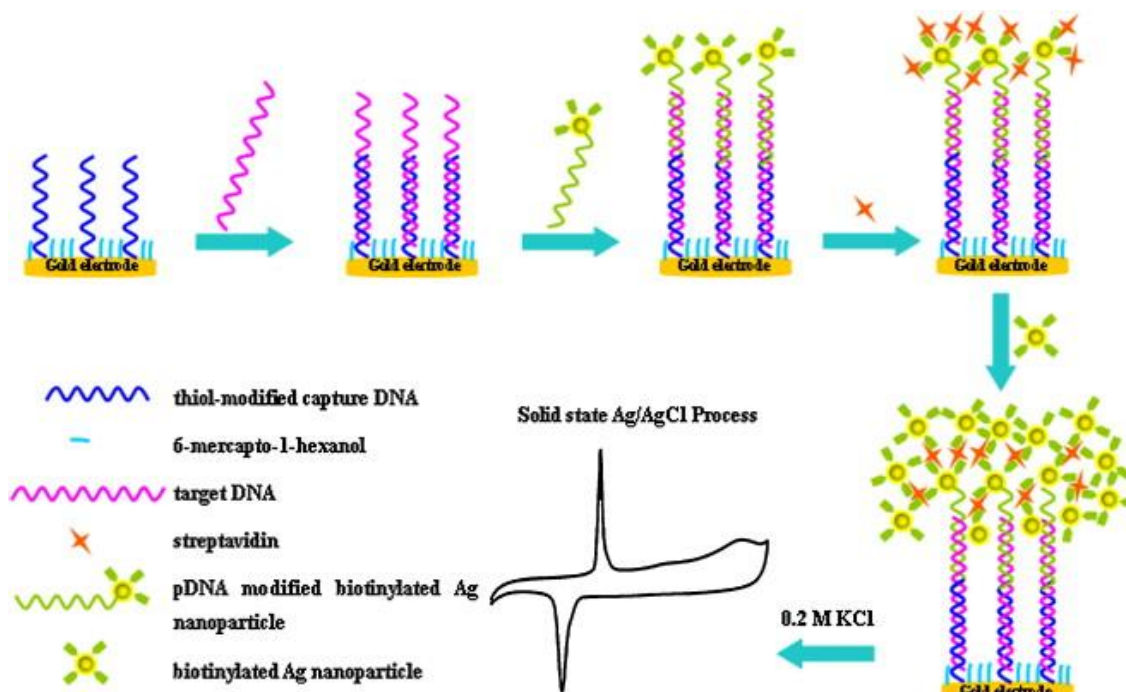


Figure 8. Schematic image of the DNA sensing strategy. Reproduced with permission from Elsevier (Jiang et al., 2011).

Other frequently reported target for gene-specific genosensor analysis is the PAT gene, which encodes an herbicide resistant protein, phosphinothricin acetyltransferase. Herein, we limit to discuss the most sensitive approaches for PAT detection. Yang et al. (2009) reported an EIS-based genosensor based on a carbon paste electrode modified by a multi-walled-nanotubes/polyaniline nanofibers (MWCNT/nano-PANI) composite dispersed in chitosan (Figure 9.I). A mixture of all these components was dripped onto the fresh surface of the electrode, followed by the adsorption of the DNA probe. The combination of these materials resulted in the synergistic effect between nano-PANI and MWCNT nanocomposites in chitosan film, giving a highly enhanced conductivity and an increase of the surface area that consequently increases the load of the DNA probe. Quantification of PAT gene was performed and a LOD of 27 fM was estimated. In the same article PCR amplification of the NOS terminator sequence, extracted from a sample of genetically modified soybean, was performed and qualitative results were reported. Another strategy reported by the same group involved the electrodeposition of L-tyrosine on a carbon electrode surface, followed by the addition of PANI-ZrO₂ nanoparticles (Yang et al., 2012a). The resulting PANI-nanoZrO₂ composite displayed an interconnected dendritic structure (Figure 9.II), which provided high specific surface area for the electron transfer and for the immobilization of the capture probe. The limit of detection for PAT sequence was 26.8 fM using EIS as electrochemical technique.

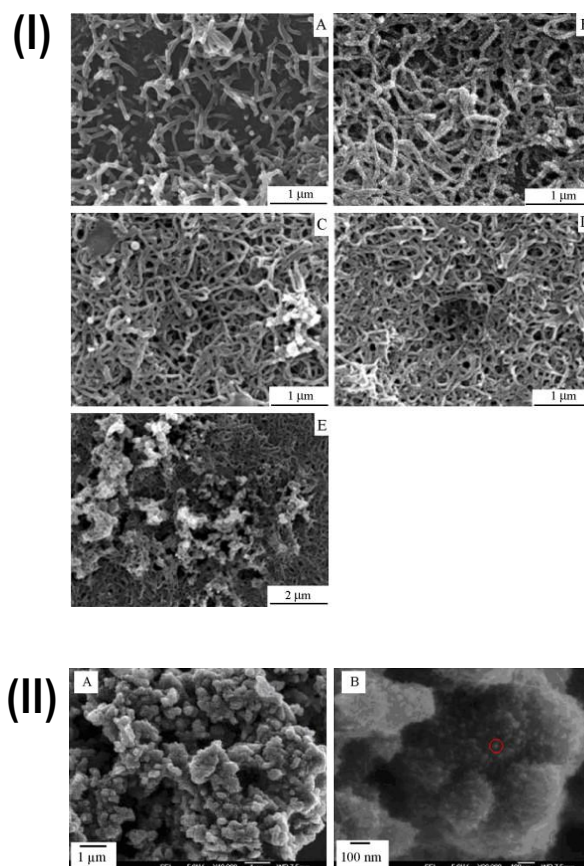


Figure 9. I) SEM images of PANI nanomaterials (A) and PANI-MWCNT nanocomposites film under different ratios of PANI to MWCNT as 1:0.01 (B), 1:0.1 (C), 1:1 (D) and 1:2 (E). Reproduced with permission from Elsevier (Yang et al., 2009). II) The SEM images at low magnification (A) and high magnification (B) of PAN-nanoZrO₂ Reproduced with permission from Elsevier (Yang et al., 2012a).

5.3. Event-specific methods

Event-specific genosensors have been the least reported group compared with other levels of GMO specificity (Barroso et al., 2015; Duwensee et al., 2009; Liao et al., 2013; Manzanares-Palenzuela et al., 2015a; Mix et al., 2012; Sun et al., 2014; Sun et al., 2013). Among these, two works based on an event-specific genosensor for a transgenic variety of maize (MON810) and its application in maize flour samples are worthy of mention because they address important issues in GMO monitoring: multi-target detection and applicability to real samples. The genosensor relied on a direct assay, where the capture probe was immobilized by mixed SAMs with MCH onto gold electrodes. The complex [OsO₄(bipy)] was utilized as an electrochemically reversible redox probe covalently bound to DNA. It reacts only with the pyrimidine bases of ssDNA. The reaction involves the oxidation of the C=C double bond in the pyrimidine ring giving a diester of osmic (VI) acid. Four different targets, two from wild type maize and two from genetically modified maize were simultaneously labeled and detected using four working electrodes, each modified with a specific capture probe. The sequences were labeled simultaneously with the osmium complex and afterwards, detection was achieved with square wave voltammetry and the signal increased proportionally to the target concentration in the nanomolar range (Duwensee et al., 2009).

The application of the previous device to detect MON810 in maize flour samples employed an asymmetric PCR, that is, a PCR that preferentially amplifies one DNA strand in a double-stranded DNA template using a great excess of one of the primers. Results showed that it was possible to discriminate between maize and GM maize in real samples from 0.6 % of GMO content. No significant detection of MON810 was possible when only 0.5% of transgenic maize was present in the sample, most likely due to insufficient amplification of the template DNA (Mix et al., 2012).

Considering the great contributions of multiplexing in GMO detection, given the increasing number of commercialized GMOs, it is important to mention the work of Liao et al. (2013). They reported a genosensor that provides a multiplex GMO analysis in a gold 16-array format. The proposed device implemented a biomolecular computational assay based on the logic gates principle. This consists on logic operations from dual or multiple inputs to produce a single logic output. Exploiting this principle, GMO-related target

sequences were used as inputs. The output was the electrochemical signal obtained if hybridization of the targets with specific probes occurred. A monolayer-modified Au electrode surface was prepared with a thiolated capture probe and 3-mercaptopropionic acid. Target DNA was incubated with two signaling probes (functionalized with FITC, or biotin) to ensure homogeneous hybridization. These hybrids were loaded over the DNA capture probe of Au surfaces. A mixture of anti-FITC-HRP and avidin-glucose oxidase solution was added followed by the addition of a mixture of enzyme substrates (glucose and TMB). When all the probes (capture and signaling probes) recognized the target, an enzymatic cascade reaction occurred in which GOx catalyzed the oxidation of glucose to produce H₂O₂, which was immediately reduced by HRP along with the resulting TMB. The latter was finally detected by chronoamperometry. Different target sequences were designed corresponding to different GM events. Each sequence was composed by fragments of three GMO regions: a promoter, a trait gene and an endogenous gene. A specific probe recognized each region: the capture probe was complementary to the promoter region and the FITC-labeled and biotinylated signaling probes were complementary to the trait and endogenous genes, respectively. According to the authors, the number of oligonucleotide bases separating the target sequences, especially trait genes from endogenous genes, may play a role in the efficiency of the cascade enzymatic reaction, which requires further attention to meet sequence diversity in various GMOs.

The system was tested with DNA extracted with a commercial kit from soybean powder containing known GM levels. The assay was able to detect concentrations as low as 25 nM of total DNA, when measuring a sample containing 0.9 % GMO. Accordingly, the LOD corresponds to 225 pM of GM DNA level. This device was capable to integrate screening and event-specific methods, and a multiplex detection toward different GM events in one analysis.

Most recently, a multiplex electrochemical platform was reported for Roundup Ready soybean quantification (Manzanares-Palenzuela et al., 2015a). The work consisted on the simultaneous detection of two DNA sequences, event and taxon-specific, via sandwich hybridization onto magnetic microparticles. Both biotinylated capture probes were anchored to the surface of streptavidin-modified magnetic beads. The target sequences were hybridized with their specific signaling probes, each one end-labeled with FITC and digoxigenin, complementary to the transgenic and taxon sequences, respectively. Dual enzymatic labeling was performed with HRP and ALP, both conjugated to Fab fragments of anti-FITC and anti-digoxigenin, using TMB and 1-naphthyl phosphate as enzymatic substrates, respectively. This dual labeling system permitted to obtain two distinct electrochemical readouts in parallel measurements, using DPV and chronoamperometry for ALP and HRP, respectively. The LOD values obtained were 0.65 pM and 0.19 pM for the

event-specific and taxon-specific targets. The transgenic-taxon ratio approach was proposed with the aim of quantifying GMOs according to the European Recommendation 2004/787/EC. To calculate the percentage of GMO in a sample, the accepted criterion is to normalize the amount of the GMO specific sequences against the amount of a plant specific gene. Based on this recommendation, the suitability of the proposed assay was assessed with synthetic mixtures of both sequences containing 0.8% and 2% of GMO. The obtained results were closed to the theoretical ones, giving low relative error values (6.3 and 5%, respectively), claiming that the assay is adequate for GMO quantification. This is the first attempt to quantify GMOs without the use of real-time PCR in terms of percentage as required by legislation.

6. KEY FEATURES IN FOOD SAFETY ASSESSMENT BY GENOSENSOR TECHNOLOGY

In recent years consumers have become very critical about food safety and food quality, especially due to modern controversies related to the hidden presence of allergens in some foods, genetically engineered crops and species authentication (Figure 10). Legal framework covers issues ranging from personal choice (GMOs), the prevention of food fraud and substitution of some food for others of lesser value (food adulteration), and to certify the safety of food to allergic or celiac individuals (allergen issue). It is clear from this review that each one of these topics deserves unique perspectives regarding legislation and analytical monitoring.



Figure 10. Hot topics of public concern related to food products

In general, food assessment related to GMOs, allergens and species authentication has been based in two analytical methodologies, immunoassays and DNA-based methods. The superior stability of DNA against food processing technologies compared to proteins, holds a major benefit for food-related applications. Among DNA methods, electrochemical genosensors are still considered as an emerging technology under ongoing and continuous research, comprising all kinds of innovative designs for DNA immobilization, labeling and detection. We have reviewed a compendium of genosensors described for specific analytical applications in food control. Detection levels as low as femto- and attomolar have been achieved with GMO and allergen sensors, respectively. Sensitivity of genosensors is mainly conditioned by the electrochemical technique and by the material used as immobilization matrices for DNA capture probes. Among electrochemical detection methods, EIS-based methods showed the best sensitivity and, in relation with the immobilization strategies, nanomaterial combinations provided best limits of detection. Also, the sensitivity is superior when the DNA sequence selected as target is a multicopy gene (Tosar et al., 2010).

It is important to highlight that the results obtained with synthetic sequences, usually used for preliminary optimization of the genosensor, and DNA from real samples, are often very different (Tosar et al., 2010). Several authors (Bettazzi et al., 2008; Del Giallo et al., 2005; Martin-Fernandez et al., 2014) have studied this difference in sensor performances and they have concluded that sometimes it is required to redesign the target-complementary probes in order to obtain adequate analytical characteristics.

However, depending on the intended application of the sensor, analytical requirements can vary, e.g. the need of obtaining very low detection limits. For instance, allergens require labeling regardless its quantity and severe reactions can be induced by very low amounts, so ultrasensitive methods are needed for allergen detection. On the contrary, in GMO analysis, sensitivity is not an issue according to the detectability required by legislation and that obtained with some electrochemical approaches. However, real quantitation aimed at evaluating compliance with legislation requires the implementation of two measurements in parallel, the taxon-specific and event-specific targets, which is rarely addressed. First trials recently have shown that this goal is achievable and deserves further development to bring genosensors and genoassays to the marketplace.

Among the three topics revised in this work, allergen detection may be the most challenging task for DNA-methods, due to the fact that they are based on its indirect detection. DNA quantity is difficult to correlate with protein content in

a given sample. This, together with the high sensitivity required to detect the lowest possible quantity of the allergenic species, makes it a defiant work. Additionally, the absence of reference materials hampers the development of quantitative methods to assess some allergens, e.g. gluten. The development of these materials would undoubtedly assist on the validation of genosensors towards this aim, raising their potential for their application in real samples.

On the other hand, genosensor applicability is often conditioned by extensive pre-treatment steps. Ideally, the genosensing technology should provide simple, direct and quick analysis of untreated samples. However, given that DNA is within cells, its concentration is usually very low in food matrices, and there are many interfering agents that prevent the direct application of genosensors to this type of samples, extraction, amplification and sometimes purification are required prior to final detection. An example regarding the low quantity of DNA in food samples is given by one of the reviewed genosensors, in which a peanut allergen-related sequence was detected with a linear response in the range of 10^{-17} to 10^{-15} M with synthetic oligonucleotides; after extracting genomic DNA from a commercial peanut sample and performing PCR amplification, a content of 10^{-11} M of the peanut sequence was quantified (Sun et al., 2015b). Considering that PCR amplifies billions of copies of DNA in a sample, the initial quantity of DNA was extremely low to be detected without PCR, especially in the case of allergens as it was previously mentioned, in which case the sensitivity needs to be higher.

From this point of view, more merit should be given to those DNA sensors capable of analyzing complex food samples, using as few sample pretreatment steps as possible. Overviewing the works reported so far, there are significant differences in relation to the complexity of analyzed samples. Some devices required post-PCR purification of amplicons, increasing the time and cost required to perform the method. Thus, great efforts should be attained in this regard, since most electrochemical sensors described to this point require, at a minimum, a pretreatment step consisting in the extraction of DNA and subsequent amplification (PCR or LAMP). Few of the reviewed works detected DNA from real food matrices without prior amplification (Liao et al., 2013; Mascini et al., 2005). In Mascini's work, the fact that the target was part of a multicopy gene makes it highly available for analysis without the need of amplification. This is why the pretreatment only was assisted by digestion with restriction enzymes, in order to cut the DNA fragment prior to the biosensing protocol. These steps, DNA extraction, amplification and/or cleavage, are time-consuming and require expensive reagents, but today are unavoidable. In the case of Liao et al., high amounts of raw material were required to detect threshold levels of GMOs. Only a few electrochemical sensors applied to other types of targets, such as clinical markers and pathogenic targets, have been able to analyze DNA samples without prior processing (Tosar et al., 2010).

However, current research aimed at finding ultrasensitive detection methods with minimized nonspecific electrochemical signals, will make it possible to provide quick and simple analysis, avoiding sample pretreatment steps in the near future.

Food safety is a greatly debated topic nowadays. We have evaluated how this has been reflected in the growing number of publications regarding novel technologies for food assessment. Genosensors can fulfill desirable characteristics for food testing, such as low cost, miniaturized and portable equipment, execution simplicity and robust results, and that is why many devices have been reported for this aim. Yet, there is still much work to do for routine application, because many drawbacks have not been overcome, especially when practical applicability is intended. However, the high sensitivity and versatility of the reviewed papers demonstrate the remarkable potential of this promising technology.

ACKNOWLEDGMENTS

B.M.F. and C.L.M.P. thank to Universidad Complutense de Madrid for the pre-doctoral grants. This work was supported by the BSCH-UCM program for “Consolidation of Research Groups” (Group 950247), Ministerio de Educación y Ciencia (Spain) Project CTQ2012-31157, and the European Regional Development Fund.

ABBREVIATIONS

ALP: Alkaline Phosphatase

Biot: Biotin

bp: Base pair

Bt: *Bacillus thuringiensis* gene

CA: Chronoamperometry

DPV: Differential Pulse Voltammetry

EIS: Electrochemical Impedance Spectroscopy

ELISA: Enzyme Linked Immunosorbent Assay

EU: European Union

FALCPA: Food Allergen Labeling and Consumer Protection Act

FITC: Fluorescein Isothiocyanate

GCE: Glassy carbon electrodes

GM: Genetically Modified

GMO: Genetically Modified Organism

HRP: Horseradish Peroxidase

LAMP: Loop-mediated isothermal amplification

LOD: Limit of Detection

LSV: Linear Sweep Voltammetry

MCH: Mercaptohexanol

MWCNT: Multiwall Carbon Nanotubes

NOS: nopaline synthase (GMO terminator sequence)

PAT: Phosphinothricin Acetyltransferase

PCR: Polymerase Chain Reaction
PEP: Phosphoenolpyruvate carboxylase promoter
SAMs: Self-Assembled Monolayers
SPEAu: Screen-printed gold electrode
SPEC: Screen-printed carbon electrode
Strp: Streptavidin
TMB: Tetramethylbenzidine
US: United States

REFERENCES

Ahmed, M. U., Hasan, Q., Mosharraf Hossain, M., Saito, M., and Tamiya, E. (2010). Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. *Food Control*. **21**: 599-605.

Akkerdaas, J. H., Schocker, F., Vieths, S., Versteeg, S., Zuidmeer, L., Hefle, S. L., Aalberse, R. C., Richter, K., Ferreira, F., and van Ree, R. (2006). Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library. *Mol. Nutr. Food Res.* **50**: 18-23.

Alonso-Lomillo, M., Dominguez-Renedo, O., and Arcos-Martinez, M. (2010). Screen-printed biosensors in microbiology; a review. *Talanta*. **82**: 1629-1636.

Alves, R. C., Barroso, M. F., González-García, M. B., Oliveira, M. B. P. P., and Delerue-Matos, M. C. (2015). New Trends in Food Allergens Detection: Towards Biosensing Strategies. *Crit. Rev. Food Sci. Nutr.* <http://dx.doi.org/10.1080/10408398.2013.831026>

Amaya-González, S., de-los-Santos-Álvarez, N., Miranda-Ordieres, A. J., and Lobo-Castañón, M. J. (2013). Aptamer-Based Analysis: A Promising Alternative for Food Safety Control. *Sensors*. **13**: 16292-16311.

Arora, P., Sindhu, A., Dilbaghi, N., and Chaudhury, A. (2011). Biosensors as innovative tools for the detection of food borne pathogens. *Biosens. Bioelectron.* **28**: 1-12.

Arugula, M. A., Zhang, Y., and Simonian, A. L. (2014). Biosensors as 21st century technology for detecting genetically modified organisms in food and feed. *Anal. Chem.* **86**: 119-129.

Asensio Gil, L. (2007). PCR-based methods for fish and fishery products authentication. *Trends Food. Sci. Technol.* **18**: 558-566.

Barroso, M. F., Freitas, M., Oliveira, M. B. P. P., de-los-Santos-Álvarez, N., Lobo-Castañón, M. J., and Delerue-Matos, C. (2015). 3D-nanostructured Au

electrodes for the event-specific detection of MON810 transgenic maize. *Talanta*. **134**: 158-164.

Berti, F., Laschi, S., Palchetti, I., Rossier, J. S., Reymond, F., Mascini, M., and Marrazza, G. (2009). Microfluidic-based electrochemical genosensor coupled to magnetic beads for hybridization detection. *Talanta*. **77**: 971-978.

Bettazzi, F., Lucarelli, F., Palchetti, I., Berti, F., Marrazza, G., and Mascini, M. (2008). Disposable electrochemical DNA-array for PCR amplified detection of hazelnut allergens in foodstuffs. *Anal. Chim. Acta*. **614**: 93-102.

Bock, S. A., Muñoz-Furlong, A., and Sampson, H. A. (2001). Fatalities due to anaphylactic reactions to foods. *J. Allergy Clin. Immunol. Pract.* **107**: 191-193.

Bonanni, A., Esplandiu, M., and Del Valle, M. (2009). Impedimetric genosensors employing COOH-modified carbon nanotube screen-printed electrodes. *Biosens. Bioelectron.* **24**: 2885-2891.

Branum, A. M., and Lukacs, S. L. (2009). Food Allergy Among Children in the United States. *Pediatr.* **124**: 1549-1555.

Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993). Four recombinant isoforms of Cor a I, the major allergen of hazel pollen, show different IgE-binding properties. *Eur. J. Biochem.* **212**: 355-362.

Briani, C., Samaroo, D., and Alaedini, A. (2008). Celiac disease: From gluten to autoimmunity. *Autoimmun. Rev.* **7**: 644-650.

Carpini, G., Lucarelli, F., Marrazza, G., and Mascini, M. (2004). Oligonucleotide-modified screen-printed gold electrodes for enzyme-amplified sensing of nucleic acids. *Biosens. Bioelectron.* **20**: 167-175.

Catassi, C., Fabiani, E., Iacono, G., D'Agate, C., Francavilla, R., Biagi, F., Volta, U., Accomando, S., Picarelli, A., De Vitis, I., Pianelli, G., Gesuita, R., Carle, F., Mandolesi, A., Bearzi, I., and Fasano, A. (2007). A prospective, double-blind, placebo-controlled trial to establish a safe gluten threshold for patients with celiac disease. *Am. J. Clin. Nutr.* **85**: 160-166.

Chaumpluk, P., Chikae, M., Takamura, Y., and Tamiya, E. (2006). Novel electrochemical identification and semi quantification of bovine constituents in feedstuffs. *Sci. Tech. Adv. Mater.* **7**: 263-269.

CODEX-STAN118 (1979). Amended in 1983 and 2008 for foods for special dietary use for persons intolerant to gluten. FAO/WHO Standards.

Available at: <http://www.codexalimentarius.org/standards/list-of-standards/> (accessed Dezember, 1).

Costa, J., Mafra, I., Kuchta, T., and Oliveira, M. (2012). Single-Tube Nested Real-Time PCR as a New Highly Sensitive Approach to Trace Hazelnut. *J. Agric. Food Chem.* **60**: 8103-8110.

Crevel, R. W. R., Ballmer-Weber, B. K., Holzhauser, T., Hourihane, J. O. B., Knulst, A. C., Mackie, A. R., Timmermans, F., and Taylor, S. L. (2008). Thresholds for food allergens and their value to different stakeholders. *Allergy.* **63**: 597-609.

Del Giallo, M., Lucarelli, F., Cosulich, E., Pistarino, E., Santamaria, B., Marrazza, G., and Mascini, M. (2005). Steric factors controlling the surface hybridization of PCR amplified sequences. *Anal. Chem.* **77**: 6324-6330.

Dennis, M. J. (1998). Recent developments in food authentication. *Analyst.* **123**: 151R-156R.

Diaz-Amigo, C., and Popping, B. (2012). Labeling Regulations, Detection Methods, and Assay Validation. *J. AOAC Int.* **95**: 337-348.

Diaz-Amigo, C., and Popping, B. (2013). Accuracy of ELISA Detection Methods for Gluten and Reference Materials: A Realistic Assessment. *J. Agric. Food Chem.* **61**: 5681-5688.

Duwensee, H., Mix, M., Broer, I., and Flechsig, G. (2009). Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine. *Electrochem. Commun.* **11**: 1487-1491.

European Recommendation 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) 1831/2003. *In*: Off. J. Eur. Union, pp. 18-26.

FALCPA (2004). Food Allergen Labeling and Consumer Protection Act. pp. 108-282.

Fasano, A., and Catassi, C. (2012). Celiac Disease. *N. Engl. J. Med.* **367**: 2419-2426.

FDA (2003). Administration Qualified Health Claims, letter of enforcement discretion-nuts and coronary heart disease (Docket No 02P-0505), Ingredients, Packaging & Labeling. Food and Drug Administration, . Available at: <http://www.fda.gov>, Washington DC.

Feng, Y., Yang, T., Zhang, W., Jiang, C., and Jiao, K. (2008). Enhanced sensitivity for deoxyribonucleic acid electrochemical impedance sensor: gold nanoparticle/polyaniline nanotube membranes. *Anal. Chim. Acta.* **616**: 144-151.

Flinterman, A. E., Van Hoffen, E., Den Hartog Jager, C. F., Koppelman, S., Pasmans, S. G., Hoekstra, M. O., Bruijnzeel-Koomen, C. A., Knulst, A. C., and Knol, E. F. (2007). Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin. Exp. Allergy.* **37**: 1221-1228.

Gallardo, J. M., Sotelo, C. G., Pineiro, C., and Perez-Martin, R. I. (1995). Use of Capillary Zone Electrophoresis for Fish Species Identification. Differentiation of Flatfish Species. *J. Agric. Food Chem.* **43**: 1238-1244.

Garino, C., Zuidmeer, L., Marsh, J., Lovegrove, A., Morati, M., Versteeg, S., Schilte, P., Shewry, P., Arlorio, M., and van Ree, R. (2010). Isolation, cloning, and characterization of the 2S albumin: A new allergen from hazelnut. *Mol. Nutr. Food Res.* **54**: 1257-1265.

Gendel, S. M. (2012). Comparison of international food allergen labeling regulations. *Regul. Toxicol. Pharmacol.* **63**: 279-285.

Green, P., and Cellier, C. (2007). Medical progress: Celiac disease. *N. Engl. J. Med.* **357**: 1731-1743.

Gupta, R. S., Springston, E. E., Warriar, M. R., Smith, B., Kumar, R., Pongratic, J., and Holl, J. L. (2011). The Prevalence, Severity, and Distribution of Childhood Food Allergy in the United States. *Pediatr.* **128**: e9-e17.

Haraszi, R., Chassaing, H., Maquet, A., and Ulberth, F. (2011). Analytical methods for detection of gluten in food--method developments in support of food labeling legislation. *J. AOAC Int.* **94**: 1006-1025.

Holzhauser, T., Wangorsch, A., and Vieths, S. (2000). Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrixes. *Eur. Food Res. Technol.* **211**: 360-365.

Iniesto, E., Jiménez, A., Prieto, N., Cabanillas, B., Burbano, C., Pedrosa, M. M., Rodríguez, J., Muzquiz, M., Crespo, J. F., Cuadrado, C., and Linacero, R. (2013). Real Time PCR to detect hazelnut allergen coding sequences in processed foods. *Food Chem.* **138**: 1976-1981.

Janssen, F. (2006). Detecting wheat gluten in food. In: *Detecting Allergens in Food*, pp. 244-272. Koppelman, S. J., and Hefle, S. L. (Eds.), Woodhead Publishing, Boca Raton.

Jiang, C., Yang, T., Jiao, K., and Gao, H. (2008). A DNA electrochemical sensor with poly-L-lysine/single-walled carbon nanotubes films and its application for the highly sensitive EIS detection of PAT gene fragment and PCR amplification of NOS gene. *Electrochim. Acta.* **53**: 2917-2924.

Jiang, X., Chen, K., and Han, H. (2011). Ultrasensitive electrochemical detection of *Bacillus thuringiensis* transgenic sequence based on in situ Ag nanoparticles aggregates induced by biotin-streptavidin system. *Biosens. Bioelectron.* **28**: 464-468.

Kagnoff, M. (2007). Celiac disease: pathogenesis of a model immunogenetic disease. *J. Clin. Invest.* **117**: 41-49.

Kerman, K., Vestergaard, M., Nagatani, N., Takamura, Y., and Tamiya, E. (2006). Electrochemical genosensor based on peptide nucleic acid-mediated PCR and asymmetric PCR techniques: Electrostatic interactions with a metal cation. *Anal. Chem.* **78**: 2182-2189.

Koppelman, S. J., and Hefle, S. L. (2006). Detecting allergens in food. Woodhead Publishing: Cambridge.

Lau, F. L., Wong, C. K., and Yip, S. H. (1995). Puffer fish poisoning. *J. Accid. Emerg. Med.* **12**: 214-215.

Liao, W., Chuang, M., and Ho, J. (2013). Electrochemical sensor for multiplex screening of genetically modified DNA: Identification of biotech crops by logic-based biomolecular analysis. *Biosens. Bioelectron.* **50**: 414-420.

Lien, T., Lam, T., An, V., Hoang, T., Quang, D., Khieu, D., Tsukahara, T., Lee, Y., and Kim, J. (2010). Multi-wall carbon nanotubes (MWCNTs)-doped polypyrrole DNA biosensor for label-free detection of genetically modified organisms by QCM and EIS. *Talanta.* **80**: 1164-1169.

Ligaj, M., Jasnowska, J., Musiał, W. G., and Filipiak, M. (2006). Covalent attachment of single-stranded DNA to carbon paste electrode modified by activated carboxyl groups. *Electrochim. Acta.* **51**: 5193-5198.

Ligaj, M., Oczkowski, T., Jasnowska, J., Musiał, W., and Filipiak, M. (2003). Electrochemical genosensors for detection of *L. monocytogenes* and genetically-modified components in food and genetically-modified components in food. *Polish Journal of Food and Nutrition Sciences.* **12**: 61-63.

Lockley, A. K., and Bardsley, R. G. (2000). DNA-based methods for food authentication. *Trends Food. Sci. Technol.* **11**: 67-77.

Lucarelli, F., Marrazza, G., and Mascini, M. (2005). Enzyme-based impedimetric detection of PCR products using oligonucleotide-modified screen-printed gold electrodes. *Biosens. Bioelectron.* **20**: 2001-2009.

Lüttkopf, D., Müller, U., Skov, P. S., Ballmer-Weber, B. K., Wüthrich, B., Skamstrup Hansen, K., Poulsen, L. K., Kästner, M., Haustein, D., and Vieths, S. (2002). Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol. Immunol.* **38**: 515-525.

Ma, Y., Jiao, K., Yang, T., and Sun, D. (2008). Sensitive PAT gene sequence detection by nano-SiO₂/p-aminothiophenol self-assembled films DNA electrochemical biosensor based on impedance measurement. *Sens. Actuators, B.* **131**: 565-571.

Mafra, I., Ferreira, I. M. P. L. V. O., and Oliveira, M. B. P. P. (2008a). Food authentication by PCR-based methods. *Eur. Food Res. Technol.* **227**: 649-665.

Mafra, I., Silva, S., Moreira, E., da Silva, C., Beatriz, M., and Oliveira, P. (2008b). Comparative study of DNA extraction methods for soybean derived food products. *Food Control.* **19**: 1183-1190.

Manzanares-Palenzuela, C. L., de-los-Santos-Álvarez, N., Lobo-Castañón, M. J., and López-Ruiz, B. (2015a). Multiplex electrochemical DNA platform for femtomolar-level quantification of genetically modified soybean. *Biosens. Bioelectron.* **68**: 259-265.

Manzanares-Palenzuela, C. L., Martín-Fernández, B., Sánchez-Paniagua López, M., and López-Ruiz, B. (2015b). Electrochemical genosensors as innovative tools for detection of genetically modified organisms. *Trends Analyt. Chem.* **66**: 19-31.

Mari, A., Scala, E., Palazzo, P., Ridolfi, S., Zennaro, D., and Carabella, G. (2006). Bioinformatics applied to allergy: Allergen databases, from collecting sequence information to data integration. The Allergome platform as a model. *Cell. Immunol.* **244**: 97-100.

Martín-Fernández, B., de-los-Santos-Álvarez, N., Lobo-Castañón, M., and López-Ruiz, B. (2015). Hairpin-based DNA electrochemical sensor for selective detection of a repetitive and structured target codifying a gliadin fragment. *Anal. Bioanal. Chem.* **407**: 3481-3488.

Martin-Fernandez, B., Miranda-Ordieres, A. J., Lobo-Castanon, M. J., Frutos-Cabanillas, G., de-los-Santos-Alvarez, N., and Lopez-Ruiz, B. (2014). Strongly structured DNA sequences as targets for genosensing: Sensing phase

design and coupling to PCR amplification for a highly specific 33-mer gliadin DNA fragment. *Biosens. Bioelectron.* **60**: 244-251.

Mascini, M., Del Carlo, M., Minunni, M., Chen, B., and Compagnone, D. (2005). Identification of mammalian species using genosensors. *Bioelectrochemistry.* **67**: 163-169.

Meric, B., Kerman, K., Marrazza, G., Palchetti, I., Mascini, M., and Ozsoz, M. (2004). Disposable genosensor, a new tool for the detection of NOS-terminator, a genetic element present in GMOs. *Food Control.* **15**: 621-626.

Mix, M., Rüger, J., Krüger, S., Broer, I., and Flechsig, G.-U. (2012). Electrochemical detection of 0.6% genetically modified maize MON810 in real flour samples. *Electrochem. commun.* **22**: 137-140.

Monaci, L., and Visconti, A. (2010). Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends Food. Sci. Technol.* **21**: 272-283.

Moore, J. C., Spink, J., and Lipp, M. (2012). Development and Application of a Database of Food Ingredient Fraud and Economically Motivated Adulteration from 1980 to 2010. *J. Food Sci.* **77**: R118-R126.

Mujico, J., Lombardia, M., Mena, M., Mendez, E., and Albar, J. (2011). A highly sensitive real-time PCR system for quantification of wheat contamination in gluten-free food for celiac patients. *Food Chem.* **128**: 795-801.

Nicolaou, N., Poorafshar, M., Murray, C., Simpson, A., Winell, H., Kerry, G., Härlin, A., Woodcock, A., Ahlstedt, S., and Custovic, A. (2010). Allergy or tolerance in children sensitized to peanut: Prevalence and differentiation using component-resolved diagnostics. *J. Allergy Clin. Immunol. Pract.* **125**: 191-197.e113.

Pastorello, E. A., Vieths, S., Pravettoni, V., Farioli, L., Trambaioli, C., Fortunato, D., Lüttkopf, D., Calamari, M., Ansaloni, R., Scibilia, J., Ballmer-Weber, B. K., Poulsen, L. K., Wütrich, B., Hansen, K. S., Robino, A. M., Ortolani, C., and Conti, A. (2002). Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J. Allergy Clin. Immunol. Pract.* **109**: 563-570.

Piñeiro, C., Barros-Velázquez, J., Pérez-Martín, R. I., Martínez, I., Jacobsen, T., Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie, I. M., and Jessen, F. (1999). Development of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis reference method for the analysis and identification of fish species in raw and heat-processed samples: A collaborative study. *Electrophoresis.* **20**: 1425-1432.

Pividori, M. I., and Alegret, S. (2010). Electrochemical biosensors for food safety. pp. 173-191. *Contributions to Science*.

Poltronieri, P., Mezzolla, V., Primiceri, E., and Maruccio, G. (2014). Biosensors for the Detection of Food Pathogens. *Foods*. **3**: 511-526.

Premanandh, J. (2011). Global consensus–Need of the hour for genetically modified organisms (GMO) labeling. *J. Commer. Biotechnol.* **17**: 37-44.

Qiao, S.-W., Bergseng, E., Molberg, Ø., Xia, J., Fleckenstein, B., Khosla, C., and Sollid, L. M. (2004). Antigen Presentation to Celiac Lesion-Derived T Cells of a 33-Mer Gliadin Peptide Naturally Formed by Gastrointestinal Digestion. *J. Immunol.* **173**: 1757-1762.

Regulation (EC) 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. **In:** *Off. J. Eur. Union*, pp. 3-5. L16.

Regulation (EC) 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. **In:** *Off. J. Eur. Union*, pp. 1-24.

Regulation (EC) 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) 1924/2006 and (EC) 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) 608/2004. **In:** *Off. J. Eur. Union*, pp. 18-63.

Regulation (EC) 1829/2003 of 22 September 2003 on genetically modified food and feed. **In:** *Off. J. Eur. Union*, pp. 1-23.

Regulation (EU) 1169/2011 of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) 1924/2006 and (EC) 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) 608/2004. **In:** *Off. J. Eur. Union*, pp. 18-63.

Ren, Y., Jiao, K., Xu, G., Sun, W., and Gao, H. (2005). An electrochemical DNA sensor based on electrodepositing aluminum ion films on stearic acid-modified carbon paste electrode and its application for the detection

of specific sequences related to bar gene and CP4 Epsps gene. *Electroanalysis*. **17**: 2182-2189.

Röhr, A., Lüddecke, K., Drusch, S., Müller, M. J., and Alvensleben, R. v. (2005). Food quality and safety—consumer perception and public health concern. *Food Control*. **16**: 649-655.

Sánchez-Paniagua López, M., Frutos Cabanillas, G., Lobo Castañón, M. J., and López-Ruiz, B. (2014). Development of a genosensor for peanut allergen ARA h 2 detection and its optimization by surface response methodology. *Biosens. Bioelectron*. **62**: 350-356.

Sathe, S. K., Teuber, S. S., and Roux, K. H. (2005). Effects of food processing on the stability of food allergens. *Biotechnol. Adv.* **23**: 423-429.

Sforza, S., Corradini, R., Tedeschi, T., and Marchelli, R. (2011). Food analysis and food authentication by peptide nucleic acid (PNA)-based technologies. *Chem. Soc. Rev.* **40**: 221-232.

Shan, L., Molberg, O., Parrot, I., Hausch, F., Filiz, F., Gray, G., Sollid, L., and Khosla, C. (2002). Structural basis for gluten intolerance in Celiac sprue. *Science*. **297**: 2275-2279.

Sicherer, S. H., and Sampson, H. A. (2006). 9. Food allergy. *J. Allergy Clin. Immunol. Pract.* **117**: S470-S475.

Soares, S., Amaral, J. S., Mafra, I., and Oliveira, M. B. P. P. (2010). Quantitative detection of poultry meat adulteration with pork by a duplex PCR assay. *Meat Science*. **85**: 531-536.

Sun, W., Lu, Y., Wu, Y., Zhang, Y., Wang, P., Chen, Y., and Li, G. (2014). Electrochemical sensor for transgenic maize MON810 sequence with electrostatic adsorption DNA on electrochemical reduced graphene modified electrode. *Sens. Actuators, B*. **202**: 160-166.

Sun, W., Zhang, Y., Hu, A., Lu, Y., Shi, F., Lei, B., and Sun, Z. (2013). Electrochemical DNA Biosensor Based on Partially Reduced Graphene Oxide Modified Carbon Ionic Liquid Electrode for the Detection of Transgenic Soybean A2704-12 Gene Sequence. *Electroanalysis*. **25**: 1417-1424.

Sun, W., Zhong, J., Qin, P., and Jiao, K. (2008). Electrochemical biosensor for the detection of cauliflower mosaic virus 35 S gene sequences using lead sulfide nanoparticles as oligonucleotide labels. *Anal. Biochem.* **377**: 115-119.

Sun, W., Zhong, J., Zhang, B., and Jiao, K. (2007). Application of cadmium sulfide nanoparticles as oligonucleotide labels for the electrochemical detection of NOS terminator gene sequences. *Anal. Bioanal. Chem.* **389**: 2179-2184.

Sun, X., Guan, L., Shan, X., Zhang, Y., and Li, Z. (2012). Electrochemical Detection of Peanut Allergen Ara h 1 Using a Sensitive DNA Biosensor Based on Stem-Loop Probe. *J. Agric. Food Chem.* **60**: 10979-10984.

Sun, X., Jia, M., Guan, L., Ji, J., Zhang, Y., Tang, L., and Li, Z. (2015a). Multilayer graphene-gold nanocomposite modified stem-loop DNA biosensor for peanut allergen-Ara h1 detection. *Food Chem.* **172**: 335-342.

Sun, X., Jia, M., Ji, J., Guan, L., Zhang, Y., Tang, L., and Li, Z. (2015b). Enzymatic amplification detection of peanut allergen Ara h1 using a stem-loop DNA biosensor modified with a chitosan-mutiwalled carbon nanotube nanocomposite and spongy gold film. *Talanta.* **131**: 521-527.

Taylor, S., Hefle, S., Bindslev-Jensen, C., Bock, S., Burks, A., Christie, L., Hill, D., Host, A., Hourihane, J., Lack, G., Metcalfe, D., Moneret-Vautrin, D., Vadas, P., Rance, F., Skrypec, D., Trautman, T., Yman, I., and Zeiger, R. (2002). Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *J. Allergy Clin. Immunol. Pract.* **109**: 24-30.

Tichoniuk, M., Ligaj, M., and Filipiak, M. (2008). Application of DNA hybridization biosensor as a screening method for the detection of genetically modified food components. *Sensors.* **8**: 2118-2135.

Tosar, J., Branas, G., and Laiz, J. (2010). Electrochemical DNA hybridization sensors applied to real and complex biological samples. *Biosens. Bioelectron.* **26**: 1205-1217.

Ulianas, A., Heng, L., Ahmad, M., Lau, H., Ishak, Z., and Ling, T. (2014). A regenerable screen-printed DNA biosensor based on acrylic microsphere-gold nanoparticle composite for genetically modified soybean determination. *Sens. Actuators, B.* **190**: 694-701.

van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Jasti, P. R., and Eisenbrand, G. (2010). A review on the beneficial aspects of food processing. *Mol. Nutr. Food Res.* **54**: 1215-1247.

van den Eede, G., Aarts, H., Buhk, H., Corthier, G., Flint, H., Hammes, W., Jacobsen, B., Midtvedt, T., van der Vossen, J., von Wright, A., Wackernagel, W., and Wilcks, A. (2004). The relevance of gene transfer to the

safety of food and feed derived from genetically modified (GM) plants. *Food Chem. Toxicol.* **42**: 1127-1156.

Van Dorst, B., Mehta, J., Bekaert, K., Rouah-Martin, E., De Coen, W., Dubruel, P., Blust, R., and Robbens, J. (2010). Recent advances in recognition elements of food and environmental biosensors: A review. *Biosens. Bioelectron.* **26**: 1178-1194.

van Hengel, A. (2007). Food allergen detection methods and the challenge to protect food-allergic consumers. *Anal. Bioanal. Chem.* **389**: 111-118.

Ventura, A., Magazzù, G., and Greco, L. (1999). Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. *Gastroenterology.* **117**: 297-303.

Wang, J., Sheng, Q., Tian, N., Chen, L., Xu, Z., and Zheng, J. (2009). Electrochemical detection of the neomycin phosphotransferase gene (NPT-II) in transgenic plants with a novel DNA biosensor. *J. Appl. Electrochem.* **39**: 935-945.

Wang, M.-Q., Du, X.-Y., Sun, Q., and Jiang, X.-C. (2008). DNA biosensor prepared by electrodeposited Pt-nanoparticles for the detection of specific deoxyribonucleic acid sequence in genetically modified soybean. *Chinese J. Anal. Chem.* **36**: 890-894.

Ward, R. D., Hanner, R., and Hebert, P. D. N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *J. Fish Biol.* **74**: 329-356.

Xie, J., Jiao, K., Liu, H., Wang, Q., Liu, S., and Fu, X. (2008). DNA electrochemical sensor based on PbSe nanoparticle for the sensitive detection of CaMV35S transgene gene sequence. *Chinese J. Anal. Chem.* **36**: 874-878.

Xu, G., Jiao, K., Fan, J., and Sun, W. (2006). Electrochemical detection of specific gene related to CaMV35S using methylene blue and ethylenediamine-modified glassy carbon electrode. *Acta Chim. Slov.* **53**: 486-491.

Yang, J., Jiao, K., and Yang, T. (2007a). A DNA electrochemical sensor prepared by electrodepositing zirconia on composite films of single-walled carbon nanotubes and poly(2,6-pyridinedicarboxylic acid), and its application to detection of the PAT gene fragment. *Anal. Bioanal. Chem.* **389**: 913-921.

Yang, J., Wang, X., and Shi, H. (2012a). An electrochemical DNA biosensor for highly sensitive detection of phosphinothricin acetyltransferase gene sequence based on polyaniline-(mesoporous nanozirconia)/poly-tyrosine film. *Sens. Actuators, B.* **162**: 178-183.

Yang, J., Yang, T., Feng, Y., and Jiao, K. (2007b). A DNA electrochemical sensor based on nanogold-modified poly-2,6-pyridinedicarboxylic acid film and detection of PAT gene fragment. *Anal. Biochem.* **365**: 24-30.

Yang, T., Zhang, W., Du, M., and Jiao, K. (2008). A PDDA/poly (2, 6-pyridinedicarboxylic acid)-CNTs composite film DNA electrochemical sensor and its application for the detection of specific sequences related to PAT gene and NOS gene. *Talanta.* **75**: 987-994.

Yang, T., Zhou, N., Li, Q., Guan, Q., Zhang, W., and Jiao, K. (2012b). Highly sensitive electrochemical impedance sensing of PEP gene based on integrated Au–Pt alloy nanoparticles and polytyramine. *Colloids Surf., B.* **97**: 150-154.

Yang, T., Zhou, N., Zhang, Y., Zhang, W., Jiao, K., and Li, G. (2009). Synergistically improved sensitivity for the detection of specific DNA sequences using polyaniline nanofibers and multi-walled carbon nanotubes composites. *Biosens. Bioelectron.* **24**: 2165-2170.

Zhang, W., Yang, T., Jiang, C., and Jiao, K. (2008). DNA hybridization and phosphinothricin acetyltransferase gene sequence detection based on zirconia/nanogold film modified electrode. *Appl. Surf. Sci.* **254**: 4750-4756.

Zhou, N., Yang, T., Jiang, C., Du, M., and Jiao, K. (2009). Highly sensitive electrochemical impedance spectroscopic detection of DNA hybridization based on Au-nano-CNT/PAN(nano) films. *Talanta.* **77**: 1021-1026.

Zhu, L., Zhao, R., Wang, K., Xiang, H., Shang, Z., and Sun, W. (2008). Electrochemical behaviors of methylene blue on DNA modified electrode and its application to the detection of PCR product from NOS sequence. *Sensors.* **8**: 5649-5660.

Zuidmeer, L., Goldhahn, K., Rona, R. J., Gislason, D., Madsen, C., Summers, C., Sodergren, E., Dahlstrom, J., Lindner, T., Sigurdardottir, S. T., McBride, D., and Keil, T. (2008). The prevalence of plant food allergies: A systematic review. *J. Allergy Clin. Immunol. Pract.* **121**: 1210-1218.e1214.