DEPARTAMENTO DE QUÍMICA ANALÍTICA

"Profesor Fermín Capitán García"

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DESARROLLO DE METODOLOGÍAS ANALÍTICAS PARA EL CONTROL DE CALIDAD Y SEGURIDAD ALIMENTARIA EN ACEITES, LÁCTEOS Y EXTRACTOS VEGETALES

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por

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MEMORIA presentada para aspirar al Grado de Doctor en Química por la Universidad de Granada

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Antonio Garballo Rubio
Licenciado en Química

A mi padre (que seguro me está viendo), mi madre y mis hermanos…

…a Antonio y Alberto -mis hijos-,…y a ti Chari

A todos vosotros por estar siempre a mi lado

Son muchas y muchos los que a lo largo de estos años me han ayudado a lograr la consecución de este trabajo. He tenido la suerte de encontrarme en este camino con personas enriquecedoras tanto a nivel técnico, como personal, y me siento en la obligación de mencionar a algunas de ellas que por su dedicación e influencia, han contribuido a este logro personal.

Quiero comenzar por agradecer en primer lugar a Monsalud del Olmo Iruela y a José Luís Vílchez Quero por guiarme en los primeros pasos. Si no se comienza recto, el camino seguramente se tuerce al final.

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A veces puedes tener la suerte de encontrarte con personas que aunque no eliges, resultan ser maravillosas, como es el caso de la familia; y a veces conoces a otras que cuando se cruzan en tu camino, pueden influir en tu vida y hacerla mejor, … como es el caso de Chari, mi esposa. Gracias por tu apoyo incondicional todos estos años.

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Por último, quiero cerrar estas letras agradeciendo de nuevo a mi familia su esfuerzo, su ayuda, su cariño y su alegría, que hacen que cada día tenga la seguridad de que se pueda conseguir cualquier cosa en la vida.

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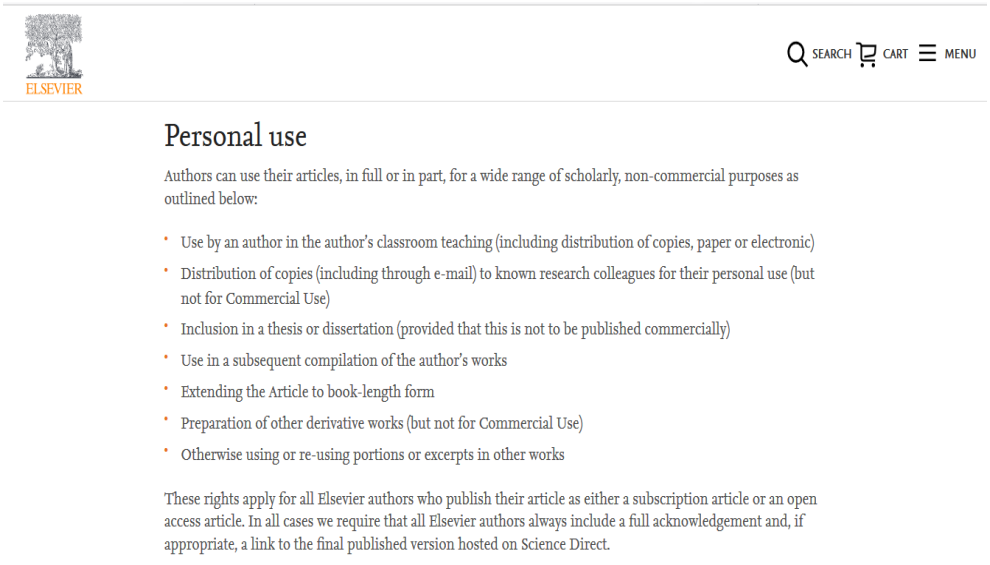

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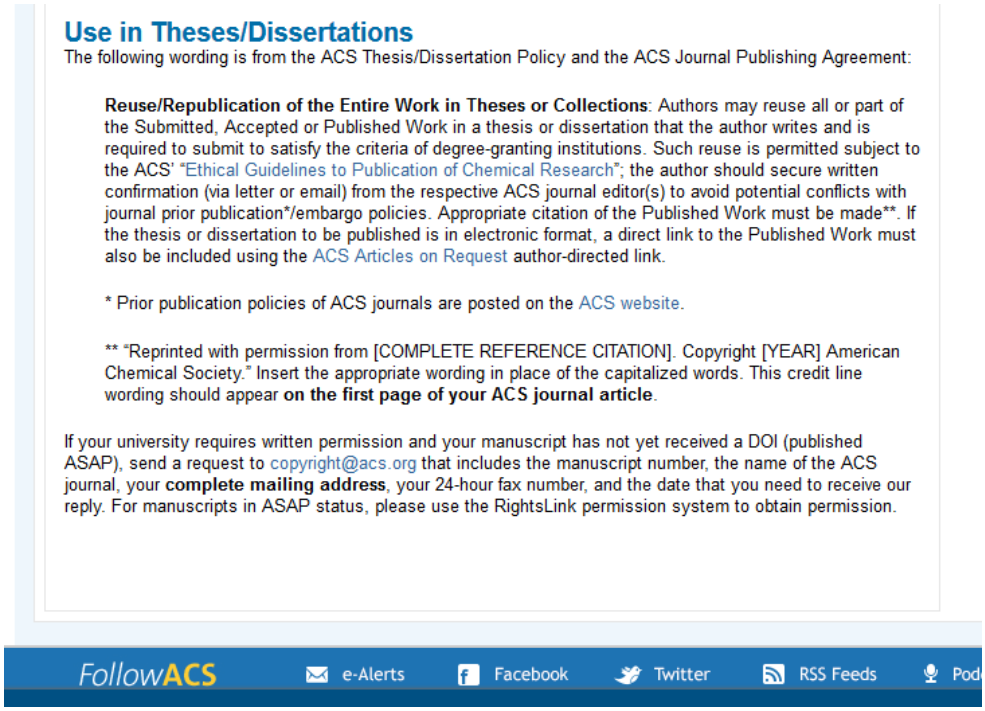

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Resumen de la Tesis

En la presente Tesis se han desarrollado metodologías analíticas de probada competencia para la determinación de diferentes familias de analitos en matrices de carácter alimentario. Estas familias se han organizado de forma que atienden a su carácter beneficioso o perjudicial desde el punto de vista de las salud humana. En este sentido, y atendiendo a sus efectos beneficiosos para la salud humana, se han seleccionado dos grupos de compuestos por su elevado nivel de uso en alimentación a nivel mundial: vitaminas hidrosolubles del grupo B y vitamina C, así como isoflavonas de soja, adicionadas ambas en matrices lácteas. Con la incorporación de este tipo de sustancias se pretende aportar funcionalidad a estos productos lácteos y por consiguiente mejorar sus propiedades nutricionales. Estos compuestos son determinados de manera rutinaria en el laboratorio donde se han puesto a punto las metodologías analíticas desarrolladas en este trabajo de tesis. Una vez desarrolladas y validadas, dichas metodologías, se han podido aplicar de manera satisfactoria en un laboratorio de análisis de rutina.

Las vitaminas son un grupo de compuestos de estructura muy heterogénea pero que tienen en común el hecho de que son fundamentales para un correcto desarrollo de las funciones biológicas, así mismo, intervienen en un elevado número de rutas metabólicas que hacen que una ingesta de las cantidades recomendadas sea muy importante para el adecuado desarrollo y prevención de enfermedades. La determinación analítica de este tipo de compuestos tan heterogéneos en matrices alimentarias presenta una serie de complicaciones derivadas de la presencia de un elevado número de interferentes, así como el diferente comportamiento que presentan cada una de ellas a cambios sutiles de pH en la separación cromatográfica. Estas peculiaridades hacen que su determinación en matrices alimentarias tales como productos lácteos sea un reto analítico.

Para el caso de las isoflavonas de soja, se ha demostrado en los últimos años que poseen una importante actividad estrogénica que beneficia la salud de la mujer desde el punto de vista de riesgo cardiovascular, osteoporosis y cáncer relacionado con problemas hormonales, sobre todo en la etapa de la menopausia.

Para promover el consumo de este tipo de compuestos, principalmente en regiones donde el consumo de soja no está muy extendido, se han desarrollado una serie de bebidas aromatizadas que sean del gusto del consumidor occidental. Así mismo, se han desarrollado bebidas lácteas a las cuales se le ha incorporado extractos de soja con un alto contenido en este tipo de compuestos (genisteina, daidzeina, gliciteina y sus respectivos derivados glicosilados – genistina, daidzina y glicitina respectivamente).

Según el Reglamento 1169/2011 sobre la información alimentaria facilitada al consumidor, se persigue ofrecer un alto nivel de protección de la salud de los consumidores y garantizar el derecho a la información para que los consumidores puedan tomar sus propias decisiones en esta materia con conocimiento de causa.

Desde el punto de vista de la seguridad alimentaria también debe tenerse en cuenta la adición de vitaminas, ya que un consumo excesivo de este tipo de nutrientes también puede tener efectos adversos para la salud humana. En este sentido, en el "*DOCUMENTO DE ORIENTACIÓN PARA LAS AUTORIDADES COMPETENTES EN MATERIA DE CONTROL DEL CUMPLIMIENTO DE LA LEGISLACIÓN DE LA UE SOBRE: Reglamento (UE) nº 1169/2011 del Parlamento Europeo y del Consejo, de 25 de octubre de 2011, sobre la información alimentaria facilitada al consumidor y por el que se modifican los Reglamentos (CE) nº 1924/2006 y (CE) nº 1925/2006 del Parlamento Europeo y del Consejo, y por el que se derogan la Directiva*

87/250/CEE de la Comisión, la Directiva 90/496/CEE del Consejo, la Directiva 1999/10/CE de la Comisión, la Directiva 2000/13/CE del Parlamento Europeo y del Consejo, las Directivas 2002/67/CE y 2008/5/CE de la Comisión, y el Reglamento (CE) nº 608/2004 de la Comisión y Directiva 90/496/CEE del Consejo, de 24 de septiembre de 1990, relativa al etiquetado sobre propiedades nutritivas de los productos Alimenticios y Directiva 2002/46/CE del Parlamento Europeo y del Consejo, de 10 de junio de 2002, relativa a la aproximación de las legislaciones de los Estados miembros en materia de complementos alimenticios por lo que respecta al establecimiento de tolerancias para los valores nutricionales declarados en la etiqueta", se establece que deben existir unos límites dentro de los cuales debe encontrarse el contenido de vitaminas de los alimentos. Este contenido puede variar debido a variaciones en el contenido natural, efectos de almacenamiento o fabricación, etc. De forma que el valor declarado en la etiqueta del producto puede no coincidir con el contenido real de la muestra. Por este motivo, existen y se establecen ciertos márgenes de seguridad dentro de los cuales debe encontrarse el contenido real de vitaminas en el producto final.

Por tanto, para tener una aproximación y control del contenido real de nutrientes en un alimento, deben desarrollarse metodologías analíticas que permitan un control rutinario de muestras y que posean una alta capacidad de procesado al tiempo que ofrezca resultados exactos (veraces y precisos). Se deben establecer una serie de medidas de control capaces de determinar el contenido de este tipo de compuestos de manera que se pueda asegurar que dicho contenido mínimo establecido en el etiquetado del producto, a final de su vida útil, siga cumpliendo con los márgenes legalmente establecidos.

En un segundo bloque de esta investigación, se han desarrollado nuevas metodologías para la adecuada cuantificación de compuestos que producen un efecto adverso para la salud humana y que se pueden encontrar en diferentes matrices alimentarias, ya sea por generación en el proceso industrial de refinado (3-MCPD, 2-MCPD o glicidilésteres derivados en matrices grasas), por una incompleta eliminación en su proceso de fabricación, como es el caso de la presencia de trazas de lactosa en productos libres de este compuesto en matrices lácteas, como consecuencia de la administración fraudulenta de ciertos compuestos (antibióticos) en la explotación animal bobina en donde no son metabolizados y son, a su vez, excretados a través de la leche, siendo susceptibles de poder ser ingeridos por la población humana y pudiendo desencadenar resistencias por parte de los microorganismos a la acción para la que fueron administradas. Así mismo, también se ha desarrollado una metodología analítica para la determinación de un tercer grupo de contaminantes como son los hidrocarburos aromáticos policíclicos (PAHs), cuyo objeto de estudio se ha centrado en su presencia en extractos vegetales, los cuales pueden estar contaminados debido tanto a la contaminación del área de cultivo de la materia prima, como a procesos industriales inadecuados a los que pueden ser sometidos para su obtención. Este grupo de contaminantes son potencialmente cancerígenos y mutagénicos, y por lo tanto, su presencia en alimentos y complementos alimenticios debe ser controlada y estudiada.

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Acrónimos y Abreviaturas

3-MCPD:	3-Monocloropropano-1,2-diol
2-MCPD:	2-Monocloropropano-1,3-diol
ACS:	American Chemical Society
AECOSAN:	Agencia española de consumo, seguridad alimentaria y nutrición
APCI:	Atmospheric pressure chemical ionization
CIP	Ciprofloxacin
CoA:	Coenzima A
DAD:	Diode array detector
DAN	Danofloxacin
DIF	Difloxacin
EFSA:	European Food Safety Authority
ELISA:	Enzyme-linked inmunosorbent assay
EMA:	European Medicines Agency
ENR	Enrofloxacin
ESI:	Electrospray ionization
FAO/WHO:	Food and Agriculture Organization of the United Nations/World Health Organization
FLD:	Fluorescence detector
FLU	Fumequine
GC:	Gas chromatography
GPC:	Gel permeation chromatography
HPLC:	High performance liquid chromatography
IM:	Incertidumbre de medida
IARC:	International Agency for Research on Cancer
LLE	Liquid-Liquid extraction

LOAEL:	Lowest observed adverse effect level
LOD:	Límite de detección
LOQ:	Límite de cuantificación
MAE:	Microwave assisted extraction
MAR	Marbofloxacin
MIP:	Molecularly Imprinted Polymer
MRM:	Multiple reaction monitoring
MS/MS:	Mass spectrometry triple quadrupole
m/z:	Relación masa/carga
NAD:	Nicotinamida adenine dinucleótido
NADH:	Nicotinamida adenina dinucleótido reducido
NADP:	Nicotinamida adenine dinucleótido fosfato
NOR	Norfloxacin
OXO	Oxolinic acid
PLE:	Pressurized liquid extraction
PRAC:	Pharmacovigilance Risk Assessment Committee - Comité Consultivo para la Determinación de Riesgos en Farmacovigilancia
PSA:	Primary Secondary Amine
QuEChERs:	Quick, Easy, Cheap, Effective, Rugged y Safe
SAR:	Sarafloxacin
SPE	Solid Phase Extraction
TOF	Time of Flight
U:	Incertidumbre de medida expandida
USP:	United States Pharmacopeia

Objeto y Justificación de la Tesis

El objetivo de la presente Tesis Doctoral ha sido el desarrollo y validación de diferentes métodos de buenas características analíticas para la determinación de varias familias de compuestos relacionados con los campos de la seguridad alimentaria y el control de calidad de los alimentos.

Los métodos se han dividido en dos grandes grupos atendiendo a su efecto para la salud desde el punto de vista del consumo humano: aquellos con efectos beneficiosos y que aportan funcionalidad al alimento al que se añaden (calidad alimentaria), y aquellos compuestos perjudiciales que se encuentran de forma intrínseca o que son generados en el procesado industrial (seguridad alimentaria).

De este modo se han establecido nuevas metodologías para el control del etiquetado de compuestos beneficiosos a las dosis recomendadas (vitaminas hidrosolubles e isoflavonas) en matrices lácteas, así como metodologías para el control de compuestos perjudiciales para la salud (3-MCPD, lactosa residual, antibióticos y PAHs) en matrices grasas, lácteas y extractos vegetales respectivamente.

En el desarrollo de estos métodos se ha tenido en cuenta la premisa de que deben ser aplicables, de forma rutinaria, en un laboratorio de control, por lo que deben conjugarse un alto grado de veracidad y precisión (exactitud) y al mismo tiempo una alta capacidad de procesamiento de muestras y coste contenido por muestra en el laboratorio. Por este motivo, se ha procurado ajustar las técnicas de preparación de muestra a esta premisa, derivándose preferiblemente hacia aquellas que emplean extracciones líquido-líquido, precipitación o derivatizaciones que ofrezcan una adecuada eliminación de potenciales interferentes al tiempo que se reducen los costes y aumenta la velocidad de procesamiento de muestra.

Como técnicas instrumentales se han empleado principalmente las cromatográficas en sus diferentes modos (de líquidos y de gases) acopladas a detectores de diversa índole (ópticos espectrofotométricos y espectrométrico de masas).

Todos los métodos han sido así mismo validados según las directrices de la norma UNE-EN ISO/IEC 17025:2005 y de la IUPAC para aseverar de este modo su aplicabilidad en las diferentes matrices seleccionadas. Como consecuencia de esta investigación, se han publicado cinco artículos en revistas internacionales de reconocido prestigio y alto impacto, y se ha redactado un sexto artículo que en la actualidad se encuentra enviado y en estado de revisión para su posterior publicación en la revista Journal of Chromatography A.

Capítulo 1. Control de Etiquetado. Marco Legal

La sociedad actual reclama un alto nivel de seguridad y conocimiento en los productos que son consumidos en general. En relación a los alimentos, este conocimiento adquiere una especial transcendencia ya que son artículos de consumo que afectan de manera directa al desarrollo, crecimiento y salud en general de los consumidores.

La opinión pública tiene un alto interés por la relación entre alimentación y salud, y por tanto, por la adecuada elección de una dieta acorde con sus necesidades particulares. En el "***Libro Blanco de la Comisión***"[1] se señala que una de la herramienta para el control de la obesidad y sobrepeso es la información nutricional que se ofrece al consumidor, de forma que éste pueda tener mayor información y capacidad de decisión con conocimiento de causa a la hora de elegir los productos que más se pueden ajustar a su forma de vida. Por tanto, desde las distintas administraciones se debe ofrecer un alto nivel de protección a la población general que demanda tener la adecuada *información* a partir de la cual se tenga la libertad de decisión. Esta libertad de decisión puede tener connotaciones éticas, religiosas, de salud, etc., que hacen que la población tenga el derecho a conocer la composición nutricional de los alimentos que consume.[2]

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1 Comisión de las Comunidades Europeas. Libro blanco - Estrategia Europea sobre problemas de salud relacionados con la alimentación, el sobrepeso y la obesidad Bruselas, 30.5.2007 COM (2007) 279 final.

2 Diario Oficial de la Unión Europea, Reglamento (UE) nº 1169/2011 del Parlamento Europeo y del Consejo de 25 de octubre de 2011 sobre la información alimentaria facilitada al consumidor y por el que se modifican los Reglamentos (CE) nº 1924/2006 y (CE) nº 1925/2006 del Parlamento Europeo y del Consejo, y por el que se derogan la Directiva 87/250/CEE de la Comisión, la Directiva 90/496/CEE del Consejo, la Directiva 1999/10/CE de la Comisión, la Directiva 2000/13/CE del Parlamento Europeo y del Consejo, las Directivas 2002/67/CE, y 2008/5/CE de la Comisión, y el Reglamento (CE) nº 608/2004 de la Comisión, 18-63.

En particular, la legislación europea ha armonizado los diferentes reglamentos y decisiones de la Comisión Europea. En la Directiva 2000/13/CE se establece la legislación de los países miembros en materia de etiquetado nutricional, sin embargo, las disposiciones que se encuentran en dicho documento se remontan al año 1978, con lo que surge la necesidad de que sea revisado y actualizado. Por su lado, la Directiva 90/496/CE relativa al etiquetado sobre propiedades nutritivas de los productos alimenticios en los países miembros, establece normas sobre el contenido y presentación de la información nutricional que debe aparecer en los alimentos envasados. En dicha directiva, se establece que la inclusión de la información nutricional no es obligatoria a menos que se realicen afirmaciones sobre las propiedades nutricionales de un producto. Esta documentación data del año 1990 y por tanto debía ser revisada y actualizada. El Reglamento 1169/2011 del Parlamento Europeo actualiza esta serie de reglamentos de forma que armoniza la información que debe aparecer en el etiquetado nutricional de los alimentos de forma que sea clara, comprensible y legible. Así mismo, moderniza la información que debe ser considerada de forma que se tengan en cuenta los nuevos avances en cuestiones del ámbito de la información alimentaria.

Se entiende como nutriente a la grasa, proteínas, hidratos de carbono, fibra, sodio, vitaminas y minerales que conforman el alimento. Estos componentes deben ser reflejados claramente en el etiquetado nutricional de productos alimenticios, evitándose inducir a error al consumidor final en particular en los siguientes aspectos:

- Naturaleza del alimento, identidad, composición, país de origen o cualidades.

- Se debe evitar atribuir al alimento cualidades que no posee.

De este modo, la información nutricional debe presentarse de una forma clara y fácilmente comprensible por el consumidor final del alimento envasado.

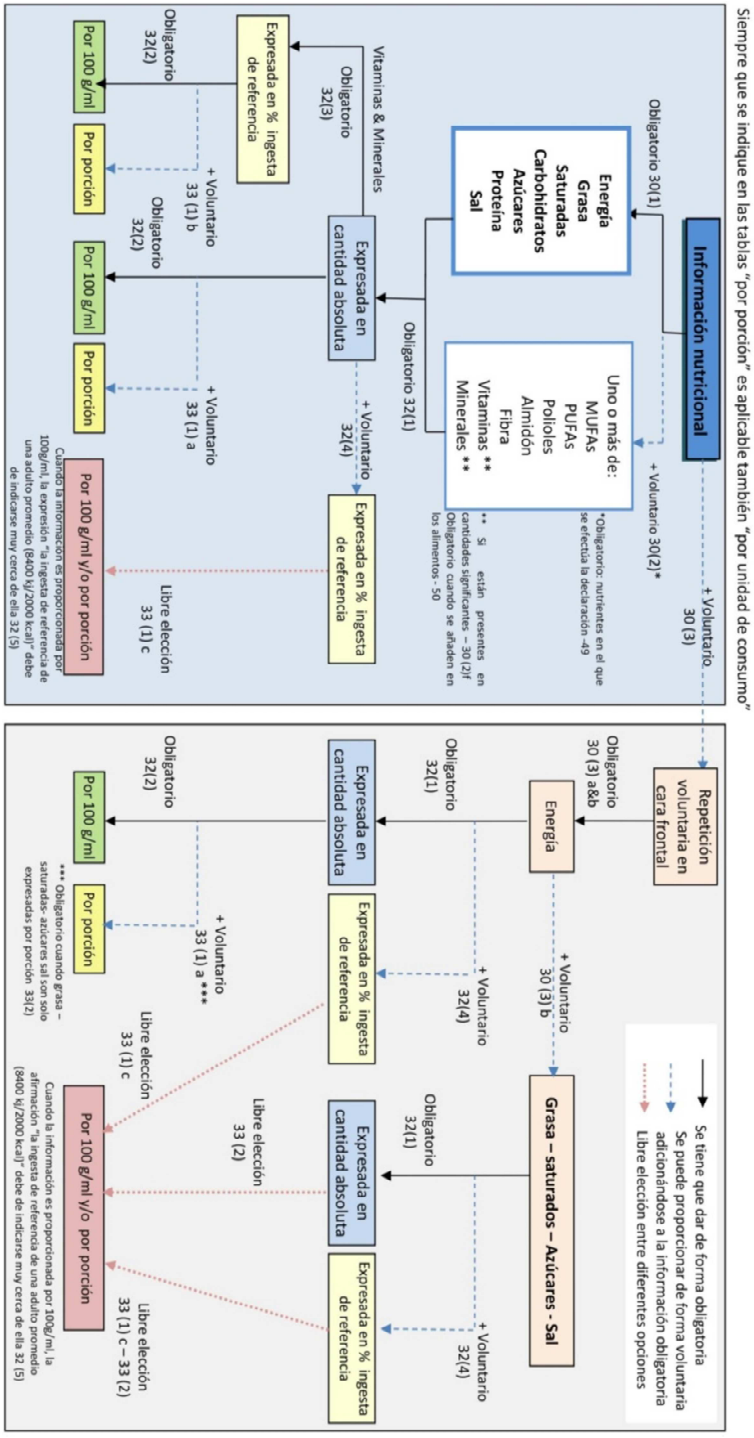
Se define en este reglamento que el contenido de nutrientes que debe estar presente obligatoriamente en el etiquetado son: grasas, ácidos grasos saturados, hidratos de carbono, azúcares, proteínas y sal (se puede indicar adicionalmente si este contenido de sal proviene exclusivamente del contenido natural de sodio que posea el alimento). Esta composición nutricional obligatoria puede ser complementada con diferentes indicaciones de la cantidad de ciertas sustancias entre las que se encuentran minerales, fibra alimentaria, ácidos grasos monoinsaturados y poliinsaturados, polialcoholes, **vitaminas** y almidón.

En la tabla 1 se resume esquemáticamente la obligatoriedad de declarar los diferentes elementos nutricionales que conforman el etiquetado nutricional[3].

En ella se indica junto a cada grupo de nutrientes, el artículo y apartado del Reglamento 1169/2011 en el cual se define la obligatoriedad o no de incluir cada nutriente en el etiquetado de producto final:

[3] Disponible en: http://eletiquetadocuentamucho.aecosan.msssi.gob.es/media/cuadro-resumen-informacion-nutricional.pdf (Visitado 27/10/2019)

Tabla 1. Cuadro resumen sobre información nutricional. *Obtenido de: http://eletiquetadocuentamucho.aecosan.msssi.gob.es/media/cuadro-resumen-informacion-nutricional.pdf*



En relación al caso de la adición de vitaminas, este reglamento establece a su vez que se debe modificar el apartado 3 del reglamento (CE) 1925/2006, donde se define que para el caso de alimentos que hayan sido enriquecidos con vitaminas o minerales, es obligatorio el etiquetado sobre propiedades nutritivas siempre y cuando las cantidades sean *significativas*. En este contexto se establece que para el caso de vitaminas, se considera que un alimento posee una cantidad *significativa* cuando supera el 15% de los valores de ingesta de referencia diaria (en adultos) en alimentos distintos de bebidas, y del 7.5% para el caso de bebidas. Estos valores de referencia son recogidos en la tabla 2:

Tabla 2. *Relación de las ingestas diarias de vitaminas recomendadas en adultos.*

Vitamina A (µg)	800
Vitamina D (µg)	5
Vitamina E (mg)	12
Vitamina K (µg)	75
Vitamina C (mg)	80
Tiamina (mg)	1.1
Riboflavina (mg)	1.4
Niacina (mg)	16
Vitamina B6 (mg)	1.4
Ácido fólico (µg)	200
Vitamina B12 (µg)	2.5
Biotina (µg)	50
Ácido pantoténico (mg)	6

Así mismo se definen también las formas químicas en la cuales se permite su adición, y que se recogen en la tabla 3:

Tabla 3. *Fórmulas vitamínicas que pueden ser añadidas a los alimentos.*

VITAMINA A	retinol
	palmitato de retinilo
	acetato de retinilo
	β-caroteno
VITAMINA D	ergocalciferol
	colecalciferol
VITAMINA E	D-α-tocoferol
	DL-α-tocoferol
	acetato de DL-α-tocoferilo
	acetato de D-α-tocoferilo
	succinato ácido de D-α-tocoferilo
VITAMINA K	filoquinona (fitomedianona)
VITAMINA B1	clorhidrato de tiamina
	mononitrato de tiamina
VITAMINA B2	riboflavina
	riboflavina 5'-fosfato sódico
NIACINA	ácido nicotínico
	nicotinamida
ÁCIDO PANTOTÉNICO	D-pantotenato sódico
	D-pantotenato cálcico
	dexpantenol
VITAMINA B6	piridoxina 5'-fosfato
	clorhidrato de piridoxina
	dipalmitato de piridoxina
ÁCIDO FÓLICO	ácido pteroilmonoglutámico
VITAMINA B12	cianocobalamina
	hidroxocobalamina
BIOTINA	D-biotina
VITAMINA C	ácido L-ascórbico
	L-ascorbato sódico
	L-ascorbato potásico
	L-ascorbato cálcico
	6-palmitato de L-ascorbilo

Por otra parte, se deben establecer de igual forma las cantidades de vitaminas que pueden ser añadidas a un alimento. En este sentido cabe destacar que estas cantidades añadidas se deben mover dentro de unos rangos máximos y mínimos. Este tipo de compuestos se degradan tanto en el procesado del alimento, como a lo largo de la vida útil del producto. Por esta razón deben ser adicionadas en exceso en el momento de su fabricación para que en el momento de la fecha de consumo preferente los valores reales que se encuentran dentro del producto, se encuentren así mismo entre los límites previamente establecidos.

Según el *Documento de orientación para las autoridades competentes en materia de control del cumplimiento de la legislación de la UE sobre: "Reglamento (UE) nº 1169/2011 del Parlamento Europeo y del Consejo, de 25 de octubre de 2011, sobre la información alimentaria facilitada al consumidor y por el que se modifican los Reglamentos (CE) nº 1924/2006 y (CE) nº 1925/2006 del Parlamento Europeo y del Consejo, y por el que se derogan la Directiva 87/250/CEE de la Comisión, la Directiva 90/496/CEE del Consejo, la Directiva 1999/10/CE de la Comisión, la Directiva 2000/13/CE del Parlamento Europeo y del Consejo, las Directivas 2002/67/CE y 2008/5/CE de la Comisión, y el Reglamento (CE) nº 608/2004 de la Comisión y Directiva 90/496/CEE del Consejo, de 24 de septiembre de 1990, relativa al etiquetado sobre propiedades nutritivas de los productos alimenticios y Directiva 2002/46/CE del Parlamento Europeo y del Consejo, de 10 de junio de 2002, relativa a la aproximación de las legislaciones de los Estados miembros en materia de complementos alimenticios por lo que respecta al establecimiento de tolerancias para los valores nutricionales declarados en la etiqueta"*, se deben cumplir las tolerancias descritas en la siguiente tabla:

Tabla 4. *Fórmulas vitamínicas que pueden ser añadidas a los alimentos. Obtenida de https://ec.europa.eu/food/sites/food/files/safety/docs/labelling_nutrition-vitamins_minerals-guidance_tolerances_summary_table_012013_en.pdf*

GUIDANCE DOCUMENT TOLERANCES: SIMPLIFIED SUMMARY TABLE — DECEMBER 2012

	Tolerances for foods including MU and for tolerances for nutrients regulated by Reg. 924/2006/EC, Reg. 1925/2006/EC when the tolerance range around the declared value given below does not overlap with the minimum or maximum level to be present		Tolerances for food supplements including MU	Tolerances for nutrients regulated by Reg. 24/2006/EC, Reg. 1925/2006/EC in foods or food supplements when the declared value=minimum or maximum level to be present or levels specified in a claim Side 1, incl. MU; range applied to minimum or maximum level to be present when this level a) ≠ declared value and b) overlaps with tolerance range around the declared value from columns 2&3			Rounding	
Vitamins	+50%**	-35%	+50%**	-20%	+ 50%**	- MU	vitamin A, folic acid: all other vitamins:	3 sig. figures 2 sig. figures
Minerals	+45%	-35%	+45%	-20%	+ 45%	- MU	Cl, Ca, P, Mg, I, K: all other minerals:	3 sig. figures 2 sig. figures
Carbohydrate*, Protein* Fibre*	<10g/100g: ±2g (fat: ±1.5g) 10-40g/100g: ±20% >40g/100g: ±8g				<10g/100g: +4g 10-40g/100g: +40% >40g/100g: +16g	- MU	≥10g/100g or ml: <10g and >0.5g/100g or ml: no detectable amounts or ≤0.5g/100g or ml:	to nearest 1g to nearest 0.1g "0g" or "<0.5g" may be declared
Sugars* Fat*					<10g/100g: -4g (fat: -3 g) 10-40g/100g: -40% >40g/100g: -16g	+ MU	(rounding also applicable to polyols*, starch*)	
Saturates*	<4g/100g: ±0.8g ≥4g/100g: ±20%				<4g/100g: -1.6g ≥4g/100g: -40%	+ MU	≥10g/100g or ml: <10 and >0.1g/100g or ml: no detectable amounts or :	to nearest 1g to nearest 0.1g "0g" or "<0.1g" may be declared
Monounsaturates* Polyunsaturates*					<4g/100g: +1.6g ≥4g/100g: +40%	- MU	≤0.1g/100g or ml:	
Sodium	<0.5g/100g: ±0.15g ≥0.5g/100g: ±20%				<0.5g/100g: -0.3g ≥0.5g/100g: -40%	+ MU	≥1g/100g or ml: <1g and >0.005g/100g or ml: no detectable amounts or : ≤ 0.005g/100g or ml:	to nearest 0.1g "0g" or "<0.01g" may be declared
Salt	<1.25g/100g: ±0.375g ≥1.25g/100g: ±20%				<1.25g/100g: -0.75g ≥1.25g/100g: -40%	+ MU	≥1g/100g or ml: <1g and >0.0125g/100g or ml: no detectable amounts or : ≤0.0125g/100g or ml	to nearest 0.1 g to nearest 0.01g "0g" or "<0.01g" may be declared
Energy								to nearest 1kJ/kcal

* not applicable to sub-categories ** for vitamin C in liquids, higher upper tolerance values could be accepted MU: measurement uncertainty sig.: significant

Adicionalmente, y desde el punto de vista de la seguridad alimentaria, el Ministerio de Sanidad, Servicios Sociales e Igualdad, a través de la Subdirección General de Promoción de la Seguridad alimentaria, de la que depende la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición, ha elaborado un documento[4] donde se establecen los niveles máximos de ingesta diaria de vitaminas a tenor de lo publicado en diferentes recomendaciones de la Unión Europea.

Estas cantidades máximas se refieren a ingestas diarias aportadas por todas las posibles fuentes, y se definen como *"el nivel más alto de ingesta de un nutriente a la que es probable que no se observe ningún efecto adverso para la salud en la mayoría de los individuos de la población general"***.** En la tabla 5 se resumen los niveles permitidos:

[4] Disponible en: http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/seguridad_alimentaria/gestion_riesgos/UL_Vitaminas_Minerales_2.pdf (Visitado 27/10/2019)

Tabla 5. *Niveles máximos de ingesta admisible (tolerable upper intake level) para vitaminas evaluados por EFSA.*[4]

Vitaminas	Nivel máximo / adulto	Referencia EFSA
Ácido fólico/folato	1 mg	SCF/CS/NUT/UPPLEV/18 28 November 2000
Ácido pantoténico	-	
Biotina	-	
Niacina	Ác. nicotínico 10 mg (No incluye embarazadas, ni mujeres en periodo de lactancia)	SCF/CS/NUT/UPPLEV/39 6 May 2002
	Nicotinamida 900 mg (No incluye embarazadas, ni mujeres en periodo de lactancia)	
Vitamina A (Retinol y Ésteres Retinilo)	3000 µg 1500 µg (Postmenopausia)	SCF/CS/NUT/UPPLEV/24 7 October 2002
Vitamina B1	-	-
Vitamina B2	-	-
Vitamina B6	25 mg	SCF/CS/NUT/UPPLEV/16 28 November 2000
Vitamina B12	-	-
Vitamina C	-	-
Vitamina D	100 µg	EFSA Journal 2012; 10(7):2813. doi:10.2903
Vitamina E	300 mg	SCF/CS/NUT/UPPLEV/31 21 april 2003

En relación a las isoflavonas de soja, no está clara la toxicidad de este tipo de compuestos. Existen estudios que concluyen que existen efectos adversos en el sistema reproductor de ratas a unos niveles de concentración (LOAEL) de 35 mg/kg para los machos y 44 mg/kg para el

caso de las hembras[5]. Sin embargo, estos valores se encuentran muy alejados de los valores encontrados en la población japonesa (0.43 mg/kg), la cual es la más expuesta a este tipo de compuestos.

De esto se desprende la necesidad de un control analítico de la composición de este tipo de compuestos, ya que se debe asegurar que la concentración que se encuentra contenida en el alimento, cumple con los rangos de tolerancia establecidos por el etiquetado de acuerdo a la legislación vigente.

Es de destacar que los métodos validados en esta investigación relacionados con este tipo de compuestos, se han aplicado sobre matrices lácteas. Este tipo de matrices presentan una serie de complejidades que hacen que la determinación de micronutrientes o compuestos a nivel de trazas presente una serie de retos que deben ser solventados. Dicha complejidad se deriva del hecho de que en la composición de la leche de vaca se encuentra una relativamente alta concentración de grasa, proteínas, azúcares, sales minerales, etc., que hacen que el riesgo de co-extracción de interferentes que puedan coeluir junto con los compuestos de interés, sea muy alto. Como valores promedios, la leche de vaca posee aproximadamente los siguientes porcentajes de cada componente (figura 1):

[5] Rozman, K.K., Bhatia, J., Calafat, A.M., Chambers, C., Culty, M., Etzel, R.A., Flaws, J.A., Hansen, D.K., Hoyer, P.B., Jeffery, E.H., Kesner, J.S., Marty, S., Thomas, J.A., Umbach, D. NTP-CERHR expert panel report on the reproductive and developmental toxicity of soy formula. Birth. Defects Res. B. Dev. Reprod. Toxicol. 77(4) (2006) 280-397.

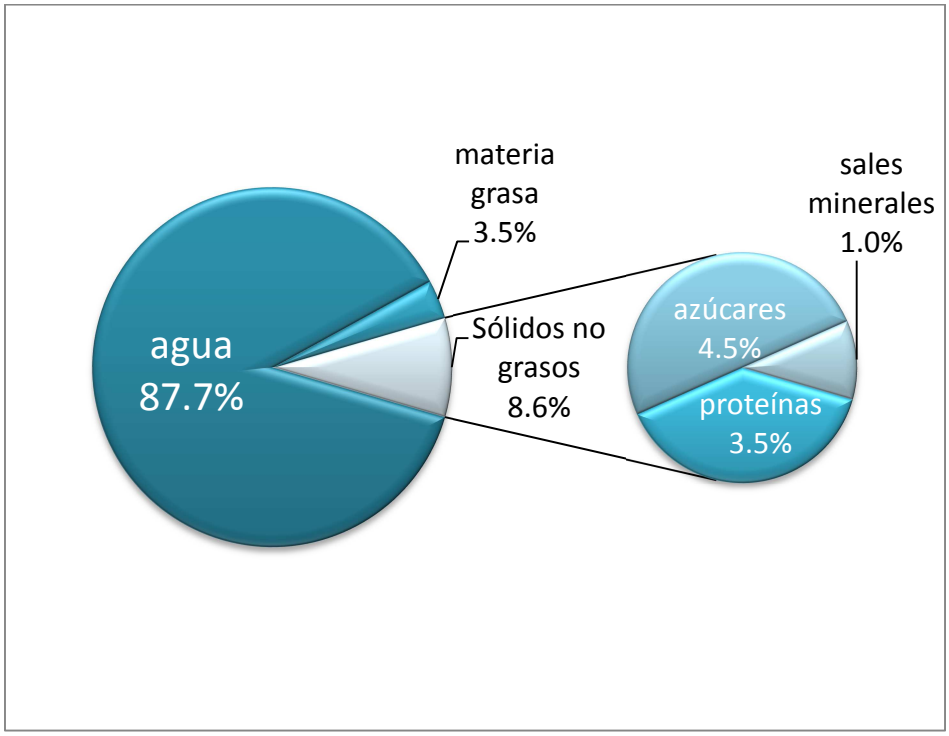


***Figura 1**. Representación de la composición nutricional media de la leche entera de vaca.*

Por esta razón, los métodos de análisis de compuestos a nivel de trazas en matrices alimentarias, comprenden etapas de preparación de muestra relativamente complejas tales como SPE, con un coste por muestra y consumo de tiempo relativamente altos, de los cuales se obtienen extractos en donde se reducen drásticamente la presencia de posibles interferentes gracias a la optimización de las diferentes etapas de lavado, donde se eliminan selectivamente familias de compuestos en base a cambios de pH, composición de mezclas de disolventes, etc.

El empleo de técnicas con alta selectividad y sensibilidad, como es el caso de la espectrometría de masas, permite emplear tratamientos de muestra más simples y menos costosos tales como la extracción líquido-

líquido, sin comprometer la robustez, precisión y exactitud del método. Esto es debido a que, a pesar de la existencia de un alto número de interferentes en el extracto obtenido tras el proceso de tratamiento de muestra, mediante una optimización de la etapa separativa previa (en este caso cromatográfica) y espectrométrica, se posibilita que se discriminen los compuestos de interés selectivamente.

Sin embargo, en este trabajo de Tesis se han optimizado métodos (vitaminas e isoflavonas de soja) donde no ha sido necesario el empleo de este tipo de detectores, y mediante una adecuada optimización de las condiciones de separación, se ha posibilitado que con detectores menos sofisticados (UV-visible y FLD) se obtengan resultados igualmente válidos analíticamente.

En los siguientes apartados se van a describir cada una de las metodologías analíticas desarrolladas para resolver los diferentes problemas analíticos planteados.

1.1. Vitaminas hidrosolubles

1.1.1. Introducción

Las vitaminas (término acuñado por Casimir Funk en 1912 debido a que al aislar la primera de ellas, esta poseía un grupo amino, y de ahí el término "vita" – "amina", hoy comúnmente aceptado[6]) son un grupo de sustancias de naturaleza muy heterogénea pero que comparten el hecho de que son fundamentales para el correcto crecimiento y desarrollo animal, incluso a muy bajas concentraciones. A principios del siglo XX, Sir Frederick Hopkins observó cómo los animales alimentados solamente a base de macronutrientes (grasa, proteínas, hidratos de carbono, etc.) no se desarrollaban adecuadamente, y aquellos en donde se incorporaba leche a su dieta sí lo hacían.[7] De ahí que se definieran unos "factores accesorios de crecimiento", como se los denominó en su época, que se sospechaban eran responsables del adecuado desarrollo del crecimiento animal.

Las vitaminas del grupo B y el ácido ascórbico, poseen una importante función en el desarrollo y metabolismo humano: la deficiencia de tiamina (*vitamina B1*) causa cambios perjudiciales en el sistema cardiovascular y nervioso que pueden ser revertidos mediante la administración de esta vitamina[8], así mismo estudios de los años 20 y 30 determinaron que actúa como coenzima en la descarboxilación oxidativa del piruvato, fundamental en el proceso de respiración celular[7].

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6 Palacios Sánchez, L. Breve historia de las vitaminas. Revista Medica Sanitas 16 (3) (2013) 142-145.

7 Bender, D.A. Nutritional Biochemistry of the Vitamins 2nd Edition, Cambridge, UK. Cambridge University Press, 2003.

8 Lu, J., Frank, E.L. Rapid HPLC measurement of thiamine and its phosphate esters in whole blood. Clinical Chem. 54 (2008) 901-906.

La riboflavina (*vitamina B2*) y el ácido fólico (*vitamina B9*) intervienen directamente en el buen funcionamiento y desarrollo del metabolismo celular y de los ritmos circadianos[9]. En particular, el papel metabólico del folato es el transporte de fragmentos de carbono en reacciones catabólicas y biosíntesis, con lo que interviene en la síntesis de nucleótidos, división celular y expresión de genes. Es importante en la función metabólica ya que actúa como portador de electrones en numerosas reacciones de oxidación y reducción de diferentes procesos metabólicos, con lo que su privación en la dieta puede provocar daños en el sistema nervioso.[7] Durante el embarazo, la OMS recomienda un consumo mínimo diario de 400µg de esta vitamina para evitar malformaciones en el tubo neuronal.

La nicotinamida (*vitamina B3*) es la parte reactiva del NAD y NADH, las cuales son coenzimas en una amplia variedad de procesos fisiológicos. Uno de los efectos más conocidos de su deficiencia en la dieta o problemas para su absorción es la enfermedad conocida como "pelagra", caracterizada por diarreas, dermatitis y demencia.

EL ácido pantoténico (*vitamina B5*) representa la parte funcional de la CoA, con lo que tiene un papel central en el metabolismo que proporciona la energía celular, biosíntesis de ácidos grasos, así como biosíntesis de esteroides, porfirinas y acetilcolinas. Aunque el ser humano no sea capaz de sintetizar el ácido pantoténico, su presencia en la dieta está muy

[9] Wang, Y., Zhu, P.H., Tian, T., Tang, J., Wang, L., Hu, X.Y. Synchronous fluorescence as a rapid method for the simultaneous determination of folic acid and riboflavin in nutritional beverages. J. Agric. Food Chem. 59 (2011) 12629-12634.

extendida, sin embargo se ha establecido una dosis recomendada de entre 3 mg/día y 7 mg/día dependiendo de la edad del individuo.[10]

La piridoxina (*vitamina B6*) tiene un papel fundamental en el metabolismo de aminoácidos ya que actúa como cofactor de enzimas involucradas en este tipo de procesos, uso de la reservas de glucógeno, síntesis hemo, formación de niacina (vitamina B3) metabolismo lipídico, síntesis de neurotransmisores y regula la expresión de genes inducidos por hormonas.[11] Aunque su presencia en alimentos está muy extendida, la EFSA ha establecido unos valores de referencia recomendados de entre 0.6 mg/día a 1.8 mg/día, dependiendo del grupo de población al que se hace referencia (niños de corta edad hasta embarazadas respectivamente).

La cianocobalamina (*vitamina B12*) contribuye al normal desarrollo de las funciones neurológicas y fisiológicas, metabolismo de la homocisteína, reducción del cansancio y la fatiga[12].

El ácido ascórbico (*vitamina C*) tiene carácter reductor, y participa en numerosas reaciones redox como cofactor tanto de forma específica en determinadas rutas metabólicas, como de forma inespecífica atenuando radicales libres. Además, actúa en el proceso de reducción de vitamina E

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[10] European Food Safety Authority. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific opinion on dietary reference values for pantothenic acid. EFSA J. 12 (2014) 3581.

[11] European Food Safety Authority. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Dietary reference values for vitamin B6. EFSA J. 14 (2016) 1-79.

[12] European Food Safety Authority (EFSA), Scientific Opinion on the substantiation of health claims related to vitamin B12 and contribution to normal neurological and psychological functions, contribution to normal homocysteine metabolism, maintenance of normal bone, maintenance of normal teeth (ID 104), maintenance of normal hair, maintenance of normal skin, maintenance of normal nails, reduction of tiredness and fatigue, and cell division pursuant to Article 13(1) of Regulation (EC) No 1924/2006, EFSA J. 8(10):1756 (2010) 1-23.

oxidada, favorece la absorción de hierro y otros metales, etc.[7] De esta forma, su actividad es fundamental en la expresión de numerosos genes que son modulados gracias a esta vitamina. Desde tiempo inmemorial, se conoce que su deficiencia prolongada causaba escorbuto, siendo esta la principal causa de muerte en largas travesías marinas, donde la ausencia de consumo de frutas frescas provocaba una importante carencia de este compuesto. Por su participación en numerosos procesos metabólicos, se recomienda el consumo de una cantidad mínima diaria de vitamina C que se encuentra entre los 25 - 120 mg/día, dependiendo del grupo de edad y sexo de la población.

Se evidencia por tanto la necesidad de disponer de metodologías de ensayo validadas capaces de determinar este tipo de compuestos en matrices alimentarias. Concretamente, para el caso de matrices lácteas, existen en el mercado numerosos productos en los que se han incorporado, entre otras, vitaminas del tipo hidrosoluble y que mejoran por tanto las propiedades nutricionales que, de forma natural, posee el alimento. Este es un ejemplo de *alimento funcional*, en el cual se ha adicionado uno o varios componentes que mejoran las propiedades nutricionales que originalmente tenía el alimento.

Para este tipo de matrices complejas, existen diferentes métodos oficiales de varias organizaciones internacionales recomendados por el Codex[13] (AOAC, USP, etc.) donde se emplean varios tipos de técnicas analíticas como son:

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[13] Recommended methods of analysis and sampling CXS234-1999 (2018) http://www.fao.org/fao-who-codexalimentarius/sh-proxy/es/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcod

- **Ensayos microbiológicos**: se analizan microorganismos cuya proliferación está directamente relacionada con la cantidad presente específicamente de cierta vitamina. Tal es el caso de vitamina B3 (AOAC 985), vitamina B5 (AOAC 992.07), vitamina B6 (AOAC 985.32; EN 4166), vitamina B9 (AOAC 992.05; EN 14131) o vitamina B12 (AOAC 986.23). En este tipo de técnica analítica la propiedad óptica que se registra es el cambio de turbidez provocado por el crecimiento en el medio acuoso de los microrganismos mediante nefelometría. Este tipo de técnica es muy sensible, aunque requiere de una adecuada formación en la manipulación de microorganismos, de lo contrario, puede provocar errores por exceso en los resultados obtenidos debidos a contaminaciones por parte de otros microorganismos no específicos en el medio de reacción. Por el contrario, se pueden dar errores por defecto debido a una inapropiada manipulación en la preparación de medios o conservación de los microorganismos. Así mismo, este tipo de técnicas tienen una respuesta no lineal frente a la concentración de analito, lo que dificulta la interpretación de los resultados y sus correspondientes intervalos de confianza.

- **Técnicas ELISA**: este tipo de técnicas rápidas se basan en la formación de un complejo antígeno-anticuerpo específico y selectivo. Un componente inmunológico, en este caso el anticuerpo específico, es inmovilizado en un soporte (normalmente las cavidades de una microplaca de 96 pocillos). A continuación, el analito de la muestra interactúa con el complejo antígeno-

ex%252FStandards%252FCODEX%2BSTAN%2B234-1999%252FCXS_234e.pdf (Visitado 27/10/2019)

anticuerpo y esta interacción es detectada mediante enzimas enlazadas a antígenos o anticuerpos secundarios que reaccionan a su vez con un sustrato que produce un cambio de color. Este cambio de color puede cuantificarse espectrofotométricamente pudiéndose por tanto establecerse funciones de calibrado.

Este tipo de técnicas requieren por tanto del uso lectores de microplacas UV-visible, los cuales representan una inversión mucho menor a otras técnicas instrumentales más sofisticadas como las cromatográficas. Otras ventajas son la alta capacidad de procesado de muestras en una misma tanda, facilidad para protocolizar el tratamiento de muestra, ya que este tipo de técnicas se realizan utilizando fases muy similares independientemente del analito a determinar: fase de incubación – lavado – conjugación – lavado – adición de sustrato – reacción – parado de reacción y medición espectrofotométrica final.

Sin embargo, la respuesta que proporciona frente a variaciones de la concentración de analito no es lineal, debiéndose emplear modelos matemáticos alternativos (cuadráticos, semi-logarítmicos, cubic spline, etc.) que dificultan la interpretación y cálculo de incertidumbre de los resultados finales. Al mismo tiempo, existe el riesgo de reacciones cruzadas con compuestos de naturaleza parecida que puedan provocar la aparición de errores por exceso.

Otro inconveniente añadido es el hecho de que normalmente debe emplearse una determinación por analito, debiéndose por tanto realizarse varios ensayos con kit ELISA diferentes para el caso de determinaciones de grupos de analitos. Esto hace que para la determinación de vitaminas hidrosolubles, deban realizarse varios

ensayos independientes, uno por cada compuesto, con el consiguiente requerimiento de tiempo y recursos necesarios.

- **Técnicas cromatográficas**: en los métodos oficiales donde se emplean este tipo de técnicas, se requiere de un tratamiento de muestra previo que consiga la liberación de las diferentes formas naturales que existen en los alimentos asociadas a proteínas u otros componentes del alimento. Esta liberación se consigue mediante autoclavado de la muestra en medio ácido[14], hidrólisis enzimática[15], etc.

  Para el caso de las vitaminas hidrosolubles en matrices lácteas, las cantidades en las que naturalmente están presentes en la leche de vaca se encuentran en el entorno del límite de cuantificación del método[16], con lo que en el método desarrollado se ha permitido la simplificación del proceso de extracción, de forma que con una única etapa de precipitación de la fracción proteica, quedan en disolución las vitaminas añadidas artificialmente y las que no se encuentran enlazadas a alguno de los diferentes componentes de la leche.

Esta etapa de precipitación única se ha logrado mediante el empleo del reactivo de Biggs-Szijarto[17], el cual precipita proteínas y grasa de la

[14] UNE-EN 14152:2015 Productos alimenticios. Determinación de vitamina B2 mediante cromatografía líquida de alta resolución.

[15] UNE-EN 14122:2014 Productos alimenticios. Determinación de vitamina B1 por cromatografía líquida de alta resolución.

[16] National Nutrient Database for Standard Reference Legacy Release, United States Department of Agriculture, Agricultural Research Service (https://fdc.nal.usda.gov/)

[17] Biggs, D.A., Szijarto, L. Method for routine determination of lactose in milk. J. Dairy Sci. 46 (1963) 1196-1200.

leche obteniéndose un extracto translúcido en donde se encuentran disueltos los componentes hidrosolubles tales como azúcares y vitaminas hidrosolubles.

1.1.2. Objetivos

El objetivo de este trabajo de investigación ha sido el desarrollo de una metodología analítica multicomponente de buenas características analíticas con una alta capacidad de procesamiento de muestras.

Los métodos oficiales de análisis de vitaminas tratan la muestra de forma específica y se determinan individualmente, haciendo este tipo de metodologías difíciles de implantar en un laboratorio de rutina donde se deben procesar un gran número de muestras diariamente, y donde además se les deben determinar un gran número de componentes.

Con la publicación aquí presentada se ha conseguido implantar en el laboratorio de rutina una metodología validada capaz de cumplir con los hitos anteriormente mencionados, ofreciendo una alta información analítica con un procesamiento de muestra rápido y simple.

1.1.3. Publicación I

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Simultaneous Determination of Eight Water-Soluble Vitamins in Supplemented Foods by Liquid Chromatography

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ABSTRACT

A fast, simple, and reliable method for the isolation and determination of the vitamins thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid in food samples is proposed. The most relevant advantages of the proposed method are the simultaneous determination of the eight more common vitamins in enriched food products and a reduction of the time required for quantitative extraction, because the method consists merely of the addition of a precipitation solution and centrifugation of the sample. Furthermore, this method saves a substantial amount of reagents as compared with official methods, and minimal sample manipulation is achieved due to the few steps required. The chromatographic separation is carried out on a reverse phase C18 column, and the vitamins are detected at different wavelengths by either fluorescence or UV- visible detection. The proposed method was applied to

the determination of water-soluble vitamins in supplemented milk, infant nutrition products, and milk powder certified reference material (CRM 421, BCR) with recoveries ranging from 90 to 100%.

KEYWORDS: Water-soluble vitamins; food labelling; supplemented foods; milk; liquid chromatography

INTRODUCTION

Vitamins are crucial for maintaining good health in humans; lack of a sufficient amount of any of them can cause serious diseases (1). The human diet does not always contain the amount of vitamins needed for normal development and maintenance of body functions. For this reason, certain food products are supplemented with vitamins, especially those directed to infant nutrition. Moreover, food processing and long periods of food storage may also lead to loss of vitamins. Thus, vitamin fortification allows the nutritional requirements of infant formulas and other baby foods to be met. A rapid and reliable analytical determination of the water-soluble vitamin content in food is needed for food laboratories, manufacturers, and regulatory authorities to confirm the percentage of the recommended dietary allowance (RDA) present in the final food products.

Current official methods (2-12) for the determination of water-soluble vitamins are based on spectroscopic, chromatographic, chemical, or microbiological techniques that are tedious and time-consuming. Different acid treatments followed by enzymatic digestion before microbiological assay or HPLC procedures have been reported, such as sulphuric acid and amylase (13), perchloric acid (14), trichloroacetic acid (15), or hydrochloric acid and taka-diastase (16) treatments. It is important to note that either official methods or HPLC multivitamer methods are not suitable for

the simultaneous determination of all the typically supplemented water-soluble vitamins in food. Separation of water-soluble vitamins has been carried out using reverse-phase liquid chromatography (RP-LC) without ion-pair reagents (16-18) and also RP-LC with ion-pair chromatography (15, 19, 20).

Specific HPLC methods have been developed to quantify vitamin C in different foods (21-26), but only a few HPLC methods simultaneously determining vitamin C and other water- soluble vitamins have been found in the literature (27). Similarly, HPLC methods for the determination of pantothenic acid in several food matrices have been reported (28-30), and attempts to overcome problems of absorbance at low wavelength have utilized post column derivatization (31) or mass spectrometry detection with electrospray ionization (32). To our knowledge, vitamin C and pantothenic acid have not been included previously in a simultaneous determination of water-soluble vitamins in infant food or vitamin-enriched food products.

In the present work, we describe a simple, fast, and reliable sample treatment procedure previous to the HPLC determination of eight water-soluble vitamins that are usually present in several supplemented food products (infant formulas, infant milk, and vitamin-enriched milks). The vitamins included are thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid.

MATERIALS AND METHODS

Instrumentation. A Megafuge 1.0 centrifuge (Heraeus, Hanau, Germany) was used for sample treatment, and the chromatographic separation was carried out in a 2695 Alliance chromatograph (Waters,

Milford, MA) equipped with a 2475 fluorescence detector, a 2996 photodiode array detector, a 20-60 °C thermostated column oven, and an automatic injector. Millenium 4.0 software was used for data treatment.

Chemicals and Reagents. All reagents were of analytical grade. Ascorbic acid (vitamin C), thiamin chloride hydrochloride, riboflavin, niacin, pantothenic acid, pyridoxine hydrochloride, folic acid, cyanocobalamin, and L-methionine were purchased from Sigma (Barcelona, Spain). HPLC-grade methanol was supplied by Panreac (Barcelona, Spain). Solvent and aqueous solutions were filtered through 0.2 µm membranes (Millipore, Bedford, MA). Triethylamine, glacial acetic acid (99%), orthophosphoric acid (85%), and potassium phosphate monobasic were obtained from Panreac. Zinc acetate hydrated, 1-octanesulfonic acid, phosphotungstic acid polyhydrated, and sodium hydroxide were obtained from Sigma. Water was purified using a Milli-Q system from Millipore.

The precipitation solution was prepared once a week by dissolving 9.10 g of zinc acetate, 5.46 g of phosphotungstic polyhydrated, and 5.8 mL of glacial acetic acid in 100 mL final volume of Milli-Q water. The aqueous mobile phase buffer (pH 2.95) was prepared daily by dissolving 6.8 g of potassium phosphate monobasic, 1.1 g of 1-octanesulfonic acid sodium salt, and 5 mL of triethylamine in 1 L of water. After that, the pH was adjusted by the addition of orthophosphoric acid.

Standards. A multistandard solution of ascorbic acid (1.0 g/L), thiamin (200 mg/L), riboflavin (30 mg/L), niacin (100 mg/L), pan- tothenic acid (100 mg/L), pyridoxine (40 mg/L), and cyanocobalamin (20 mg/L) was prepared in 2.4% (v/v) acetic acid (solution 1). Folic acid standard (50 mg/L) was prepared by dilution in sodium hydroxide (0.1 M) (solution 2). Solutions were kept in dark bottles at -20°C to avoid vitamin degradation. Working

standard were prepared by mixing both solutions 1:1 (v:v) and diluting with Milli-Q water just before use.

Samples. Vitamin-enriched milk and infant nutrition products were obtained from different supermarkets in Granada (Spain). Enriched milk 1 is a multivitamin ultrahigh-temperature (UHT) dairy drink for children, enriched milk 2 is a multivitamin UHT dairy drink for children over 1 year of age, and the infant milk is a supplemented milk powder. The proposed method was validated using a CRM 421 milk powder certified reference material (Community Bureau of Reference-BCR, Brussels, Belgium).

Sample Treatment. All operations were performed in subdued light. For solid samples (e.g., infant formula), 0.5 g of sample was placed in a glass or plastic tube (15 mL) and 4.5 g of heated water (40°C) was added. The mixture was homogenized by vortex-stirring for 1 min, sonicated for 5 min, and kept for 60 min at room temperature and in darkness. For liquid samples, 5.0 g of homogenized sample was accurately weighed into tubes.

Then, 1.0 g of the precipitation solution was added into the sample tube, and the mixture was vortexed for 1 min at room temperature. After 15 min under darkness, tubes were centrifuged at 3500 g for 5 min, and the supernatants were filtered through 0.22 µm nylon Millipore filters before injection into the HPLC column.

Chromatographic Conditions. The HPLC procedure is based on conditions described by Albalá-Hurtado et al. (10) with several modifications in order to be able to obtain a proper separation of the eight water-soluble vitamins. The separation was carried out in a Waters Alliance 2695 separation module using a gradient elution on a C18 Waters Spherisorb ODS-2 column (25 cm 4.6 mm, 3 µm) set at 40°C.

The mobile phase was a modified buffer phosphate (solvent A) and methanol (solvent B), and the initial values were 99.4% of A and 0.6% B followed by a 0.5 min hold; then four linear gradients were established to reach, first, a 94.0% A and 6.0% B composition at 4 min, a 70.0% A and 30.0% B composition at 12 min, and a 60.0% A and 40.0% B composition at 17 min followed by a return to initial conditions at 22 min and a hold for 8 min to get column conditioning. The flow rate was 1.0 mL/min during the entire run. The injection volume was 50 µL, and the autosampler temperature was set at 5 °C to avoid degradation.

The fluorescence detection was set at 290/410 nm (excitation/ emission) for pyridoxine, held for 10 min, and changed to 400/520 nm (excitation/emission) for riboflavin determination. The optimal wave lengths in UV-vis detection were set at 245 nm (thiamin), 261 nm (niacin), 195 nm (pantothenic acid), 282 nm (folic acid and vitamin C), and 370 nm (cyanocobalamin).

RESULTS AND DISCUSSION

Sample Treatment. Determination of water-soluble vitamins by official methods based on chemical or microbiological techniques is laborious and time-consuming. Available fast methods for enriched food products, such as protein precipitation with trichloroacetic acid (15), have shown, in our hands, poor extraction capacity for some vitamins, such as niacin or ascorbic acid. At the same time, we have noticed the appearance of impurities that interfere with the chromatographic determination of pantothenic acid and folic acid, possibly due to the use of strong acids, such as sulfuric acid (33), hydrochloric acid (16), or trichloroacetic acid (15), for protein precipitation.

Investigation of possible sample treatments of milk-related products turned us to a precipitation solution containing zinc and wolframium salts in an acid medium. This solution is currently used for milk treatment in the analysis of lactulose as described in the method by the International Dairy Federation (34). We decided to evaluate similar treatment conditions to extract water-soluble vitamins by precipitation of proteins and fat in vitamin-enriched milks. By using the chromatographic conditions described below, we were able to verify that all eight supplemented vitamins could be isolated, separated, and quantified. Next, the ratio of precipitation solution and sample was optimized for maximum recovery. The volumes were decreased to minimize final residues on the analysis because heavy metals are present in the precipitation solution. The final conditions for sample treatment are simple and fast and can be extended to other products such as infant formulas, infant milk, and enriched fruit juices.

Chromatographic Separation. Separation is based on methodology described by Albalá-Hurtado et al. (15) using ion pair liquid chromatography, where 1-octanesulfonic acid sodium salt was added to the aqueous mobile phase. Several modifications were introduced in order to be able to obtain a proper separation of the eight water-soluble vitamins. Triethylamine was also added to improve peak symmetry. The total mobile phase was optimized using mixtures of the aqueous mobile phase and methanol. Acetonitrile was also assayed without good results. The pH was an extremely critical factor for the separations of vitamins, as has been previously reported (15, 35). pH values from 2.5 to 4.0 were tested by the addition of orthophosphoric acid to the mobile phase, and it was concluded that the pH should be lower than 3.0 to resolve the critical vitamin pairs niacin/pyridoxine and riboflavin/cyanocobalamin. The initial methanol concentration in the mobile phase must be lower than 1% to

obtain good separation of all vitamins under study but, nevertheless, present in order to avoid an excessive retention time for thiamine. Therefore, pH 2.95 and 0.6% of initial methanol concentration were selected.

Analytical Characteristics of the HPLC Method. Scan analysis of standard vitamins was performed to check the optimum conditions for detection. Wavelengths for UV-visible and fluorescence detection were selected according to the elution time of each vitamin (see Materials and Methods). To obtain adequate linear range to measure vitamin C in selected samples, 282 nm was selected as the wavelength instead of the maximum at 244 nm. Calibration graphs for samples treated according to the analytical procedure described above were carried out. The calibration graphs are linear for the concentration range shown in Table 1. Linearity of the calibration graphs was tested according to the recommendation of the Analytical Methods Committee (36); the lack-of-fit (lof) test was applied to two replicates and three injections of each standard. The results for the intercept (a), slope (b), correlation coefficient (R^2), and probability level of the lof test [Plof (%)] are also summarized in Table 1. Thus, the data yield good linearity within the stated range. The precision, as repeatability, determined as relative standard deviation (RSD) of 10 independent determinations of the same sample, was measured for a concentration of 75.0 mg kg^{-1} for vitamin C; 12.5 mg kg^{-1} for thiamine, niacin, and pantothenic acid; 2.5 mg kg^{-1} for riboflavin, pyridoxine, and cyanocobalamin; and 3.5 mg kg^{-1} for folic acid. The RSD is below 4% in all cases (Table 1).

A fundamental aspect that needs to be examined in the validation of any analytical method is its limit of detection in order to determine if an

analyte is present in the sample. In this paper, a criterion for method performance has been used that includes the decision limit, CCα, and the detection capability, CCβ (37). The decision limit is the limit from which it can be decided that a sample is contaminated with an error probability of α. The detection capability is the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of β. Decision limit and detection capacity, which are better adjusted to a statistical evaluation, are implemented. Thus, CCα (α) (5%), CCβ (β) (5%), and quantification limits were calculated, and the results obtained are also summarized in Table 1.

Table 1. *Analytical Characteristics of the HPLC Method*

parameter[a]	vitamin C	thiamin	riboflavin	niacin	panthotenic acid	pyridoxine	folic acid	cyanocobalamin
k	9	10	9	10	9	9	8	9
a	$1.25 \cdot 10^4$	$2.35 \cdot 10^3$	$7.39 \cdot 10^4$	$1.12 \cdot 10^3$	$2.68 \cdot 10^3$	$-6.47 \cdot 10^4$	$4.05 \cdot 10^3$	$-1.08 \cdot 10^2$
sa	$1.22 \cdot 10^4$	$3.30 \cdot 10^3$	$1.28 \cdot 10^5$	$2.13 \cdot 10^3$	$4.34 \cdot 10^2$	$6.99 \cdot 10^4$	$6.52 \cdot 10^2$	$2.12 \cdot 10^2$
b (kg mg-1)	$4.89 \cdot 10^4$	$6.09 \cdot 10^5$	$9.73 \cdot 10^6$	$1.01 \cdot 10^5$	$8.26 \cdot 10^4$	$6.51 \cdot 10^6$	$1.50 \cdot 10^5$	$5.05 \cdot 10^4$
sb (kg mg-1)	$2.02 \cdot 10^2$	$2.22 \cdot 10^2$	$7.74 \cdot 10^4$	$2.62 \cdot 10^2$	$5.05 \cdot 10^2$	$3.05 \cdot 10^4$	$2.31 \cdot 10^3$	$1.26 \cdot 10^2$
LDR (mg kg-1)	2.5-150.0	0.06-25.0	0.01-5.00	0.03-25.0	0.05-25.0	0.04-6.00	0.04-7.50	0.02-5.00
R 2 (%)	99.98	99.99	99.95	99.99	99.97	99.98	99.84	99.99
SRC	$2.89 \cdot 10^4$	$8.13 \cdot 10^3$	$2.97 \cdot 10^5$	$5.21 \cdot 10^3$	$9.68 \cdot 10^3$	$1.63 \cdot 10^5$	$1.37 \cdot 10^4$	$4.73 \cdot 10^2$
CCα (mg kg-1)	0.580	0.01	0.003	0.006	0.01	0.03	0.01	0.01
CCβ (mg kg-1)	0.950	0.02	0.005	0.010	0.02	0.04	0.02	0.02
LOQ (mg kg-1)	2.49	0.06	0.01	0.030	0.05	0.11	0.04	0.04
RSD (%)	2.4	0.8	0.9	0.6	0.9	3.7	1.3	0.5
Plof (%)	36.9	54.5	50.2	52.7	62.6	48.8	15.2	36.4

[a] k, calibration levels; a, intercept; sa, intercept standard deviation; b, slope; sb, slope standard deviation; R2, determination coefficient; LDR, linear dynamic range; CCα,0.05, decision limit; CCβ,0.05, detection capability; RSD, relative standard deviation; SRC, regression standard deviation; Plof, P value for lack of fit test.

Table 2. *Determination of Vitamins in Reference Material*

	milk powder CRM 421				
	certified	found[a]	t_{calc}	t_{tab} [b]	Conclusion
Ascorbic acid	769.0 ± 42.0	744.0 ± 35.0	1.727	2.120	Ho
Thiamin	6.51 ± 0.48	6.16 ± 0.50	1.529	2.074	Ho
Riboflavin	14.5 ± 1.0	13.1 ± 2.0	2.172	2.179	Ho
Niacin	68.0 ± 2.4	65.2 ± 4.5	1.979	2.131	Ho
Pantothenic acid	not certified	61.2 ± 3.0			
Pyridoxine	6.66 ± 0.85[c]	6.08 ± 0.95	1.626	2.111	Ho
Folic acid	1.40 ± 0.2[d]	1.35 ± 0.20	0.275	2.074	Ho
Cyanocobalamin	0.034 ± 0.005	<LD			

[a] Mean of 10 determinations ± standard deviation (mg kg^{-1}). [b] t_{tab} (R) 0.05; $n_1 + n_2 - 2$ fd). [c] Certified material includes pyridoxal, pyridoxamine, and pyridoxine. [d] Certified material includes total folates.

Table 3. *Recovery Assay for B5 and B12*

	Recovery assay for pantothenic acid and cyanocobalamin		
	added (mg kg^{-1})	found[a] (mg kg^{-1})	% recovery
Pantothenic acid	0.100	0.095	95.0
	1.000	0.980	98.0
	5.000	4.850	97.0
Cyanocobalamin	0.050	0.047	95.3
	1.000	1.040	104.0
	5.000	5.120	102.4

[a] Mean of 10 determinations

Table 4. *Results obtained for commercial milk products*

		Ascorbic acid	Thiamin	Riboflavin	Niacin	Pantothenic acid	Pyridoxine	Folic acid	Cyano-cobalamin
enriched milk 1	labeled	90	2.1	2.4	27.0	9.0	3.0	0.3	0.0015
	found	110.0	2.1	2.5	30.0	11.2	3.1	0.4	nd
enriched milk 2	labeled	100	0.8	2.0	14.0	3.0	1.1	0.15	4.5
	found	120.0	0.7	1.7	19.4	3.5	0.7	0.30	nd
infant milk	labeled	500	6	12	80	30	4	0.7	0.021
	found	490.0	7.3	14.8	67.8	35	5.0	0.75	nd
UHT milk	ref[b]	12.8	0.33	1.800	0.900	0.350	0.410	0.046	0.004
	found	0.0	0.33	1.679	0.780	0.356	0.376	0.042	nd

[a] *Mean of 10 determinations (mg kg^{-1}); nd, not detected.* [b] *Reference value from Soucci (39)*

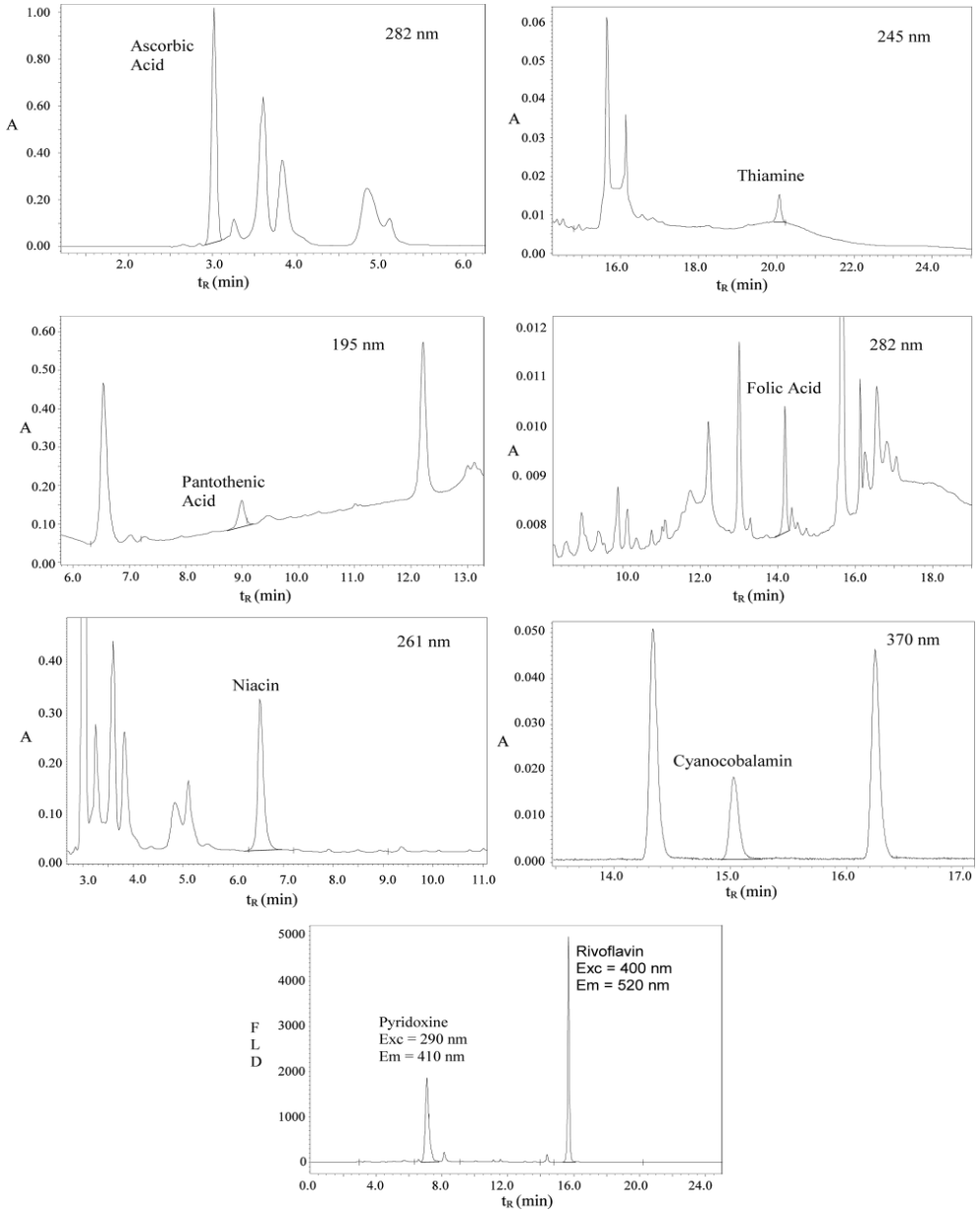

***Figure 1**. Representative chromatograms of a supplemented commercial milk sample.*

Validation of the Method Using Certified Reference Materials. The reliability of the proposed method for the determination of water-soluble

vitamins in milk and infant formulas was confirmed by using a certified reference material milk powder, CRM 421. All vitamins are certified in CRM 421, except for pantothenic acid and cyanocobalamin. Ten independent determinations were carried out for the same certified material, and a mean was obtained. The results are shown in Table 2. A statistical test was carried out to compare obtained and certified values. The test includes a variance comparison (Snedecor F test) and means comparison based on Student's t test (38). In all cases, it can be concluded by Ho; therefore, there were no significant differences between the results obtained by the chromatographic method and the certified values (test performed at significance level of $\alpha < 0.05$). These data also confirm the efficiency of the sample treatment procedure for the recovery of all studied vitamins from the samples.

A recovery assay was carried out according to the standard addition procedure due to the absence of pantothenic acid and the low level of cyanocobalamin present in the certified material. Three addition levels (0.10, 1.00, and 5.00 mg kg^{-1} for pantothenic acid and 0.05, 1.00, and 5.00mg kg^{-1} for cyanocobalamin, respectively) were used for each vitamin. Ten determinations were carried out for each addition level. Mean recoveries obtained were in all cases higher than 95% for both compounds (Table 3).

Analysis of Food Samples. The water-soluble vitamins specified previously were quantified in different food samples. Several commercial products, including infant food, infant milk, and enriched milk products, were analyzed. The chromatographic peaks in the samples analyzed were identified by comparing spectra and retention data obtained for the standards and spiked samples under optimized method conditions. Figure

1 shows an example of UV-vis at different wavelengths and a fluorescence chromatogram obtained for a commercial enriched milk sample. Values obtained for four specific food samples, three supplemented and a non-supplemented milk, are shown in Table 4. Most of the results are slightly higher than the labeled amounts in the commercial products. Actually, most vitamin- enriched products are manufactured with a small overaddition of these vitamers, so that the labeled amounts are still present in the food at the expiration date. It is important to point out that the present methodology is adequate for the eight previously specified vitamin forms but not for other natural forms, such as nicotinic acid, pyridoxamine, and pyridoxal, which could be present in these samples.

In conclusion, the proposed method is a powerful tool to improve the throughput and reliability of any food laboratory for the determination of the typical eight water-soluble vitamins found in supplemented food samples. It is a simple, rapid, and economical procedure. It is important to remark that ascorbic and pantothenic acid can also be measured with the same analysis.

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1.2. Isoflavonas de Soja

1.2.1. Introducción Isoflavonas de Soja

Las isoflavonas de soja son una familia de compuestos polifenólicos con una estructura análoga al estradiol. Principalmente existen 3 formas libres (agluconas) de isoflavonas: daidzeína, genisteína y gliciteína, encontrándose también asociadas a diferentes tipos de azúcares (normalmente glucosa), siendo estas sus derivados glicosilados: daidzina, genistina y glicitina[18].

Estas formas glicosiladas son hidrolizadas en el organismo resultando las formas agluconas, las cuales son fácilmente absorbibles debido a su hidrofobicidad y bajo peso molecular[19-21].

[18] Informe del Comité Científico de la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) en relación con las consecuencias asociadas al consumo de isoflavonas. Número de referencia: AESAN-2007-002 Documento aprobado por el Comité Científico en sesión plenaria de 27 de febrero de 2007 (visitado 11/09/2019) http://solgenisoflavones.com/packages/syscover/cms/storage/attachment/3/es/AESAN.pdf (Visitado 21/10/2019).

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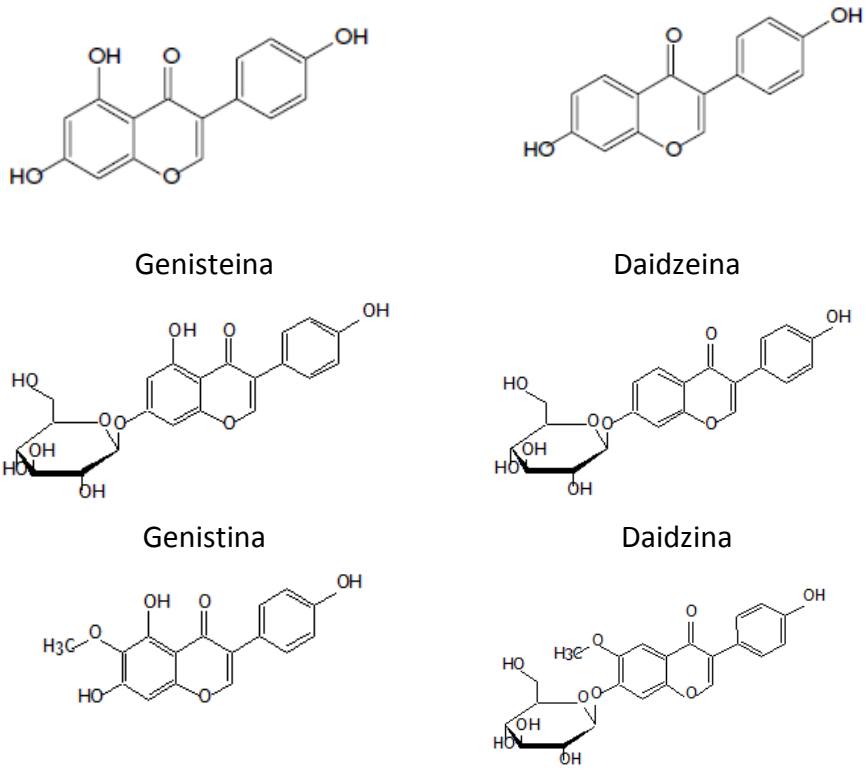

***Figura 2**. Estructura química de las isoflavonas estudiadas.*

Una vez estas sustancias se encuentran en el interior del organismo, entre sus mecanismos de acción destaca su capacidad para unirse a receptores estrogénicos[22]. De esta forma, el complejo resultante de la unión entre isoflavona y receptor, tiene una actividad similar a la ejercida por el complejo 17 β estradiol – receptor.

Otra actividad descrita para este tipo de sustancias es su capacidad antiestrogénica, ya que actúa como agonista o antagonista de estrógenos

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dependiendo de la concentración en que se encuentran: a bajas concentraciones de estrógenos endógenos actuaría como agonista y a altas concentraciones actuaría como antagonista[23]. Otros estudios también muestran que las isoflavonas inhiben la conversión de estrona en estrógeno[18].

En los alimentos, este tipo de compuestos se encuentran principalmente en las legumbres en su forma conjugada como glucósidos[24], destacando la soja como una de las principales fuentes[25]. Los efectos de las isoflavonas sobre la salud han sido ampliamente estudiados, destacando los siguientes aspectos:

- **Menopausia**: normalmente se asocia a un incremento de la concentración de hormona estimulante de los folículos, fallo ovárico y disminución de los niveles de estradiol. Las isoflavonas han sido estudiadas como una alternativa natural a la terapia sustitutiva hormonal, la cual podría estar asociada a un aumento del riesgo de padecer enfermedades cardiovasculares y cáncer de mama. Sin embargo, los resultados obtenidos no son concluyentes[24]. Por otra parte, sí que se evidencian efectos

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beneficiosos para la salud de la mujer, en concreto para la disminución significativa de sofocos[26-28].

- **Enfermedad cardiovascular**: no existen estudios que concluyan que las isoflavonas son responsables de variaciones en las concentraciones de colesterol tanto LDL como HDL, así como que provoquen variaciones en la concentración de triglicéridos o presión arterial[29]. Sin embargo, sí que existen estudios que demuestran que las isoflavonas disminuyen la agregación plaquetaria y la trombogénesis[30].

- **Diabetes y obesidad:** no se ha concluido que el efecto beneficioso descrito en varios estudios gracias al consumo de proteínas de soja sea debido a su contenido en isoflavonas, o a otro tipo de componentes como fitoestrógenos o saponinas que tengan un efecto sinérgico[31-33].

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- **Osteoporosis**: existen estudios que sugieren que la genisteína estimula la acción de la osteoprogerina, responsable de regular el mecanismo de generación de hueso a través de la inhibición de la actividad de los osteoclastos, responsables de la reabsorción, y por tanto favoreciendo la actividad de los osteoblastos, responsables de la generación del hueso.[18]

Tal y como se ha comentado con anterioridad, para promover el consumo isoflavonas, principalmente en regiones donde el consumo de soja no está muy extendido, se han desarrollado una serie de bebidas aromatizadas que sean del gusto del consumidor occidental. Dentro de este tipo de alimentos funcionales se han desarrollado bebidas lácteas a las cuales se les han incorporado extractos de soja con un alto contenido en isoflavonas de soja.

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1.2.2. Objetivo

El objetivo de este trabajo es el establecimiento de metodologías analíticas de buenas características, capaces de ofrecer resultados precisos y exactos con un tratamiento de muestra adaptado para procesar un gran número de muestras diarias.

Los métodos oficiales se aplican principalmente a matrices de extractos vegetales de soja, no hay metodologías adaptadas a la de terminación de este tipo de sustancias en matrices lácteas[34,35]. Además, estas metodologías requieren tiempos de análisis instrumental de hasta 70 min por muestra debido a la similitud que existe entre las diferentes estructuras moleculares de las isoflavonas en estudio.

Por este motivo, se ha desarrollado un método cromatográfico donde se ha logrado reducir el tiempo de análisis, además de adaptar el tratamiento de muestra a volúmenes y cantidades de reactivos que permitan una alta capacidad de procesado de muestras.

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1.2.3. Publicación II

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Improved Sample Treatment and Chromatographic Method for the Determination of Isoflavones in Supplemented Foods

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Abstract

Isoflavones are a group of substances that belong to the family of phytoestrogens. These natural substances may offer several benefits to human health. One of the most important sources for human isoflavone intake is soy and soybean food derivatives. An improved sample treatment followed by a high performance liquid chromatographic method for the determination of isoflavones in supplemented milk and juices is proposed and compared to the AOAC official method. Detection limits found were between 0.2 and 0.3 mg L^{-1} for daidzein and genistein respectively.

Quantification limits found were between 0.7 mg L^{-1} for daidzein to 1.0 mg L^{-1} for genistin, while inter and intra-day variability was under 10 % in all cases. Recoveries for spiked samples were over 90% and under 110 %. The method was validated by comparison with the AOAC method and by recovery assay methodology.

Introduction

Isoflavones are a group of substances that belong to the family of phytoestrogens (Xiao et al., 2009). Many flavonoids are present as flower pigments in most angiosperm families. However, their occurrence is not restricted to the flowers but includes all parts of the plant (Gunatilaka, 2009). From a chemical point of view they comprise a wide group of structurally related compounds with a chromane skeleton provided with a phenyl substituent at the C2 (flavones) or C3 (isoflavones) position (Gikas, Alesta, Economou, Karamanos, & Tsarbopoulos, 2008; Patil, Mahajan, & Katti, 2009).

Isoflavones are usually found in plants as glycosides, i.e. provided with sugar substituents such as galactose, rhamnose or glucose, or glycoside malonates. The malonates are of biological interest because the plant can use this conjugated form to store the less soluble flavonoid aglycons (Crozier, Jaganath, & Clifford, 2009; de Rijke, Zafra-Gomez, Ariese, Brinkman, & Googier, 2001). If necessary, the organisms generate the aglycon form from these precursors by hydrolysis of the stored form (Sumner, Paiva, Dixon, & Geno, 1996).

These natural substances offer important benefits to human health, such as reduction of cardiovascular risk (Rimbach et al., 2008), osteoporosis (Zhang, Chen, Lai, & Wong, 2008) or hormone-dependent

cancers (Messina & Wood, 2008). They have also been shown to stimulate the immune system and to prevent nitration of tyrosine (Oldreive, Zhao, Paganga, Halliwell, & Rice-Evans, 1998). Some other beneficial aspects that have been ascribed to isoflavones include antioxidant activity, metal chelation (Rice-Evans, Miller, & Paganga, 1996) and anticarcinogenic, antiallergic and antiviral effects (Middelton & Kandaswami, 1994, Chapter 15). Ryan-Borchers et al. (2006) have also described that soy isoflavones modulate im- mune function in health postmenopausal women. In recent years, some authors have described the use of this family of compounds (mainly genistein) in the treatment of genetic diseases like mucopolysaccharidosis or cystic fibrosis (Melin et al., 2004; Piotrowska et al., 2006, 2008; Vandebrouck et al., 2006).

The isoflavones genistein, daidzein, and their glycosides, found in high concentrations in soybeans and soy-protein foods, may have beneficial effects in the prevention or treatment of many hormone-dependent diseases. These bioactive phytoestrogens possess a wide range of hormonal and nonhormonal activities (Setchell, Zimmer-Nechemias, Cai, & Heubi, 1997).

Nevertheless, there is a high controversy in the scientific com- munity about the real health effects of these natural compounds since recent human's studies are inconclusive about the decrease of breast cancer risk on isoflavone high intake (Keinan-Boker, Van Der Schouw, Grobbee, & Peeters, 2004).

One of the most important sources for human isoflavone intake is soy and soybean food derivatives. In the United States, about 25% of infant formula sold is based on soy extracts (Cao et al., 2009). Some of these substances are naturally found in soy grains and are also found in soy

extracts (containing daidzin, genistin, daidzein and genistein) that are added to drinks.

In the last years, several methods for analysis of isoflavones have been described for different matrices (Nguyenle, Wang, & Cheung, 1995). HPLC has widely been used as the main analytical technique. The determination of content of the soybean isoflavones in functional food has been proposed (Mantovani, Filho, Santos, Ferreira de Souza, & Watanabe, 2009). Isoflavones have been also studied in meat products (Vanha, Hinkova, Slukova, & Kvasnicka, 2009), soy foods (Chan et al., 2009), fruits and vegetables (Kuhnle et al., 2009; Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003), health foodstuffs (Schwartz & Sontag, 2009) and foods and dietary supplements (Delmonte & Rader, 2006; Setchell, Zimmer-Nechemias, Cai, & Heubi, 1998). Gas chromatography has been also used to quantify isoflavone content in different matrices (Grace et al., 2003; Liggins, Bluck, Coward, & Bingham, 1998; Mazur et al., 1996; Morton, Arisaka, Miyake, & Evans, 1999).

The majority of the proposed methods to quantify isoflavones in food samples described in the literature include tedious, expensive and time-consuming extraction procedures to isolate the analytes from the real samples. These methodologies include different sample treatments such as hydrolysis, microwaves, reflux and soxhlet or sonication steps. In this paper, an improved extraction method based on the simple addition of a precipitation mixture to remove proteins and lipids and a filtration followed by HPLC–DAD has been developed to quantify the isoflavone content (daidzin, daidzein, genistin and genistein) in supplemented cow milk and juices. The method is a powerful tool to improve the throughput and reliability of any quality control laboratory for the determination of the

typical isoflavones found in supplemented food samples. It is a simple, rapid and economical procedure in comparison with those proposed in the literature. The method has been compared with the AOAC Official Method, 2001.10: ''Determination of Isoflavones in Soy and selected Food containing Soy" AOAC, 2001, Chapter 45.4.14.

Experimental

Reagents and standards

All reagents were analytical grade unless specified otherwise. Water was purified with a Milli-Q plus system (Millipore, Bedford, USA). Daidzin, daidzein, genistin, genistein were purchased from Sigma (Barcelona, Spain). HPLC-grade methanol and acetonitrile were supplied by Panreac (Barcelona, Spain). Solvent and aqueous solutions were filtered through 0.2 µm membranes (Millipore, Bedford, MA). Glacial acetic acid (99%) and o-phosphoric acid (85%) were obtained from Panreac (Barcelona, Spain). Zinc acetate hydrated, phosphotungstic acid polyhydrated and sodium hydroxide were obtained from Sigma (Barcelona, Spain). The precipitation solution was prepared once a week by dissolving 9.10 g of zinc acetate, 5.46 g of phosphotungstic acid polyhydrated and 5.8 mL of glacial acetic acid in 100 mL final volume of Milli-Q water. The aqueous mobile phase was prepared by mixing 700 mL of methanol and 300 mL of distilled water and 0.2 mL of o-phosphoric acid were added. A multi-standard solution of isoflavones (1.0 g L^{-1}) was prepared in methanol. Solutions were kept in dark at 20 °C in order to avoid sample degradation. Working standard solutions were prepared by dilution just before use.

Sample preparation

Isoflavone enriched milk and juice were obtained from different supermarkets in the city of Granada (Spain). Samples were stored in the dark at 4 °C until treatment was performed, which occurred within 48 h of sample collection in all cases. The analysis was performed as described under Section 2.3.

Fortified milk or juice samples were prepared by spiking 100 g (accurately weighed) of minced blank adding the adequate volumes of working solutions of studied isoflavones. Before sample treatment and analysis, all samples were allowed to stand in the dark for 60 min at room temperature to permit the total interaction between the compounds and sample. All samples were pre- pared in duplicate. The recovery (parameter considered in order to optimise all the parameters) was evaluated by comparison with spiked amount.

Isoflavones isolation

For solid samples (e.g., powder cow milk), 0.5 g of sample were placed in a glass or plastic tube (50 mL) and 4.5 g of heated water (40 °C) were added. The mixture was homogenised by vortex-stirring for 1 min, sonicated for 5 min and kept for 60 min at room temperature and darkness. For liquid samples, samples were accurately homogenised before weight into flasks.

Newly developed method

Four grams of sample were placed in a 10 mL flask. Then, 2 mL of the precipitation solution (see Section 2.1) and 2 mL of acetonitrile were added into the sample flask and the sample was diluted to 10 mL with Milli-Q

water. The mixture was vortexed for 1 min at room temperature and after 15 min under darkness; samples were centrifuged at 3500 g for 10 min. The supernatant was filtered through 0.22 µm nylon Millipore filter before injection into the HPLC column.

AOAC methodology

Five grams of sample were placed in a 50 mL flask and 40 mL of a mixture methanol/water (1:1, v/v) were added. The mixture was mechanically shaken at 65 °C for 2 h and then 3 mL of sodium hydroxide solution (2 M) were added. The mixture was shaken for 10 min at room temperature and 1 mL of glacial acetic acid was added. The sample was diluted to 50 mL with methanol/water mixture and 1 mL was centrifuged for 5 min at 7000 g. The supernatant was filtered through 0.22 lm nylon Millipore filter before injection into the HPLC column.

Apparatus and software

Instrumentation: A Megafuge 1.0 centrifuge (Heraeus, Hanau, Germany) was used for sample treatment and the chromatographic separation was carried out in a 2695 Alliance chromatograph (Waters, Milford, MA) equipped with a 2996 photodiode array detector, a 20-60^{o}C thermostated column oven and an automatic injector. Millenium software 4.0 was used for data treatment. Statgraphics software package (Statgraphics, 2000) was used for the statistical analysis of data.

Chromatographic conditions: Newly developed method

An Hypersil C18 (100 x 4 mm, 3 µm) from Agilent Technologies (Santa Clara, CA, USA) column was used. The mobile phase consisted in a mixture of methanol/water/o-phosphoric acid (70:30:0.2, v/v/v) as solvent A, and

pure methanol as solvent B. The initial values were 0% of A and 100% B, hold for 0.0 min, then a linear gradient were established in order to reach a 20% A and 80.0% B composition at 20 min, hold for 1 min and returning to initial conditions at 22 min and hold for 3 min to get column conditioning. The flow rate was 0.4 mL min^{-1} during the entire run. The injection volume was 20 µL and the autosampler temperature was set at 5 °C in order to avoid degradation.

AOAC methodology

A Silica C18 based column (200 × 4.6 mm, 5 lm) column is recommended. In our work, a Spherisorb ODS-2 (200x4.6 mm, 5 µm) column from Waters Chromatography (Milford, MA, USA) was used. The mobile phase consisted in a mixture of methanol/ water/acetic acid (10:88:2) as solvent A, and a methanolic solution of acetic acid (2%, v/v) as solvent B. The initial values were 90% of A and 10% B, hold for 0.1 min, then four linear gradients were established in order to reach first, a 40% A and 60.0% B composition at 30 min, a 0% A and 100.0% B composition at 31 min, a 20.0% A and 80.0% B composition at 31.5 min returning to initial conditions at 37 min and hold for 7.5 min to get column conditioning. The flow rate was 1.5 mL/min during the entire run. The injection volume was 20 µL and the autosampler temperature was set at 5 °C in order to avoid degradation.

Results and discussion

Isolation procedure

Sample treatment techniques for isoflavone isolation from food samples published in literature or in official methods are laborious and time-

consuming. Investigation on possible sample treatments of milk-related products turned us to a precipitation solution containing zinc and wolframium salts in an acidic media. This solution is currently used for milk treatment in the analysis of lactulose as described in the method by the International Dairy Federation (FIL-IDF Standard 147B, 1998). We decided to evaluate similar treatment conditions to extract isoflavones by precipitation of proteins and fat in isoflavones-enriched milks. By using chromatographic conditions described below, we were able to verify that all four iso- flavones could be isolated, separated and quantified. Next, the ratio of precipitation solution and sample was optimised for maximum recovery. The volumes were decreased to minimise final residues on the analysis since heavy metals are present in the precipitation solution. The final conditions for sample treatment are simple, fast and can be extended to other products such as juices with milk.

HPLC analysis

A Luna C18 (150 mm × 4.6 mm i.d., 5 µm particle size) from Phenomenex (Torrance, USA), a Spherisorb ODS-2 (250 mm × 4.6 mm, 5 lm), an Atlantis dC18 (150 mm × 4.6 mm, 5 µm), a XTerra (150 mm × 2.1 mm, 5 µm) from Waters (Milford, MA, USA) and a Hypersil C18 (100 x 4 mm, 3 µm) from Agilent Technologies (Santa Clara, CA, USA) liquid chromatographic columns were tested. Hypersil C18 provides the best results for these compounds. In addition, this column showed the best resolution and the shortest chromatographic times. Therefore, the column was selected as the most appropriate for our purposes.

Different mobile phases were studied in order to optimise the separation and peak shapes (mainly referring to critical pair daidzin and an interference peak). With this objective in mind, two organic solvents

(methanol and acetonitrile) commonly used in reversed-phase liquid chromatography (RP-LC) were evaluated. Methanol gave better results than acetonitrile. Moreover, acidification of the LC eluent was necessary to allow the formation of protonated species. Formic acid, acetic acid, trifluoroacetic acid and o-phosphoric acid were tested as additives. The best separation of compounds was obtained using a mobile phase composed of 0.2% (v/v) o-phosphoric acid aqueous solution modified with 70% of methanol (v/v) (solvent A) and methanol (solvent B). A linear gradient, described previously, was used to obtain a good resolution for all compounds.

Lastly, in order to improve the detection limits of the method, a study was performed to evaluate the possibility of increasing the injection volume. A range from 10 to 50 µL was studied and an extra broadening of the peaks was observed at volumes higher than 20. Accordingly, 20 µL was chosen as the injection volume.

Scan analysis of standard isoflavones was performed to check the optimum conditions for detection. Wavelength was selected in order to obtain adequate linear range to measure. The optimal wavelength for UV–vis detection was set at 265 nm. The chromatograms of isoflavones in a mixture of standards and in a supplemented milk sample are shown in Fig. 1.

Analytical performance

Calibration graphs for samples treated according to the analytical procedure described above were prepared. Linearity of the calibration graphs was tested according to the Analytical Methods Committee (Analytical Methods Committee, 1994); the lack-of-fit test was applied to

two replicates and two injections of each standard. The results for the intercept (a), slope (b), correlation coefficient (R^2) and probability level of the lack-of-fit test, Plof (%), are summarised in Table 1.

The analytical performance parameters assessed for the overall assay were linearity, precision, accuracy, sensitivity, and selectivity (SANCO/1805/2000 Rev. 1, 2000).

Linearity

The response of compounds was checked in the range of application of the analytical method by linear regression analysis by the least-squares method of peak area ratio of analyte against different analyte concentrations. The responses were linear in the range of concentrations evaluated for all compounds.

Accuracy

The accuracy was determined for the overall assay by measuring the percentage of recovery after the addition of known amounts of standard in samples. The recovery studies were carried out (five replicates) by spiking 100 mL of samples with the analytes, at three concentration levels. The recoveries of the tested compounds were between 90% and 110% in all cases as shown in Table 2. These recoveries were very good considering the amount of sample and the low concentration of analytes. The values indicate that compounds are quantitatively extracted using this method.

Precision

The precision expressed as relative standard deviation (RSD), was obtained from the repeated analysis, five times, of a 10 mg L^{-1} spiked

sample during the same day (repeatability) and in five different days (reproducibility). Repeatability, as relative standard deviation, is lower than 10% in all cases as is shown in Table 1. Data indicate that the analytical method is repeatable and reproducible.

Sensitivity

Limits of detection and quantitation were calculated in order to determine if analytes are present in real samples. The results obtained are shown in Table 1.

Selectivity

Analytes appear to be well resolved and free from interference peaks (Fig. 1). The identity of the chromatographic peak was confirmed by its retention time.

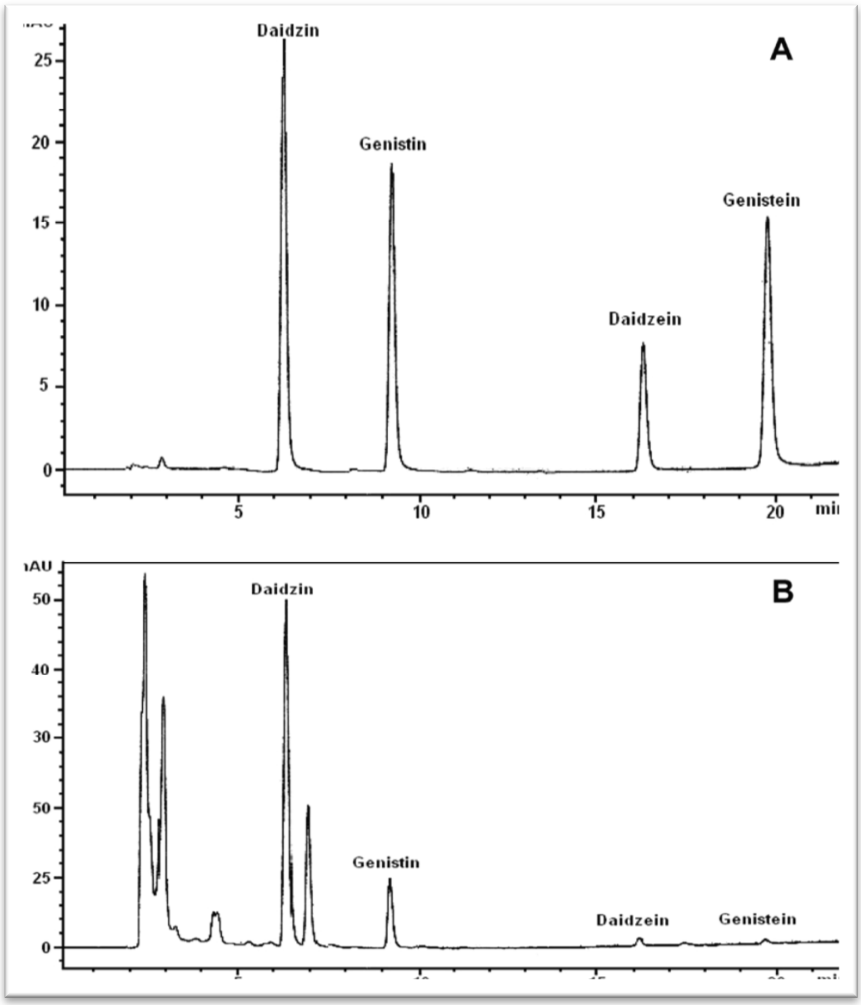

Fig. 1. LC Chromatogram obtained with the newly proposed methodology of: (A) isoflavones in a standard mixture (10 mg L^{-1}) and (B) isoflavones in a supplemented real milk sample.

Table 1. *Analytical parameters of the newly developed method.*

	Determined value			
Parameter[a]	Daidzin	Genistin	Daidzein	Genistein
Lineal dynamic range (mg L^{-1})	0.9–50.0	1.0–50.0	0.7–50.0	0.9–50.0
b (L mg^{-1})	59.7	84.1	34.9	35.2
Sb (L mg^{-1})	0.41	0.47	0.36	0.60
a (mAU)	- 1.62	- 1.29	1.19	4.32
S_a (mAU)	5.31	4.84	0.20	0.26
R^2 (%)	98.2	99.0	98.4	97.8
LOD (mg L^{-1}, a = 1%; b = 5%)	0.3	0.3	0.2	0.3

[a] *b, slope; S_b, slope standard deviation; a, intercept; S_a, intercept standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ: limit of quantification; RSD: relative standard deviation, calculated for 25 aliquots (five replicates/ day for five days); P_{lof}, P-value for lack-of-fit test.*

Validation and application of the method

Validation was performed according to the USA Food and Drugs Administration (FDA) guideline for bioanalytical assay validation (US Department of Health and Human Services, 2001). A recovery assay was performed by comparing the analytical results for extracted samples, free of analytes, spiked at three concentration levels for the four compounds studied. The concentration of each compound was determined by interpolation from the standard calibration curve within the linear dynamic range and compared with the added amount. A total absence of matrix interference was con- firmed through analysis of two different batches of blank extract. The results are shown in Table 2.

As well, the proposed analytical methodology was compared with the official method of the AOAC (Table 2). A statistical test was carried out in order to compare obtained values with both methodologies. The test includes a variance comparison (Snedecor F-test) and means comparison based in t-student test. In all cases, it can be concluded by H0; therefore, there were no significant differences between the results obtained by the proposed method and the AOAC method (test performed at significance level of $a < 0.05$). These data also confirm the efficiency of the sample treatment procedure for the recovery of isoflavones from the samples.

The proposed methodology improves the AOAC official method- ology. Firstly, the complexity of analyte isolation used in the official method has been removed. On the other hand, the time for complete analysis is much lower in the new methodology (about 30 min) in comparison with more than 2 h. It is also highly remarkable the use of low amounts of reagents and solvents for isoflavone isolation. All those point are important in order to minimise the cost of the analysis.

Table 2 Comparison of the newly developed and the AOAC methods in the recovery assay for isoflavones in spiked milk (mean of five determinations ± standard deviation).

Isoflavone	Amount added (mg L^{-1})	Recovery		[a] t^{cal}	[a] t_{tab}	Conclusion[a]
		AOAC method	New method			
Daidzin	1.0	0.97 ± 0.12	0.99 ± 0.10	2.082	2.306	H_0
Genistin	1.0	1.05 ± 0.12	1.07 ± 0.11	1.927	2.306	H_0
Daidzein	1.0	1.09 ± 0.08	1.08 ± 0.07	2.258	2.306	H_0
Genistein	1.0	0.96 ± 0.09	0.95 ± 0.08	1.752	2.306	H_0
Daidzin	10.0	9.5 ± 0.4	9.8 ± 0.5	1.868	2.306	H_0
Genistin	10.0	9.8 ± 0.5	10.1 ± 0.4	1.868	2.306	H_0
Daidzein	10.0	9.6 ± 0.3	9.7 ± 0.4	1.026	2.306	H_0
Genistein	10.0	9.8 ± 0.8	10.5 ± 0.6	1.788	2.306	H_0
Daidzin	25.0	24.1 ± 0.5	24.2 ± 0.6	0.426	2.306	H_0
Genistin	25.0	23.9 ± 0.6	24.3 ± 0.7	1.205	2.306	H_0
Daidzein	25.0	25.4 ± 0.5	26.2 ± 0.8	2.283	2.306	H_0
Genistein	25.0	26.0 ± 0.4	25.8 ± 0.5	1.249	2.306	H_0

[a] t_{cal}, calculated value for statistical t-student test; t_{tab}, tabulated value for a significance level of 0.05 and $n_1 + n_2 — 2$ freedom degrees; H_0: conclusion for the test if there are no significant differences between the results obtained by the proposed methods at significance level of a <0.05.

Table 3. *Application of the proposed methodology to supplemented milk and juices.*

	Concentration (mg L^{-1})[a]			
Product	Daidzin	Daidzein	Genistin	Genistein
Orange juice with added soy	3.4	0.9	7.6	1.0
Peach juice with added soy	18.2	1.0	28.4	1.0
Peach juice with added soy	5.8	1.0	15.7	2.9
Orange-mango juice with added soy	5.1	0.0	9.9	0.9
Pine-apple juice with added soy	16.7	0.7	23.9	0.9
Flavoured soy drink	44.1	2.1	99.5	3.6
Soy drink, supplemented milk	73.4	2.6	102.0	3.1
Soy drink, supplemented milk	120.0	13.2	126.0	1.9
Soy drink, supplemented milk	83.6	1.2	96.5	1.5
Soy drink, supplemented milk	93.0	1.4	91.8	1.4

[a] *Mean of five determinations*

Finally, liquid chromatographic analysis has also been improved. Chromatographic time (25 min) is clearly shorter than in the official method (44.5 min) and the flow of the mobile phase is about three times lower 0.4 mL/min (1.5 mL in AOAC method). These two points reports a high benefit in cost terms of time and money.

In order to carry out the application of the proposed methodology, the isoflavones daidzin, genistin, daidzein and genistein were quantified in different food samples. Several commercial products, including isoflavone enriched-milk products and juices, were analysed. The chromatographic peaks in the samples analysed were identified by comparing UV-visible

spectra and retention data obtained for the standards and spiked samples under optimised method conditions. Figure 1 shows an example of UV-vis chromatogram obtained for a commercial enriched-milk sample (A) and a standard mixture (B). The results obtained are summarised in Table 3. It can be observed that the selected samples contain different amounts of daidzin and genistin, lower amounts of daidzein and genistein, and also different total amount of isoflavones in each sample.

Conclusions

The proposed method is a powerful tool to improve the throughput and reliability of any quality control milk laboratory for the determination of the typical isoflavones found in supplemented food samples. It is a simple, rapid and economical procedure. The simplicity of the new method makes it a possible alternative to measure isoflavones in food matrices. This method has been compared with the AOAC, 2001.10: *''Determination of isoflavones in Soy and selected Food containing Soy"* and no significant differences in results have been found.

The method has been successfully applied to spiked and unspiked milk and juices with milk samples from different supermarkets of the city of Granada, in the south of Spain, with different composition, consequently. Proper sample collection in conjunction with sound conservation practices prior to analysis allows for good recovery values in all cases as demonstrated by the validation procedure employed.

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Capítulo 2. Seguridad Alimentaria Marco Legal

En el marco de la Comunidad Europea, se deben establecer normas que armonicen los criterios de los diferentes miembros. Dentro de estos criterios, existen algunos referentes a la salud de la población que requieren una especial atención por la gran sensibilidad que despierta e importancia que genera.

Según el Codex Alimentarius, una sustancia contaminante se define como *"Cualquier sustancia no añadida intencionalmente al alimento, que está presente en dicho alimento como resultado de la producción (incluidas las operaciones realizadas en agricultura, zootecnia y medicina veterinaria), fabricación, elaboración, preparación, tratamiento, envasado, empaquetado, transporte o almacenamiento de dicho alimento o como resultado de contaminación ambiental. Este término no abarca fragmentos de insectos, pelo de roedores y otras materias extrañas"*.[36]

Como medida para la protección de la salud de la población en general, se redactó el Reglamento 466/2001, en donde se fija el contenido máximo de ciertas sustancias contaminantes. Sin embargo, este documento ha sido modificado de manera sustancial en numerosas ocasiones. Este hecho, unido a la evolución del Codex Alimentarius y a la nueva información que se está continuamente estudiando, es la razón por la cual se ha sustituido por un nuevo documento: *Reglamento 1881/2006 DE LA COMISIÓN de 19 de diciembre de 2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios*.

[36] Codex Alimentarius. Norma General para Los Contaminantes y Las Toxinas Presentes en los Alimentos y Piensos. CXS 193-1995 Adoptada en 1995, Revisada en 1997, 2006, 2008, 2009 Enmendada en 2018.

En dicho documento se recogen los diferentes niveles máximos permitidos de diversas familias de sustancias, teniendo en cuenta su toxicidad al mismo tiempo que se deben tener en cuenta los niveles que razonablemente pueden ser obtenidos si se aplican buenas prácticas pesqueras, agrícolas y de producción. Así mismo, se tiene en cuenta la exposición probable de una cierta sustancia contaminante a los diferentes grupos de población.

Para el caso de sustancias potencialmente cancerígenas genotóxicas, o en aquellos casos donde la exposición a grupos de población vulnerables se aproxime a la ingesta tolerable o se supere, deben establecerse contenidos máximos tan bajos como sea razonablemente posible ("as low as reasonably achievable, ALARA"). De esta forma se emplaza a la industria productora a que apliquen medidas para minimizar y prevenir la contaminación para así proteger la salud pública.

En este documento se establece además que aquellos alimentos que contengan una concentración de sustancias contaminantes superior al límite, no pueden ser comercializados como tales, ni diluidos con otros componentes ni como ingredientes en otros alimentos. De ahí la importancia del control analítico en matrices tan complejas como las alimentarias.

Existen ciertas excepciones derivadas de circunstancias específicas propias de ciertos productos o regiones, como ejemplo de ello, se permite que se supere el límite para dioxinas y PCBs similares a dioxinas en pescado de la región del Báltico debido a que una exclusión de este tipo de alimento

en la dieta, tendría una repercusión negativa en la salud de la población de la zona. Sin embargo, se debe controlar que aquel pescado con un contenido superior en este tipo de sustancias, no debe ser comercializado fuera de los países de esta región: Suecia y Finlandia.

Precisamente para asegurar el libre comercio y evitar desigualdades entre países miembros, el Reglamento 178/2002 estableció normas para la libre circulación de alimentos seguros y saludables, armonizando los requisitos para esta libre circulación siempre que se cumpla con los elevados niveles de protección para la salud. Así mismo, establece una Autoridad Europea de Seguridad Alimentaria, encargada de "facilitar el *asesoramiento científico y apoyo científico y técnico de cara a la labor legislativa y política de la Comunidad en todos aquellos ámbitos que, directa o indirectamente, influyen en la seguridad de los alimentos y los piensos. La Autoridad facilitará información independiente acerca de todos los temas comprendidos en estos ámbitos e informarán sobre riesgos"*.[37]

Del mismo modo, se crea un sistema de alertas rápidas (RASFF – Rapid Alert System for Food and Feed) el cual es una pieza clave que asegura el flujo de información que permite una rápida reacción cuando se detecta un riesgo para la salud pública a través de la cadena alimentaria.

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[37] Reglamento (CE) No 178/2002 del Parlamento Europeo y del Consejo de 28 de enero de 2002 por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria. Diario Oficial de las Comunidades Europeas 1-24.

Por tanto, para el control de la presencia de sustancias contaminantes en los alimentos, se requiere del desarrollo de metodologías de buenas características analíticas que adicionalmente ofrezcan una gran agilidad a la hora de procesar muestras en laboratorios de rutina.

Idealmente, las características que deben tener este tipo de metodologías son principalmente: bajos límites de detección y cuantificación, minimizar el riesgo de falsos positivos o negativos, robustez y bajo riesgo de efecto matriz y finalmente que requieran de tratamientos de muestra rápidos que permitan una alta capacidad de procesamiento de muestras.

Para alcanzar estos requerimientos, normalmente se requieren técnicas instrumentales potentes, tales como la cromatografía tanto de líquidos como de gases acoplada a espectrometría de masas, con alta sensibilidad y selectividad, capaces de detectar y cuantificar sustancias en presencia de un gran número de posibles sustancias interferentes.

En los laboratorios de Control de Calidad se tiende, siempre que sea posible, a emplear tratamientos de muestra rápidos, tales como extracciones líquido-líquido con volúmenes reducidos o precipitaciones de proteínas, en donde se trata de evitar el empleo de cartuchos de extracción en fase sólida por su coste y tiempo requerido para un adecuado acondicionamiento, retención de analitos de interés y posterior lavado de interferentes. Sin embargo, este tipo de tratamientos de muestra rápidos tienen normalmente la contrapartida de que son co-extraídos un elevado número de compuestos de naturaleza parecida a los analitos de interés.

Este hecho hace que las técnicas instrumentales que se emplean en este tipo de metodologías, deban tener una elevada selectividad capaz de minimizar el efecto matriz que puedan provocar estas sustancias co-extraídas. La cromatografía acoplada a detectores de espectrometría de masas ofrece este tipo de características, sin embargo, se necesita un bajo límite de cuantificación, lo que hace que se requiera una puesta a punto de los diferentes parámetros instrumentales muy optimizada para conseguir métodos robustos, sensibles y precisos.

En el caso particular de este trabajo de Tesis, se han desarrollado metodologías para determinación de antibióticos de la familia de las quinolonas en matrices lácteas, cloropropanoles en aceites, lactosa residual en productos lácteos hidrolizados sin lactosa e hidrocarburos policíclicos aromáticos (PAHs) en extractos vegetales.

Se han empleado técnicas cromatográficas tanto de líquidos como de gases, acopladas a detectores de espetrometría de masas. Como se ha comentado anteriormente, este tipo de técnicas instrumentales permiten una adecuada determinación de los compuestos de interés, a los niveles de concentración exigidos por la legislación europea, con tratamientos de muestra relativamente simples que, además de los compuestos de interés, son capaces de extraer un gran número de compuestos que pueden interferir en la calidad de la medición, pero que con una adecuada optimización de los parámetros instrumentales que afectan a la separación y detección, permiten la determinación resultando en metodologías con buenas características analíticas.

Para el caso de los métodos desarrollados en este trabajo, las técnicas disponibles para el análisis rápido de quinolonas en leche se basan principalmente en kits de reacción rápida, donde a través de tiras reactivas y mediante incubación y posterior lectura de absorbancia, se pueden obtener lecturas cualitativas o semicuantitativas de la concentración presente de este tipo de compuestos[38]. Por otra parte, otra familia de métodos son aquellos donde es necesario el empleo de un tratamiento de muestra previo usando principalmente extracción en fase sólida[39], microextracción líquido-líquido dispersiva[40], QuECheRS[41], etc., seguido de determinación por cromatografía de líquidos acoplada a espectrometría de masas o fluorescencia.

Para la determinación del 3-MCPD, 2-MCPD y glicidilésteres, la técnica más habitualmente empleada, en la que se basan los métodos de referencia que se están desarrollando, es la cromatografía de gases con

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[38] Charm Sciences, Inc. Charm, Charm Quinolone Test (QUIN). Visitado 26/10/2019 (https://www.charm.com/products/test-and-kits/antibiotic-tests/rosa-lateral-flow/quin-charm-quinolone-test/)

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[41] Amelin, V.G.; Volkova, N.M.; Timofeev, A.A.; Tret'yakov, A.V. QuEChERS sample preparation in the simultaneous determination of residual amounts of quinolones, sulfanilamides, and amphenicols in food using HPLC with a diode-array detector. J. Anal. Chem. 70 (2015) 1076-1084.

detección por espectrometría de masas. Se trata de medidas indirectas, en las que se lleva a cabo una hidrólisis previa de los derivados ésteres y la posterior determinación singular de 3-MCPD, 2-MCPD y glicidol.

Por último, en relación a la lactosa residual, existen en el mercado diferentes tipos de kits de determinación rápidos basados en reacciones específicas de ciertas enzimas con los monosacáridos resultantes de la hidrólisis de la lactosa, así como métodos cromatográficos con detección electroquímica, que sin embargo, presentan una serie de inconvenientes que en el trabajo realizado en la presente Tesis Doctoral, se han tratado de eliminar.

2.1. Quinolonas en Lácteos

2.1.1. Introducción

Las quinolonas son un grupo de sustancias antibacterianas de amplio espectro para el tratamiento contra bacterias tanto gram-positivas como gram-negativas. Se emplean en el tratamiento tanto humano como veterinario de afecciones respiratorias, urinarias y del tracto gastrointestinal, así como de infecciones cutáneas, óseas y articulares desde hace más de 40 años.

Están disponibles en la Unión Europea desde 1962 y han sido empleadas para tratar millones de pacientes con infecciones bacterianas graves o potencialmente mortales y cuando se deben tratar infecciones resistentes a otro tipo de medicamentos[42].

En general, el empleo tan extendido de este tipo de sustancias hace que la Organización Mundial de la Salud alerte del riesgo de desarrollo de resistencias a este tipo de sustancias por parte de bacterias que impidan el adecuado tratamiento y consiguiente riesgo para la salud al no poder emplearse este tipo de tratamientos por su ineficacia[43].

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[42] Agencia Europea de Medicamentos, Resumen de la audiencia pública de la EMA sobre antibióticos quinolonas y fluoroquinolonas, celebrada el 13 de junio de 2018. 20 de junio de 2018 (2018) 1-7.

[43] Resistencia a los antibióticos. Disponible en: https://www.who.int/es/news-room/fact-sheets/detail/resistencia-a-los-antibi%C3%B3ticos (Visitada 26/10/2019)

Esta resistencia se debe tanto al abuso en el consumo de este tipo de sustancias por parte de la población en general, como por su presencia en alimentos debido a su empleo en tratamientos veterinarios. En este último caso, los antimicrobianos se emplean para tratar infecciones bacterianas tanto en animales de compañía como en productores de alimentos. En el caso de ganado y aves de corral, los antibióticos son fundamentales para asegurar un suministro seguro de alimentos a través de animales más sanos. En todos los casos, el veterinario que prescribe y supervisa debe seleccionar y administrar el tratamiento más eficaz que se considere apropiado.

Actualmente los medicamentos disponibles tienen deficiencias en el tratamiento de algunas enfermedades bacterianas importantes que afectan al ganado y aves de corral tales como infecciones respiratorias y sistémicas (causadas por ejemplo por *Pasteurella, E. coli, Actinobacillus, Mycoplasma*)[44]. Las quinolonas representan un avance significativo en este sentido, aliviando de este modo el sufrimiento animal, al mismo tiempo que se reduce la duración de la infección y se previene la muerte del animal, con el consiguiente beneficio económico para los productores.

Para el caso particular de las fluoroquinolonas, estudios recientes advierten de que la tasa de resistencia es del 59% para *Moraxella*

[44] Guidelines for the use of quinolones in veterinary medicine. Disponible en: https://www.bayer-chikusan.jp/products/pdf/baito_e_guidelines.pdf (Visitada 26/10/2019)

catarrhalis[45], la cual es una de las bacterias patógenas del tracto respiratorio más frecuentes[46].

Adicionalmente, se han publicado importantes efectos secundarios que están siendo estudiados en la actualidad. Según se recoge en la audiencia pública de la Agencia Europea del Medicamento (EMA) celebrada en junio de 2018, y donde participaron representantes de pacientes sometidos a tratamiento con este tipo de sustancias, empresas fabricantes, académicos y profesionales de la salud, el Comité Consultivo para la Determinación de Riesgos en Farmacovigilancia (PRAC) de la EMA inició una investigación a raíz de los informes recibidos donde se alertaban de efectos graves y duraderos tras el tratamiento con este tipo de sustancias que afectan principalmente a tendones, músculos, articulaciones y sistema nervioso. Estos informes recogen discapacidades que afectan al normal desarrollo de la vida cotidiana ya que provocan dolor a largo plazo. Aunque los efectos adversos ya eran conocidos por los facultativos, en esta audiencia se trató de abordar y discutir el hecho de que estos efectos perduraran y tengan un carácter duradero.

Los pacientes describieron cómo a raíz de su tratamiento con este tipo de sustancias, comenzaron a sufrir dolor y discapacidades durante varios

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[45] Shaikh, S.B., Ahmed, Z., Arsalan, S.A., Shafiq, S. Prevalence and resistance pattern of Moraxella catarrhalis in community-acquired lower respiratory tract infections. Infect. Drug Resist. 8 (2015) 263-267.

[46] Hoban, D., Doern, G., Fluit, A., Roussel-Delvallez, M., Jones, R. Worldwide prevalence of antimicrobial resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae* and Moraxella catarrhalis in the SENTRY Antimicrobial Surveillance Program. 1997-1999. Clin. Infect. Dis. 32 (2001) 81-93.

años, y algunos tan sólo experimentaron una ligera mejoría con el paso del tiempo. Estos efectos han provocado que en muchos casos no se puedan realizar actividades cotidianas como atarse los zapatos o abotonarse una camisa.

En esta línea, el PRAC de la EMA ha emitido recientemente un comunicado donde recomienda la restricción en el uso de este tipo de medicamentos administrados por vía oral, intramuscular o por inhalación[47]. Como consecuencia de los efectos secundarios de larga duración aparecidos en raras ocasiones, en pacientes sometidos a tratamientos con este tipo de sustancias, se ha recomendado su eliminación del mercado ya que fueron autorizadas solamente para el tratamiento de infecciones que ya no deberían ser tratadas con este tipo de medicamentos. En este sentido, **no** se recomienda su uso en el tratamiento de infecciones no graves, para prevenir diarreas e infecciones urinarias que no se extienden más allá de la vejiga y en pacientes donde previamente se hayan detectados episodios de este tipo de efectos secundarios.

Tampoco se recomienda su uso en tratamientos de infecciones más severas salvo para aquellos casos donde se haya comprobado que otros tipos de antibióticos no han sido eficaces.

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[47] Fluoroquinolone and quinolone antibiotics: PRAC recommends restrictions on use. New restrictions follow review of disabling and potentially long-lasting side effects. 5 October 2018 EMA/668915/2018. Disponible en: https://www.ema.europa.eu/en/news/fluoroquinolone-quinolone-antibiotics-prac-recommends-new-restrictions-use-following-review (Visitada: 26/10/2019)

Por otra parte, se recomienda su uso aunque extremando la precaución en pacientes con afecciones hepáticas, edad avanzada, pacientes que hayan sufrido trasplante o aquellos que hayan sido tratados con corticoesteroides sistémicos, ya que este tipo de pacientes presentan un alto riesgo de sufrir daños en tendones causados por quinolonas.

El PRAC recomienda así mismo que los profesionales de la salud adviertan a sus pacientes de que interrumpan el tratamiento en caso de que aparezca algún síntoma relacionado con músculos, tendones o huesos, o del sistema nervioso.

Desde el punto de vista analítico, para controlar y detectar la presencia de este tipo de sustancias en alimentos, existen en el mercado numerosos kits de determinación, basados en la formación de complejos antígeno – anticuerpo[48-52].

Este tipo de determinaciones tiene la ventaja de que pueden ser procesadas un gran número de muestras en una misma tanda con una alta

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[48] Charm Scientific Charm Quinolone Test for Milk. 2017. Disponible en: https://www.charm.com/wp-content/uploads/2018/06/MRK-665.pdf (Visitada: 26/10/2019)

[49] Life Technologies (India) Pvt. Ltd. ATZ Labs. Quinolone rapid test strip (Tissue) Disponible en: https://www.atzlabs.com/pdf/Quinolone-rapid-test-strip.pdf (Visitada: 26/10/2019)

[50] Creative Diagnostics. Quinolones Residue Rapid Test Strip (Milk) Disponible en: https://www.creative-diagnostics.com/Quinolones-Residue-Rapid-Test-4066-452.htm (Visitada: 26/10/2019)

[51] Unisensor. QuinoSensor - KIT038. Disponible en: https://unisensor.be/products/KIT038 (Visitada: 26/10/2019)

[52] Neogen. Veratox® for Fluoroquinolone. Disponible en: https://foodsafety.neogen.com/en/veratox-fluoroquinolone (Visitada: 26/10/2019)

sensibilidad, sin embargo, la respuesta suele ser o semicuantitativa (visualmente por comparación de intensidad del color desarrollado de una banda - en el caso de tiras reactivas) o cuantitativa no lineal mediante medida de absorbancia (UV-visible). Esta respuesta no se ajustaría a un modelo lineal, sino que deben emplearse modelos matemáticos alternativos (semilogarítmicos, cubic spline, etc.) que dificultan el cálculo de la incertidumbre asociada al resultado, así como y la interpretación de los resultados.

Adicionalmente, la respuesta de estos sistemas de determinación no es específica a un compuesto concreto, sino que normalmente los anticuerpos reaccionan indistintamente a cualquier sustancia de la misma familia con respuestas similares. Este aspecto hace que no pueda ser determinado el compuesto concreto responsable de la contaminación de una muestra dada. Debido a este tipo de dificultades, se hace necesario el desarrollo de metodologías analíticas con mejores características analíticas.

2.1.2. Objetivos

Las quinolonas se emplean ampliamente en tratamientos veterinarios tanto desde el punto de vista terapéutico como preventivo. Tras el tratamiento, se deben aplicar los períodos de seguridad establecidos por el especialista antes de proceder a la explotación del animal. En este sentido, cabe el riesgo de que estos periodos de seguridad no sean aplicados correctamente, con el consiguiente riesgo de que este tipo de sustancias pasen a la cadena trófica.

Como se ha comentado previamente, la ingesta incontrolada de este tipo de sustancias puede provocar resistencias de los agentes patógenos frente a la acción de los antibióticos, con el consiguiente riesgo para la salud pública.

Por ello, se hace necesario el desarrollo de metodologías analíticas de buenas características capaces al mismo tiempo de procesar un gran número de muestras con costes contenidos. Así, se puede emplear esta metodología en la industria tanto en la fase de recepción de producto bruto como en producto terminado, pudiéndose así aumentar el número de controles disponibles, y mejorando por tanto la probabilidad de detectar posibles muestras contaminadas. Se aumenta así el control sobre los alimentos susceptibles de ser consumidos por la población en general y se contribuye a la mejora de la salud pública.

La metodología desarrollada es aplicada en leche. Se trata de una matriz con un gran número de posibles interferentes por su alto contenido

en grasas, proteínas, azúcares, sales minerales y micronutrientes tales como vitaminas que pueden afectar a la adecuada cuantificación.

En este trabajo se ha conseguido desarrollar y validar un método analítico que al mismo tiempo es capaz de contener los costes económicos y procesar un alto número de muestras por serie (hasta 96). Esto ha sido posible gracias al empleo de la cromatografía de líquidos acoplada a la espectrometría de masas como sistema de detección. El empleo de esta técnica ha permitido la detección y cuantificación de los compuestos a muy bajas concentraciones, característica necesaria para poder alcanzar los límites legales establecidos para este tipo de sustancias, recogidos en el Reglamento 37/2010 de 22 de diciembre de 2009 por el que se establecen los límites máximos de residuos de sustancias farmacológicamente activas en productos alimenticios de origen animal[53].

[53] Diario Oficial de la Unión Europea, Reglamento (UE) N o 37/2010 de la Comisión de 22 de diciembre de 2009 relativo a las sustancias farmacológicamente activas y su clasificación por lo que se refiere a los límites máximos de residuos en los productos alimenticios de origen animal, 1-72.

2.1.3. Publicación III

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Simultaneous Determination of Quinolone Antibacterials in Bovine Milk by Liquid Chromatography-Mass spectrometry

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ABSTRACT

A new liquid chromatography–mass spectrometry (LC–MS) method has been developed and validated for the simultaneous determination of eight quinolone antibacterials for veterinary use in processed bovine milk samples. The quinolones studied included marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid and flumequine. Also, a new sample-treatment procedure was used for extraction and preconcentration of these compounds. It involved defatting by centrifugation, protein precipitation by adding a mixture of glacial acetic

acid–acetonitrile and removing acetonitrile with dichloromethane; finally, the acidified aqueous layer was evaporated to dryness in a speed vac system, resuspended in the mobile phase and filtered prior to LC injection. The mobile phase was composed of a formic acid aqueous solution 0.1% (v/v) and acetonitrile, with an initial composition of water–acetonitrile 95: 5 (v/v) and using linear gradient elution. Norfloxacin was used as internal standard. The limits of quantification found (2–7 ng g□1) were in all cases lower than the maximum residue limits tolerated by the European Union for these compounds in milk.

KEYWORDS: quinolones; processed milk; liquid chromatography–mass spectrometry (LC–MS)

INTRODUCTION

Quinolones are a group of structurally related antibiotics that are widely used in human and veterinary medicine for the treatment and prevention of pulmonary, urinary and digestive infections (Andriole, 1998; Kuhlmann et al., 1997; Kaartinen et al., 1995; Delépine and Hurtaud-Pessel, 2000). They were introduced for human use in Europe and the USA in the mid-1980s and approved for livestock treatment in the mid-1990s (Anderson et al., 2001). The occurrence of pharmaceuticals—particularly antibiotics—in the environment and in food has generated increasing attention (Tuerk et al., 2006; Kummerer, 2004). Long-term exposure to antimicrobial agents has been associated with an increased risk of the development and spread of antibiotic resistance (Neu, 1992). The 1998 World Health Report of the World Health Organization (WHO) de- scribed

the increasing occurrence of resistant bacteria and their rapid spread in the world population as one of the biggest health problems of the twenty-first century (World Health Organization, 1998 a,b).

In order to ensure the safety of human foodstuffs, the European Union (EU) has set tolerance levels for quinolones in products of animal origin (EU Com- mission, 1996). Thus, the establishment of sensitive methods for the analysis of residual amounts of these drugs is required for the quality control of food products for consumers and to evaluate the correct application of withdrawal times.

Many studies have been published in the literature on multiresidue analysis of quinolones in biological samples and animal tissues (Carlucci, 1998; Hernández-Arteseros et al., 2002; Samanidou et al., 2005). Most of them involve liquid chromatography with ultraviolet (LC–UV; Gigosos et al., 2000; Pecorelli et al., 2003; Bailac et al., 2004; Christodoulou and Samanidou, 2007), fluorescence (LC–FD; Eng et al., 1998; Hernández-Arteseros et al., 2000; Chu et al., 2002; Espinosa- Mansilla et al., 2005; Hassouan et al., 2007) or mass spectrometric detection (Van Vyncht et al., 2002; Toussaint et al., 2002, 2005a,b; Schneider et al., 2005; Van Hoof et al., 2005; Hermo et al., 2006; Bailac et al., 2006; Rubies et al., 2007).

Only a few methods have focused, however, on the determination of quinolone residues in milk. The method proposed by Roybal required a complex milk extraction procedure and periodic column regeneration due to the reaction of the milk proteins with the stationary phase (Roybal et al., 1997). Holtzapple used on-line immunoaffinity extraction sample clean-up

and preconcentration, and reported contamination of the analytical column after 15–20 milk sample injections (Holtzapple et al., 1999). Cinquina determined enrofloxacin and its metabolite, ciprofloxacin, in goat milk by LC–UV, combined with LC–MS for confirmation; the limit of quantification found for both quinolones was 20 ng mL^{-1} (Cinquina et al., 2003). Marazuela developed a LC method with fluorescence and UV-diode array detection for the determination of five fluoroquinolones (Marazuela and Moreno-Bondi, 2004) and Van Hoof proposed a LC–MS method for the analysis of eight quinolones in bovine milk (Van Hoof et al., 2005); both methods shown good recoveries for all compounds but required a solid-phase extraction (SPE) step for clean-up and preconcentration of the analytes. Volmer studied 4-quinolone antibiotics in biological samples (Volmer et al., 1997).

An LC–FD method reported in the bibliography permitted the rapid determination of enrofloxacin and ciprofloxacin in bovine milk using a simple pretreatment of the samples; the limit of quantification was 2 ng mL^{-1} for both compounds (Idowu and Peggins, 2004).

Verdon developed a very good LC–FD method for the determination of 10 quinolone residues in multimatrix/multispecies animal tissues including bovine raw milk (Verdon et al., 2005). It involved the extraction of residues from biological tissues and fluids by acidic aqueous solution, centrifugation and filtration prior to injection into a C18 narrow-bore column, and detection through a three-step-mode fluorescence detector. The limits of detection found ranged from 4 to 11 µg kg^{-1} but they did not achieve very high recoveries (between 64 and 89%).

Recently, an LC-UV has been proposed for the determination of 10 quinolones in milk (Christodoulou and Samanidou, 2007). It involves a solid-phase extraction procedure using LiChrolut RP18 cartridges. Achieved recoveries varied between 75 and 92%. The limits of detection found ranged from 1.5 to 6.8 μg mL^{-1}.

This paper describes the development of a simple, selective and sensitive LC–MS method for the simultaneous determination of quinolones regulated and forbidden by the European Union in milk: danofloxacin (MRL = 30 μg kg^{-1}) difloxacin (forbidden), enrofloxacin (MRL = 100 μg kg^{-1}, as enrofloxacin + ciprofloxacin), flumequine (MRL = 50 μg kg^{-1}), marbofloxacin (MRL = 75 μg kg^{-1}) and oxolinic acid (forbidden). Sarafloxacin has also been included because, even though it does not yet have an assigned MRL, it is the main metabolite of difloxacin. A new sample-treatment was used for isolation of compounds from samples. The proposed method was applied for the determination of these compounds in a large number of processed milk samples obtained from different supermarkets in Granada (South of Spain).

EXPERIMENTAL

Materials, chemicals and reagents.

All reagents were analytical grade. Water (18.2 MΩ cm^{-1}) was purified using a Milli-Q system from Millipore (Millipore, Bedford, MA, USA). Acetonitrile (HPLC-gradient grade), dichloromethane (HPLC-grade), ethanol (99.9%, v/v) and glacial acetic acid (99%, w/v) were purchased from Panreac (Barcelona, Spain).

The quinolones were obtained from different pharmaceutical firms: marbofloxacin (MAR) from Vetoquinol (Aartselaar, Belgium), ciprofloxacin (CIP) from Ipsen Pharma (Barcelona, Spain), enrofloxacin (ENR) from Cenavisa (Tarragona, Spain), danofloxacin (DAN) from Pfizer (Karlsruhe, Germany), sarafloxacin (SAR) and difloxacin (DIF) from Abbott (Madrid, Spain), and norfloxacin (NOR), flumequine (FLU) and oxolinic acid (OXO) from Sigma-Aldrich (Madrid, Spain).

Stock solutions of individual compounds were prepared in ethanol at a concentration of 100 µg mL^{-1}. These solutions were stored at 4°C in the dark for no longer than 2 months. Individual working solutions were prepared by diluting suit- ably with a water–ethanol mixture (95: 5, v/v). All solvents and solutions prepared for LC were filtered through 0.22 µm nylon filter membranes (Millipore) before use.

Instrumentation and software. Analyses were performed with a Waters (Milford, MA, USA) Alliance 2695 HPLC coupled with a Waters Micromass ZQ single quadrupole mass spectrometer (MS). The electrospray ionisation (ESI) source was operated in the positive mode. MassLynx 4.0 software (Milford, MA, USA) was used for instrument control and data acquisition.

Chromatograms were acquired in SCAN mode (50–600 m/z range) and SIM (selected-ion monitoring) mode. The nebulizing gas (N2) flow was 70 L h^{-1}. Nitrogen drying gas was used at a flow rate of 300 L h^{-1} and 250°C. MS parameters were optimized to the following values: capillary voltage, 3.50

kV; extractor cone, 3 V; RF lens, 0.5 V; source temperature, 130°C; sample cone voltage, 35 V; and multiplier voltage, 600 V.

A Megafuge 1.0 centrifuge (Heraeus, Hanau, Germany) and a Speed Vac evaporator (Thermo Electron Corporation, Philadelphia, USA) were also used. Statgraphics software package (Statgrapics, 2000) was used for statistical analysis of the data and for regression analysis (linear model).

Chromatographic conditions. Chromatographic separation of studied quinolones was performed on an XTerra (150 x 2.1 mm i.d., 5 µm particle size) with a guard column XTerra MS C18 (100 x 2.1 mm i.d., 5 µm particle size) from Waters (Milford, MA, USA).

A gradient program with the mobile phase combining 0.1% (v/v) formic acid aqueous solution (solvent A) and acetonitrile (solvent B) was used. The initial values were 95% of A and 5% B and held for 7 min. A linear gradient was then established in order to reach a 50% A and 50% B composition at 15 min, kept stable for 2 min, returned to initial conditions at 20 min and held for 10 min for column conditioning.

The flow-rate was 0.5 mL min^{-1} with postcolumn splitting so that 0.30 mL min^{-1} entered the detector; the injection volume was 100 µL and the column temperature was maintained at 40°C.

Using these chromatographic conditions, the separation of these compounds was achieved in less than 20 min.

Sample preparation. Processed milk was obtained at different grocery stores in Granada (Spain). For powdered milk, 0.6 g of sample were

accurately weighed and placed in a glass tube (15 mL) and 5.4 g of heated deionized water (~40°C) were added. The mixture was vortexed for 1 min and sonicated for 5 min, and 100 µL of 6.0 µg mL^{-1} norfloxacin (internal standard) solution was added. The mixture was vortexed for 1 min and sonicated for 5 min.

For liquid milk, 6.0 g of adequately homogenized sample was accurately weighed and placed into a glass tube (15 mL); 100 µL of 6 µg mL^{-1} norfloxacin (internal standard) solution was added. The mixture was vortexed for 1 min and sonicated for 5 min.

Preparation of fortified samples. Fortified samples were prepared by spiking 0.6 or 6 g of blank sample adding adequate volumes of working solutions of the studied quinolones and 100 µL of 6 µg mL^{-1} internal standard solution (norfloxacin). Before sample treatment and analysis, all samples were allowed to stand in the dark for 30 min at room temperature to allow full interaction between the antibiotics and matrix studied. All samples were prepared in duplicate.

The recovery (parameter used to optimize all parameters) was evaluated by comparison with samples that were spiked after the extraction procedure and that were considered 100% extracted.

Sample treatment. Fat from the above prepared samples was removed by centrifugation at 4500 rpm (2400g) for 10 min. The upper fat layer was carefully removed using a Pasteur pipette. The operation was repeated twice.

After fat removal, each sample was filtered through a 0.45 µm nylon membrane (Millipore) and 1 mL of glacial acetic acid and 10 mL of acetonitrile were added to filtrate in order to precipitate proteins. The mixture was vortexed for 1 min and centrifuged for 5 min at 4500 rpm (2400g). The upper liquid layer was transferred to a new glass tube and 10 mL of dichloromethane were added to remove acetonitrile. After gently shaking, sample was centrifuged at 4500 rpm (2400 g) for 2 min and the upper aqueous layer (about 3.5 mL) was transferred to a clean glass tube and evaporated to dryness in a Speed Vac evaporator (96 samples per hour). Finally, 250 µL of mobile phase was added and, after shaking for 2 min in the vortex, the supernatant was filtered through a 0.22 µm nylon membrane (Millipore) before injection into the LC column.

RESULTS AND DISCUSSION

Chromatographic separation

A Luna C18 (150 x 4.6 mm i.d., 5 µm particle size) from Phenomenex (Torrance, USA), a Spherisorb ODS-2 (250 x 4.6 mm i.d., 5 µm particle size), an Atlantis dC18 (150 x 4.6 mm i.d., 5 µm particle size) and an XTerra (150 x 2.1 mm i.d., 5 µm particle size) from Waters liquid chromatographic columns were tested. Waters XTerra provided the best results for these compounds. In addition, this column gave the best resolution for critical pair OXO and FLU at shorter times. Therefore, the column was selected as the most appropriate for our purposes.

Different mobile phases were studied in order to optimize the separation and peak shapes (mainly referring to critical pair OXO and FLU).

With this objective in mind, two organic solvents (methanol and acetonitrile) commonly used in reversed-phase liquid chromatography (RP-LC) were evaluated. Acetonitrile gave better results than methanol. Moreover, acidification of the LC eluent was necessary to suppress the ionic mobility of the analytes and to allow the formation of protonated species. Formic acid, acetic acid and trifluoroacetic acid were tested as additives. The best separation and ionization yield of compounds was obtained using a mobile phase composed of 0.1% (v/v) formic acid aqueous solution (solvent A) and acetonitrile (solvent B). A linear gradient, described previously, was used to obtain a good resolution for all studied compounds.

Lastly, in order to improve the detection limits of the method, a study was performed to evaluate the possibility of increasing the injection volume. A range from 10 to 100 µL was studied and no extra broadening of the peaks was observed, even at maximum value. Accordingly, 100 µL was chosen as the injection volume.

LC-MS method

SCAN analysis of a standard mixture was performed to check the optimum conditions for detection. The ESI interface in positive and negative mode was evaluated for all studied compounds. Positive mode was selected because of its higher sensitivity to these compounds. Selected ion monitoring (SIM) mode was used for quantitative analysis. Figure 1 shows the mass spectra of studied quinolones. MAR, NOR, CIP, DAN, ENR, SAR and DIF showed a peak at 363, 320, 332, 358, 360 386 and 400 m/z,

respectively, corresponding in all cases to the molecular ion in positive ionization mode $[M + H]^+$. Similarly, OXO and FLU showed a base peak at 262 m/z corresponding to the molecular ion $[M + H]^+$ Finally, it is important to remark that mass spectra showed important peaks corresponding to the lost of water molecules $[M + H -18]^+$, in all cases. Table 1 shows the parameter used for SIM mode establishment. Four fragments were selected for MAR, NOR, CIP, DAN, ENR, SAR and DIF, and only three fragments could be selected for OXO and FLU.

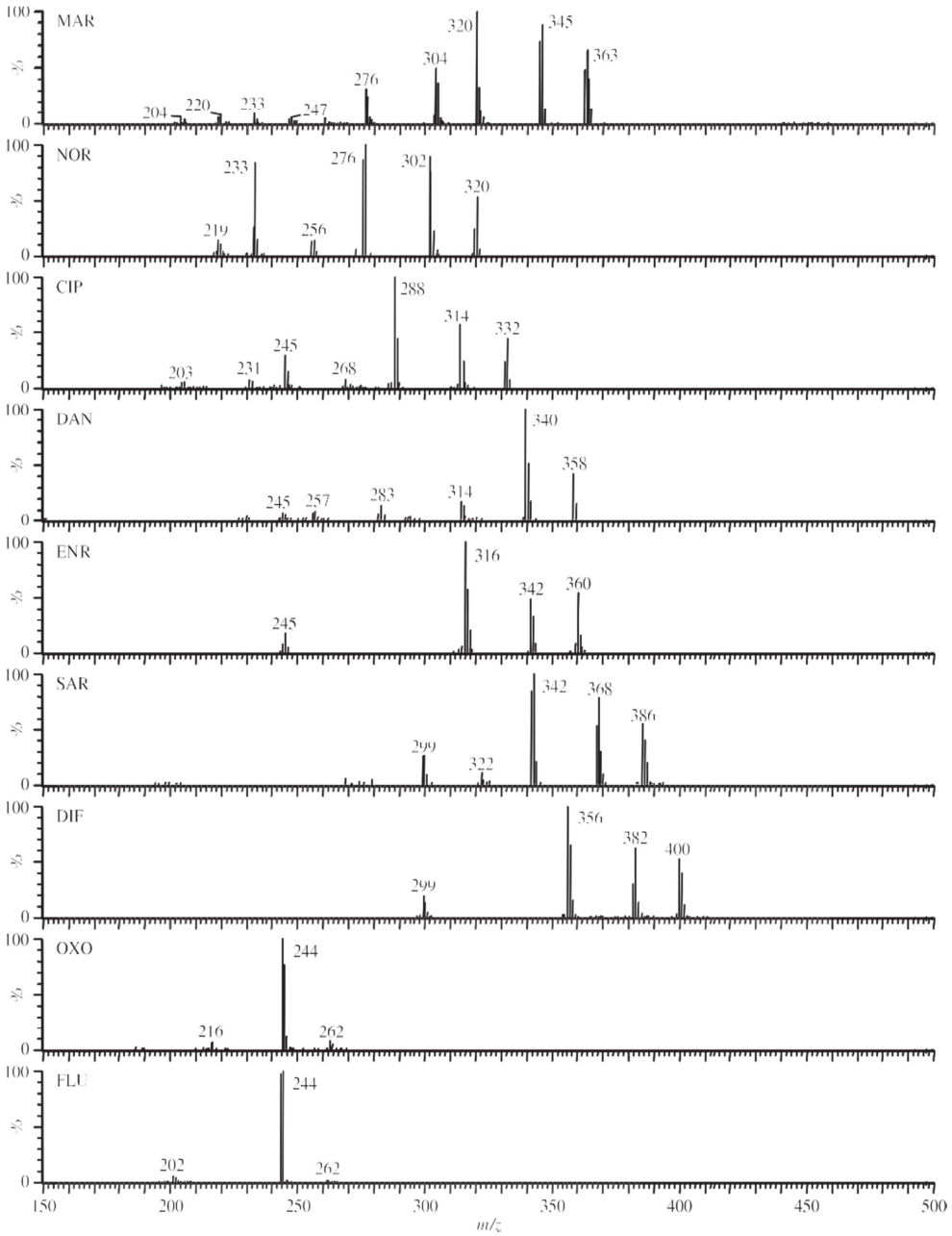

Figure 1. Mass spectra of the quinolones studied (ESI interface in positive mode).

Method validation

The whole analytical method was validated in terms of linearity, decision limit, detection capability, limit of quantification, intra-day and inter-day repeatability and trueness by means of recovery studies.

For the calibration, spiked standard samples at six concentration levels were extracted following the extraction procedure previously described (each level was prepared in duplicate, and each calibration sample was analyzed twice). Calibration curves were constructed using analyte/internal standard peak area ratio vs concentration of analyte. Linearity of the calibration graphs was tested in accordance with the Analytical Methods Committee; the lack-of-fit test (Analytical Methods Committee, 1994) was applied to two replicates and two injections of each standard.

A fundamental aspect that must be examined in the validation of any analytical method is its limit of detection in order to determine if an analyte is present in the sample. In this paper, a criterion for method performance was used that includes the decision limit (CC_α) the detection capability (CC_β) and the limit of quantification (LOQ; EU Commission, 2002). The decision limit is the limit from which it can be decided that a sample is contaminated with an error probability of α. The detection capability is the smallest content of the analyte that may be detected, identified and/or quantified in a sample with an error probability β. The decision limit and detection capacity, which are better adjusted to a statistical evaluation, were implemented. Thus, CC_α (α = 5%), CC_β (β = 5%) and LOQ were

calculated. The analytical and statistical parameters for each quinolone studied are summarized in Table 2.

Table 1. SIM mode for studied quinolones

Compound	t_r (min)	Mode	Voltage	Dwell time	m/z
NOR (IS)	11.25	ESI +	35 V	0.1 s	320 + 302 + 276 + 233
MAR	10.07	ESI +	35 V	0.1 s	363 + 345 + 320 + 277
CIP	11.49	ESI +	35 V	0.1 s	332 + 314 + 288 + 245
ENR	11.83	ESI +	35 V	0.1 s	360 + 342 + 316 + 245
DAN	11.97	ESI +	35 V	0.1 s	358 + 340 + 314 + 283
SAR	12.31	ESI +	35 V	0.1 s	386 + 368 + 342 + 299
DIF	12.34	ESI +	35 V	0.1 s	400 + 382 + 356 + 299
OXO	14.57	ESI +	35 V	0.1 s	262 + 244 + 216
FLU	16.25	ESI +	35 V	0.1 s	262 + 244 + 202

Table 2. *Analytical and statistical parameters*

Parameter	MAR	CIP	DAN	ENR
k	6	6	6	6
a	$-2.03\cdot10^{-2}$	$1.40\cdot10^{-3}$	$5.60\cdot10^{-3}$	$9.30\cdot10^{-3}$
s_a	$2.05\cdot10^{-2}$	$1.42\cdot10^{-2}$	$1.19\cdot10^{-2}$	$8.65\cdot10^{-3}$
b (g ng $^{-1}$)	$6.50\cdot10^{-2}$	$2.18\cdot10^{-2}$	$2.91\cdot10^{-2}$	$2.26\cdot10^{-2}$
s_b	$1.05\cdot10^{-3}$	$5.74\cdot10^{-4}$	$4.86\cdot10^{-4}$	$3.43\cdot10^{-4}$
LDR (ng g^{-1})	5-50	7-50	4-50	4-50
R^2 (%)	99.8	99.7	99.9	99.9
S_{rc}	$4.10\cdot10^{-2}$	$1.15\cdot10^{-2}$	$1.87\cdot10^{-2}$	$1.18\cdot10^{-2}$
CC_α (ng g^{-1})	0.8	1.6	1.7	1,0
CC_β (ng g^{-1})	1.3	2.7	2.7	1,6
LOQ (ng g^{-1})	3,2	6,5	6,5	3,8
P_{lof} (%)	37.9	55.8	48.9	56.4
	SAR	DIF	OXO	FLU
k	6	6	6	6
a	$2.00\cdot10^{-3}$	$3.71\cdot10^{-3}$	$4.25\cdot10^{-2}$	$9.00\cdot10^{-4}$
s_a	$1.34\cdot10^{-2}$	$1.31\cdot10^{-2}$	$1.29\cdot10^{-2}$	$1.49\cdot10^{-2}$
b (g ng $^{-1}$)	$2.58\cdot10^{-2}$	$3.71\cdot10^{-2}$	$5.80\cdot10^{-2}$	$7.54\cdot10^{-2}$
s_b	$5.42\cdot10^{-4}$	$5.41\cdot10^{-4}$	$5.15\cdot10^{-4}$	$5.41\cdot10^{-4}$
LDR (ng g^{-1})	7-50	5-50	3-50	2-50
R^2 (%)	99.7	99.9	99.9	99.9
S_{rc}	$2.05\cdot10^{-2}$	$1.95\cdot10^{-2}$	$1.79\cdot10^{-2}$	$2.21\cdot10^{-2}$
CC_α (ng g^{-1})	1,3	0,9	0,6	0,5
CC_β (ng g^{-1})	2,2	1,5	0,9	0,8
LOQ (ng g^{-1})	5,2	3,5	2,2	2,0
P_{lof} (%)	50.9	39.6	34.8	92.4

k, calibration levels; a, intercept; Sa, intercept standard deviation; b, slope; S_b, slope standard deviation; LDR, linear dynamic range; R^2, determination coefficient; S_{rc}, regression standard deviation; $CC_{\alpha,0.05}$, decision limit; $CC_{\beta,0.05}$, detection capability; LOQ, limit of quantification; P_{lof}, P-value for lack-of-fit test.

In order to determine the intra-day and inter-day repeatability, blank milk samples were spiked at three concentration levels (10, 25 and 50 ng g^{-1} for each quinolone studied) and six analyses were performed over 3 days. Recoveries were achieved by comparing the analytical results for extracted standard samples of bovine milk at the aforementioned concentrations with unextracted standards prepared at the same concentrations in blank extract representing 100% recovery.

The results obtained, summarized in Table 3, meet the requirements defined by the European Union legislation (EU Commission, 2002).

Table 3. Intra-day and inter-day recovery (%) and precision (RSD, %). Data obtained for the determination of studied quinolones in milk samples (n=6)

Compound		Concentration Level (ng g^{-1})		
		10	25	50
MAR	Day 1	94(3)	96 (4)	99 (4)
	Day 2	95(4)	98 (4)	97 (3)
	Day 3	96(3)	95 (3)	94 (3)
CIP	Day 1	96(3)	94 (3)	93 (4)
	Day 2	94(4)	99 (3)	95 (4)
	Day 3	98(4)	96 (3)	97 (3)
DAN	Day 1	99(3)	101(3)	96 (3)
	Day 2	96(3)	97 (3)	95 (3)
	Day 3	97(3)	97 (4)	100 (2)
ENR	Day 1	98(4)	95 (3)	97 (4)
	Day 2	93(3)	94 (3)	95 (3)
	Day 3	96(4)	99 (4)	93 (4)
SAR	Day 1	99(4)	95 (3)	97 (3)
	Day 2	94(4)	96 (4)	93 (3)
	Day 3	98(4)	94 (3)	96 (4)
DIF	Day 1	97(3)	99 (4)	95 (4)
	Day 2	94(4)	95 (3)	97 (4)
	Day 3	95(4)	98 (3)	96 (3)
OXO	Day 1	67(4)	68 (4)	71 (3)
	Day 2	69(5)	70 (5)	67 (4)
	Day 3	70(4)	71 (4)	68 (4)
FLU	Day 1	70(3)	68 (4)	71 (5)
	Day 2	67(5)	69 (3)	68 (4)
	Day 3	69(5)	67 (4)	70 (4)

The proposed method was applied to the determination of studied quinolones in different samples of liquid and powder milk. Thirty samples of whole milk, 30 of skimmed milk and 30 of powder milk were analysed and none of them gave a positive result for these compounds. The quinolone content in samples was found to be smaller than the above-sated detection capabilities.

The chromatographic peaks in the samples analyzed were identified by comparing mass spectra and retention data obtained for the standards and spiked samples under optimized method conditions. Without the detection of the compounds studied in the samples assayed, these samples were spiked at different levels (10, 25 and 50 ng g^{-1} for each compound). A representative chromatogram, using SIM mode, of a spiked milk sample is shown in Fig. 2.

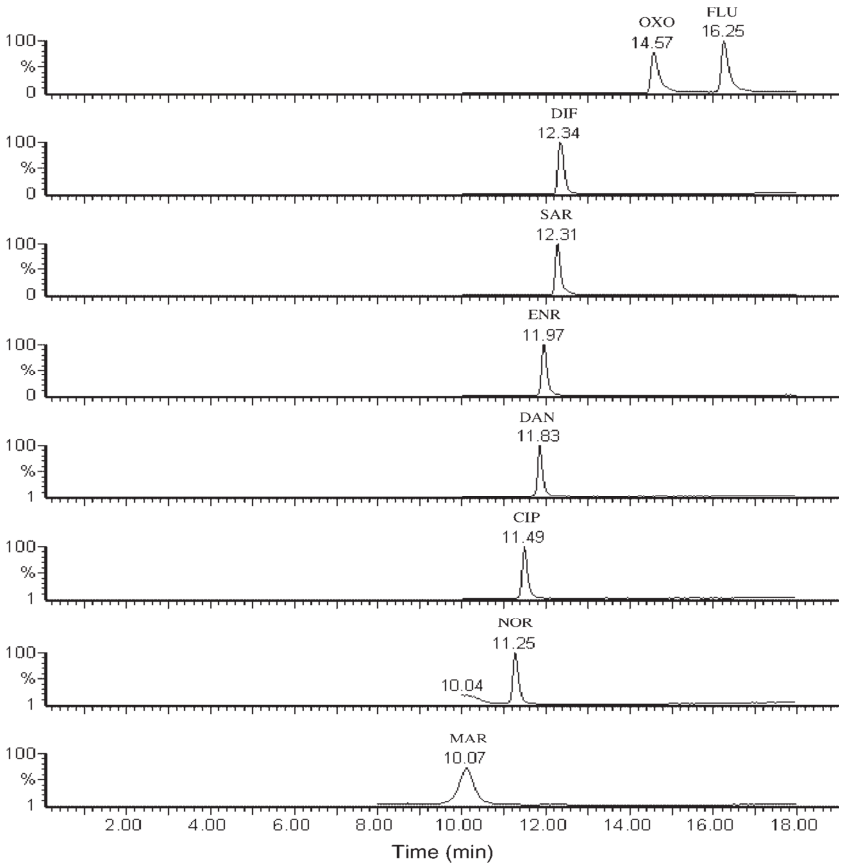

Figure 2. Representative chromatogram of a spiked milk sample using SIM mode. Chromatographic conditions are described in the text. Concentration level: 25 ng g^{-1} for each quinolone and 100 ng g^{-1} for the internal standard (norfloxacin)

The validation of the proposed method was tested using a recovery test (Student t-test; Miller and Miller, 1993). Ten determinations were carried out for each additional level. As the p-values calculated in all cases were greater than 0.05, the null hypothesis appears to be valid, i.e. recoveries are close to 100%. The results obtained are shown in Table 4.

Table 4. Recovery assay, precision (repeatability) and accuracy of target compounds

		Whole milk		Skimmed milk		Powder milk	
	Added ($ng \cdot g^{-1}$)	Found[a] ±SD (RSD, %)	Recovery (%)	Found[a] ± SD (RSD, %)	Recovery (%)	Found[a] ± SD (RSD, %)	Recovery (%)
MAR	10.0	10.8 ± 0.5 (4.6)	108.0	9.6 ± 0.4 (4.2)	96.0	9.4 ± 0.4 (4.3)	94.0
	25.0	23.9 ± 0.8 (3.3)	95.6	24.2 ± 1.5 (3.3)	96.8	25.9 ± 1.2 (4.6)	103.6
	50.0	52.6 ± 1.5 (2.8)	104.8	52.5 ± 3.3 (4.4)	105.0	48.3 ± 1.2 (2.5)	96.6
CIP	10.0	10.4 ± 0.4 (3.8)	104.0	9.8 ± 0.3 (3.1)	98.0	9.6 ± 0.4 (4.2)	96.0
	25.0	24.2 ± 0.9 (3.7)	96.8	23.9 ± 1.2 (5.0)	95.6	26.2 ± 1.0 (3.8)	104.8
	50.0	45.0 ± 1.6 (3.4)	95.0	49.0 ± 1.8 (3.7)	98.0	48.6 ± 1.8 (3.7)	97.2
ENR	10.0	10.4 ± 0.4 (3.8)	104.0	9.8 ± 0.4 (4.1)	98.0	10.4 ± 0.3 (2.9)	104.0
	25.0	26.0 ± 1.2 (4.6)	104.0	24.1 ± 0.9 (3.7)	96.4	25.8 ± 1.0 (3.9)	103.2
	50.0	48.7 ± 2.4 (4.9)	97.4	48.7 ± 2.1 (4.3)	97.4	48.9 ± 1.8 (3.6)	97.8
DAN	10.0	10.5 ± 0.3 (2.9)	105.0	10.4 ± 0.4 (3.8)	104.0	10.6 ± 0.4 (3.8)	106.0
	25.0	23.8 ± 1.2 (3.8)	95.2	24.2 ± 1.2 (5.0)	96.8	25.9 ± 0.9 (3.5)	103.6
	50.0	48.0 ± 3.1 (3.5)	96.0	51.7 ± 2.1 (4.1)	103.4	51.3 ± 1.6 (3.1)	102.6
SAR	10.0	9.8 ± 0.3 (3.1)	98.0	9.6 ± 0.6 (4.2)	96.0	9.4 ± 0.2 (2.1)	94.0
	25.0	24.3 ± 0.6 (2.5)	97.2	26.2 ± 1.1 (4.2)	104.8	24.6 ± 0.6 (2.4)	97.6
	50.0	51.8 ± 1.9 (3.7)	103.6	50.3 ± 1.9 (3.8)	100.6	50.9 ± 1.4 (2.8)	98.2
DIF	10.0	10.5 ± 0.2 (2.9)	105.0	10.2 ± 0.2 (2.0)	102.0	9.8 ± 0.3 (3.1)	98.0
	25.0	23.8 ± 0.9 (3.8)	95.2	24.2 ± 1.2 (3.3)	96.8	25.6 ± 0.8 (3.1)	97.6
	50.0	49.2 ± 2.3 (4.7)	98.4	51.7 ± 1.2 (2.3)	103.4	49.1 ± 1.3 (2.6)	98.2
OXO	10.0	10.2 ± 0.2 (3.9)	102.0	9.6 ± 0.4 (4.2)	96.0	9.4 ± 0.3 (3.2)	94.0
	25.0	26.0 ± 1.1 (4.2)	104.0	24.8 ± 1.0 (4.0)	99.2	26.1 ± 0.9 (3.4)	95.6
	50.0	47.7 ± 1.9 (4.0)	95.4	48.5 ± 2.9 (4.1)	97.0	52.8 ± 2.1 (4.0)	94.4
FLU	10.0	10.5 ± 0.4 (3.8)	105.0	9.6 ± 0.4 (4.2)	96.0	10.2 ± 0.3 (2.9)	102.0
	25.0	23.8 ± 1.0 (4.2)	95.2	24.3 ± 1.2 (5.0)	97.2	24.6 ± 0.6 (2.4)	98.4
	50.0	51.6 ± 1.8 (3.5)	103.2	51.5 ± 2.5 (4.8)	96.8	50.3 ± 1.2 (2.4)	99.4

[a] Mean value ($ng\ g^{-1}$) ± standard deviation of 10 determinations; (RSD, %), relative standard deviation

CONCLUSIONS

The identification and quantification of eight quinolones by liquid chromatography with mass spectrometric detection in milk samples was successfully performed. A new sample-treatment procedure was used in order to isolate quinolones from the matrix. The advantages of this procedure included the simplicity of the quantitative extraction (not required solid- phase extraction) and the simultaneous determination of these eight quinolones (zwitterionic and acidic) in a single chromatographic run. The quantification limits were found to be low enough to determine quinolone residues in milk samples below the permissible MRLs established by the European Union (EU Commission, 1990, 1996).

The method was validated using a recovery assay with spiked samples and it was applied to a large number of milk samples obtained from different supermarkets in Granada (Spain). None of the samples were contaminated with compounds, as expected.

In conclusion, the proposed method is a useful tool for assuring the reliability and veracity of food laboratories in determining quinolone residues found in these food samples. It is a fast, simple and inexpensive procedure.

Acknowledgments

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2.2. Ésteres de Cloropropanol y Glicidil Ésteres

2.2.1. Introducción

El glicidol y sus compuestos relacionados, principalmente ésteres derivados, así como los cloropropanoles (2-monocloropropanodiol (2-MCPD) y 3-monocloropropanodiol (3-MCPD)) y sus ésteres derivados, son una familia de compuestos que aparecen en los alimentos debido principalmente al procesado y refinado de estos cuando son sometidos a altas temperaturas. Para el caso de 2 y 3-MCPD, la presencia de iones cloruros en medio ácido provoca su reacción con residuos de acilglicéridos que desencadenan la formación de ésteres derivados de este tipo de compuestos:

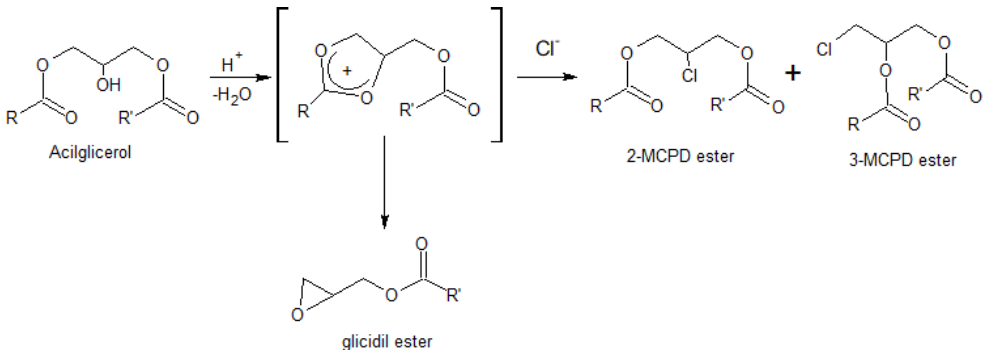

Figura 3. *Representación del mecanismo de formación de derivados de Cloropropanol y glicidilésteres.*

Tanto el 3-MCPD como el 2-MCPD se han postulado como posibles agentes carcinógenos, enmarcándose dentro del grupo 2B por la Agencia

Internacional de investigación contra el cáncer (IARC)[54] , ya que se han encontrado evidencias experimentales suficientes en animales como para atribuirles este riesgo. Por su parte, el glicidol se ha incluido en el grupo 2A como probable agente carcinógeno por esta misma Agencia ya que se ha demostrado que posee una actividad como agente alquilante de acción directa que es mutagénico en una amplia gama de ensayos tanto in-vivo como in-vitro[55].

Actualmente este tipo de sustancias se contemplan en el Reglamento 1881/2006 por el que se establecen los contenidos máximos de ciertos contaminantes en matrices alimentarias. Este Reglamento fue revisado recientemente para incluir los aceites y grasas vegetales, alimentos infantiles y alimentos infantiles para usos médicos especiales.

Inicialmente, la presencia de 3-MCPD fue descrita y legislada en matrices de proteínas vegetales hidrolizadas producidas por hidrólisis ácida tales como salsas de soja. En este tipo de matrices, el 3-MCPD se encuentra en su forma libre, no asociada a esteres de ácidos grasos. Sin embargo, en

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[54] International Agency for Research of Cancer, (2012), Some Chemicals Present in Industrial and Consumer Products, Food and Drinking-water IARC Monographs – 101. 3-Monochloro-1,2-propanediol. Lyon, World Health Organization Press, 349-374. Disponible en: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono101-010.pdf (Visitado 26/10/2019)

[55] International Agency for Research of Cancer, (2000) Some Industrial Chemicals, IARC Monographs–77, Lyon, World Health Organization Press. Disponible en: https://monographs.iarc.fr/wp-content/uploads/2018/06/mono77-19.pdf (Visitado 26/10/2019)

2013 la EFSA publicó un informe[56] sobre la presencia de este tipo de contaminantes en Europa con una evaluación de la exposición a la que es sometida la población. Se analizaron unas 1.235 muestras de alimentos de diferente naturaleza. De este estudio se desprende que los alimentos que principalmente contribuyen a la exposición total entre los diferentes grupos de población son la margarina y productos análogos, así como grasas y aceites vegetales, seguido de bollería fina y carne en conserva ahumada.

Se ha estimado que el 100% de los ésteres de 2 y 3-MCPD son liberados en su forma libre en el tracto digestivo humano[57], con lo cual para evaluar el riesgo y determinar los niveles seguros, se estima conjuntamente tanto el contenido de forma libre como asociado en forma de ésteres. Por su parte, para el caso del glicidol y ésteres derivados se considera que igualmente la liberación en humanos es casi completa.

Desde el punto de vista analítico, este tipo de compuestos fueron separados inicialmente por cromatografía en capa fina (TLC)[58], técnica

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[56] European Food Safety Authority. Analysis of occurrence of 3-monochloro propane-1,2-diol (3-MCPD) in food in Europe in the years 2009-2011 and preliminary exposure assessment. EFSA J. 11 (2013) 1-45.

[57] European Food Safety Authority. EFSA Panel on Contaminants in the Food Chain (CONTAM). Risks for human health related to the presence of 3- and 2 -monochloropropanediol (MCPD), and their fatty acid esters, and glycidyl fatty acid esters in food. EFSA J. 14 (2016) 1-159.

[58] Crews, C., Chiodini, A., Granvogl, M., Hamlet, C., Hrnčiřík, K., Kuhlmann, J., Lampen, A., Scholz, G., Weisshaar, R., Wenzl, T., Jasti, P.R., Seefelder, W. Analytical approaches for MCPD esters and glycidyl esters in food and biological samples: a review and future perspectives. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 30 (2012) 11-45.

mediante al cual se separan las diferentes fracciones de acilglicéridos que conforman la grasa. Una vez aislado, se determina mediante cromatografía de gases acoplada a espectrometría de masas.

Existen actualmente dos grandes grupos de técnicas analíticas para determinar estas familias de compuestos:

- *Métodos directos*: son aquellos en donde se determinan los diferentes ésteres derivados de glicidol y 2- y 3-MCPD directamente, es decir, las especies intactas sin ningún tipo de hidrólisis previa. Este tipo de métodos emplea principalmente la cromatografía de líquidos acoplada a espectrometría de masas (principalmente TOF). Sin embargo, los cromatogramas resultantes se componen de un gran número de picos, provenientes de las posibles combinaciones de los diferentes ácidos grasos que pueden formar parte de los derivados mono-acilglicéridos y di-acilglicéridos clorados. Para el caso de aceites vegetales, donde el número de ácidos grasos mayoritarios que componen el perfil lipídico es relativamente bajo, este tipo de cromatogramas puede ser interpretado, sin embargo, para el caso de grasas con un perfil de ácidos grasos con un mayor número de estos, los posibles compuestos derivados de las posibles combinaciones de ácidos grasos es muy alta, por lo que representa un verdadero reto el conseguir una adecuada separación y cuantificación de este tipo de contaminantes. Adicionalmente, un problema añadido en relación a la adecuada cuantificación es el hecho de poder obtener

patrones de pureza adecuada para poder calibrar y obtener la respuesta del equipo para cada uno de los diferentes compuestos.

- *Métodos indirectos:* en este tipo de métodos se somete la muestra de grasa a un tratamiento previo donde se libera el 2-, 3-MCPD y glicidol de sus esteres derivados. De este modo, todos los mono- o diacilgliceroles contribuyen a la formación de un único compuesto en su caso, independientemente de cuál era el derivado del que partía originalmente. En este tipo de métodos se determina por tanto glicidol, 2-MCPD o 3-MCPD liberado mediante hidrólisis, y no puede ser distinguido de la porción libre que previamente pudiera existir en la muestra original. Sin embargo, tal y como se ha descrito previamente, los derivados mono y diacilglicéridos de los cloropropanoles, así como de los glicidilésteres, son hidrolizados en el tracto digestivo, con lo que desde el punto de vista de la salud pública, el dato que más pueda interesar es el contenido total de este tipo de compuesto, sin importar la forma en que se encontraba originalmente en la matriz natural. Desde el punto de vista analítico, al contribuir todas los esteres derivados a un mismo compuesto, se mejora la sensibilidad de este tipo de métodos indirectos con respecto a los directos. Adicionalmente, sí que se disponen de sustancias patrón perfectamente definidas de estas formas libres que permiten por tanto una adecuada cuantificación.

Dentro de esta familia de métodos indirectos, se pueden diferenciar 2 subgrupos:

- M*étodos indirectos mediante hidrólisis ácida*. La liberación del 2-MCPD, 3-MCPD y glicidol se lleva a cabo mediante la adición de ácidos minerales fuertes diluidos durante varias horas. Este tipo de métodos tienen la desventaja de que son muy dependientes de la cantidad de agua presente en el medio además de necesitar un tiempo de reacción para la liberación de unas 16 h[59].

- *Métodos indirectos mediante hidrólisis básica*. La liberación del 2-MCPD, 3MCPD y glicidol se realiza mediante la adición de hidróxido sódico para conseguir de este modo la trans-esterificación de los mono y di acilglicéridos clorados y de los esteres de glicidol. Este tipo de métodos son en los que están basados los métodos oficiales que actualmente están disponibles. Sin embargo, tienen el inconveniente de que el 3-MCPD liberado en la reacción sufre una conversión a glicidol en el medio básico donde se lleva a cabo la reacción. Por este motivo existen diferentes alternativas para poder controlar o evitar este efecto: hidrólisis lenta a baja temperatura (entre -20 y -25°C) durante 16h[60], así se minimiza la conversión de 3-MCPD en glicidol en medio básico mediante la disminución de la cinética de reacción; o

[59] Ermacora A., Hrncirik, K. A novel method for simultaneous monitoring of 2-MCPD, 3-MCPD and glycidyl esters in oils and fats. J. Am. Oil Chem. Soc. 90 (2013) 1-8.

[60] Kuhlmann, J. Determination of bound 2,3-epoxy-1-propanol (glycidol) and bound monochloropropanediol (MCPD) in refined oils. Eur. J. Lipid Sci. Technol. 113 (2011) 335-344.

por otra parte, la hidrólisis rápida a temperatura ambiente mediante la realización de dos ensayos en paralelo por muestra: una en ausencia y otra en presencia de cloruros[61]; de esta forma, en la determinación en ausencia de cloruros en el medio de reacción se cuantifica el contenido exclusivamente de 3-MCPD presente en la muestra, y en el ensayo paralelo en presencia de cloruros, tanto el glicidol presente originalmente en la muestra como el derivado de la conversión del 3-MCPD, son cuantificados conjuntamente, de forma que el resultado de esta segunda determinación representa el contenido de 3-MCPD más el contenido de glicidol que se ha transformado totalmente en 3-MCPD. De esta forma, el contenido en glicidol se obtiene por diferencia de los resultados obtenidos en ambas determinaciones.

Los métodos indirectos son los más empleados en laboratorios de control debido a las ventajas anteriormente descritas, sin embargo, al mismo tiempo tienen importantes inconvenientes como largos tiempos de tratamiento de muestra o necesidad de duplicar ensayos, aspectos ambos que representan impedimentos para el acceso a resultados de manera ágil.

[61] DGF: DGF-Einheitsmethoden C-VI 18 (10). Standard Method C-VI 18 (10) Fatt-acid-bound 3-chloropropane- 1,2-diol (3-MCPD) and 2,3-epoxipropane-1-ol (glycidol). Determination in oils and fats by GC/MS (Differential measurement). Deutsche Einheitsmethoden zur Untersuchung von Fetten, Fettprodukten, Tensiden und verwandten Stoffen, 2011.

2.2.2. Objetivos

El objetivo de este trabajo es el desarrollo de una metodología de buenas características que posibilite la adecuada cuantificación de 3-MCPD, 2-MCPD y glicidol en matrices grasas pero que al mismo tiempo posibilite el procesado de un gran número de muestras por tanda.

Se ha optado por la metodología indirecta mediante hidrólisis rápida en medio básico a temperatura ambiente, pero con el desarrollo de un cálculo matemático que posibilita la cuantificación y corrección de la extensión del grado de conversión de 3-MCPD en glicidol. De esta forma se posibilita la cuantificación rápida, directa y en un solo paso de 3-MCPD, 2-MCPD y glicidol en aceites.

Este trabajo ha sido la base para la implantación de un método analítico acreditado por ENAC según la norma UNE-EN ISO/IEC 17025 en las instalaciones del Departamento de Servicios Analíticos de Biosearch S.A.

2.2.3. Publicación IV

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A novel method for the determination of glycidyl and 3-monochloropropanediol esters in fish oil by gas chromatography tandem mass spectrometry

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Abstract

Today, food security is one of the most important global issues with food quality control and identification of contaminants in foods and beverages, being crucial for human health and safety. In this paper, a novel single-step method for the simultaneous determination of 3-monochloropropanediol (3-MCPD) and glycidol esters in samples of winterized and non-winterized fish oil by using gas chromatography tandem mass spectrometry (GC-MS/MS) is validated. The method is based on alkaline hydrolysis of esters

at room temperature, using only 3-MCPD-d5 as internal standard, and a derivatization step with phenylboronic acid (PBA) at 90 ºC. The use of GC-MS/MS results in a simplified sample treatment and improvement of the limits of quantification and precision of the analytical method with no need of additional concentration of the extracts. A backflush tee placed between two HP-5 MS UI columns (15 m × 0.25 µm × 0.25 mm) was used in order to minimize matrix effects and peak shape degradation usually observed in routine analyses. The method was validated in winterized and non-winterized fish oil, achieving a limit of quantification of 100 ng g^{-1} and 50 ng g^{-1} for 3-MCPD and glycidol, respectively. Method validation was accomplished by comparing our laboratory results with results obtained by an accredited reference laboratory (SGS Germany GmbH) and by calculating the recoveries obtained in an assay with spiked samples. For glycidol quantification, a mathematical equation was developed in order to compensate for the partial conversion of 3-MCPD into glycidol. This expression involves the quantification of 3-MBPD-d5 generated during hydrolysis reaction.

Keywords: Food Safety; 3-Monochloropropanediol, Glycidol; Fish oil; GC-MS/MS

1. Introduction

Food security is a “flexible concept” as is reflected in the many attempts to define it proposed over the years. The United Nations’ Committee on World Food Security defines food security as the condition in which all people, at all times, have physical, social and economic access

to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life. Over the coming decades, a changing climate, growing global population, rising food prices, and environmental stressors will have significant yet highly uncertain impacts on food security. Economic growth is only sustainable if all countries have food security. Without country-owned food security strategies, there will be obstacles and additional costs to economic growth. The continuing evolution of food security as an operational concept in public policy has reflected the wider recognition of the complexities of the technical and policy issues involved [1]. Foodborne illnesses are a preventable and underreported public health problem. These illnesses are a burden on public health and contribute significantly to the cost of health care. Although anyone can get a foodborne illness, some groups like young children and older adults are at greater risk [2]. In this context, it is crucial to have reliable methods capable of detecting defects or contaminants in foods, either from an external source or formed during food processing and that might pose a hazard to consumers.

Fatty acid esters of 3-monochloropropanediol (3-MCPD) and glycidol are process-induced contaminants found in refined edible oils. The International Agency for Research Cancer (IARC) considers these esters as “possibly carcinogenic to humans (Group 2B)”, making their presence in edible oils a potential health risk [3,4]. These compounds are mainly formed during the refining process in the presence of chloride ions at low pH and high temperature [3,4]. Industry is currently working to improve

the refining processes in order to reduce the concentration of these contaminants in the final products.

A number of analytical methods have been developed over the years aimed at the detection and control of the concentrations of 3-MCPD and glycidol esters in processed foods [6,7]. The evaluation of the scientific literature shows that there are two main approaches for the determination of these compounds: indirect methods and direct methods of analysis. Directs methods determine the content of intact ester derivatives by using mainly liquid chromatography coupled to time-of-flight mass spectrometry (HPLC-MSTOF) [9-11]. However, in fish oil samples, where there are a high number of different fatty acids present, direct determination is not viable due to the high number of possible ester combinations [12]. Therefore, the limit of detection (LOD) of the total content of 3-MCPD would be too high and the chromatographic separation would require to be really studied and improved. Indirect methods quantify the total content of 3-MCPD and glycidol released from the ester bond, irrespective of the original molecule. In this respect, indirect methods are preferred because only a few peaks are generated which allows for higher sensitivity. Within indirect methods, different ways for ester release have been reported: acid catalysis [13,14] alkaline catalysis at low temperature [15,16], alkaline catalysis at room temperature [17], and hydrolysis by lipase [18]. Acid catalysis requires long reaction times (about 16 h) and a pre-treatment to transform glycidol into the more stable 3-MBPD derivative. This pre-treatment is strongly dependent on the reaction medium and can result in a loss of sensitivity and precision. Alkaline catalysis at low temperature also requires long

reaction times in order to minimize the conversion of 3-MCPD into glycidol, and the addition of three different internal standards and two parallel determinations in order to determine the undesired conversion of 3-MCPD into glycidol. On the other hand, alkaline catalysis at room temperature, based on one of the most extended official methods (Deutsche Gesellschaft für Fettwissenschaft e.V., DGF), are performed in duplicate (in the presence and absence of chloride ions) to determine the glycidol content by estimating the difference between the analysis used to determine total content of 3-MCPD plus glycidol and the analysis used to determine only 3-MCPD content. The difference between these two analyses is used as an indirect measure of glycidol content of the sample. Additionally, it has been reported that the DGF method gives inconsistent results when salting out conditions are varied because this method destroys and reforms MCPD during the cleavage process [19]. Other treatments, such as the hydrolysis of esters using lipase, do not give the expected results and the limit of quantification (LOQ) obtained was too high. Also, additional cleaning steps as solid phase extraction (SPE) before cleavage of sample have been described [20], but to our experience this does not provide significant improvement.

Glycidol reacts quantitatively in the presence of halogenated ions in aqueous acid media to form its halogenated derivative [15,16]. Usually bromide ion is used for the simultaneous determination of 3-MCPD and glycidol, since both 3-MCPD and 3-MBPD derivatives are easily separated by GC once derivatized with phenylboronic acid (PBA) reagent. The present work describes a rapid, sensitive indirect method for the simultaneous and

routine analysis of 3-MCPD and glycidol in fish oil using only one internal standard solution (3-MCPD-d5) and one assay per sample. This drastically cuts cost and analysis time while maintaining the reliability and robustness of the results. A short alkaline catalytic reaction at room temperature is used. The main problem of this determination in alkaline media is the conversion of 3-MCPD into glycidol. In the present work, a way to monitor this conversion during esterification is also proposed. This monitoring is done by quantifying the glycidol derivatives formed from the internal standard, taking into account that the deuterated glycidol derivative can be formed from the conversion of 3-MCPD-d5. It is assumed that there are no significant differences between the response factor of each compound and its deuterated derivatives, and that the transformation of glycidol into its brominated derivative is quantitative [15,16].

2. Materials and methods

2.1. Chemicals and reagents

Water (18.2 MW cm) was purified using a MilliQ system from Millipore (Molsheim, France). Analytical grade standards: 3-MCPD, 3-monobromopropanediol (3-MBPD), glycidol and deuterated 3-monochloropropanediol d5 (3-MCPD-d5) used as internal standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycidyl stearate and 1-palmitoyl-3-chloropropanediol were purchased from Cymit Química (Barcelona, Spain). Glacial acetic acid (>99.85%), acetone, n-hexane, diethyl ether and methanol (HPLC-grade), sodium bromide, sodium hydroxide, ammonium sulfate and PBA were purchased from Sigma-Aldrich (St. Louis,

MO, USA). Stock standard solutions (1.0 mg mL^{-1}) of 3-MCPD (1), glycidol (2) and 3-MBPD (3) were prepared by weighing 25 mg of each compound into a 25 mL flask and adding methanol (MeOH) to the final volume. Standard solutions remained stable for at least 9 months at -20 ºC in the darkness. Working standards were prepared fresh from the MeOH solutions prior to the experiments. For 1-palmitoyl-3-chloropropanediol solution, 25 mg of the compound were accurately weighed into a 25 mL volumetric flask and dissolved in n-hexane (equivalent to 316 mg L^{-1} of 3-MCPD) (4). Similarly, 25 mg of glycidylstearate were accurately weighed into a 50 mL volumetric flask and dissolved in n-hexane (equivalent to 109 mg L^{-1} of glycidol) (5). For internal standard 3-MCPD-d5, 20 mg of the pure standard were transferred to a 25 mL volumetric flask and dissolved in MeOH. This solution was diluted by adding 100 µL of the stock solution to a 25 mL volumetric flask with methanol. For the experiments, 140 µL of this solution was used for each assay. To stop the alkaline hydrolysis reaction, a solution of sodium bromide was used. The bromide solution was prepared by dissolving 7.5 g of the salt in a final volume of 25 mL of milli-Q water containing 825 µL of acetic acid. Finally, the derivatization solution was prepared by dissolving 2 g of PBA in 19 mL of acetone and adding 1 mL of deionized water.

2.2. Instrumentation and software

GC-MS/MS analyses were performed using a 7890A GC system coupled to an Agilent 7000B triple quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). A backflush system was employed in order to minimize the effects from the excess of derivatizing agent present in the final extract

of the samples. Separation was achieved with two HP-5MS columns (15 m × 0.25 µm × 0.25 mm) and placing a backflush tee between them. The derivatization reaction was carried out in a Stuart block heater (Bibby Sci. Ltd, Staffordshire, UK). A vortex-mixer (Heidolph, Schwabach, Germany) and a Mettler-Toledo analytical balance were also used.

2.3. Basic procedure

An aliquot of 100 mg ± 20 mg accurately weighed is placed in a 15 mL plastic tube. After adding 140 µL of the internal standard solution, 5 mL of diethyl ether is added and the tube vigorously shaken. After adding 200 µL of NaOH (0.5 N) in methanol and mixing, the mixture is left to react for 2.5 min. To stop the reaction, 3 mL of acid bromide solution is added. The organic phase is discarded and 3 mL of n-hexane are added. After shaking the tube, allow the phases to separate and discard the organic phase. The procedure is repeated twice. For derivatization of the analytes, 300 µL of PBA solution is added and the mixture heated at 90 ºC for 30 min in a block heater. Finally, the derivatized extracts are cooled to room temperature and 2.5 mL of n-hexane are added. After vortexing the mixture during 1 min, the organic phase is placed in a chromatographic vial and injected into the GC-MS/MS system.

2.3.1. Preparation of calibration and spiked samples

For calibration purposes, 1 mL of 3-MCPD solution (1) and 0.25 mL of glycidol solution (2) are transferred into a 50 mL volumetric flask that is filled to the mark with methanol. For the standard solutions, 25 µL to 1000 µL of the resulting solution are transferred to in separate 10 mL volumetric

flasks which are filled to the mark with methanol. Finally, 100 µL of each standard solution are added to 100 mg of a blank oil in a reaction tube and the solutions undergo the previously described sample treatment.

For the standard addition procedure, 1 mL of both solutions (4) and (5) are transferred into a 10 mL volumetric flask and that is filled to the mark with n-hexane. Each standard solution is prepared by adding from 0.1 mL to 3 mL of the resulting solution into 25 ml volumetric flasks, and finally 100 µL of each standard solution are added to 100 mg of a blank oil in a reaction tube and the solutions undergo the previously described sample treatment.

2.3.2. Glycidol-d5 synthesis

Glycidol-d5 could not be purchased by our laboratory due to its high cost and the lack of a local distributor. Because of this, to test the similarity of the response factors between glycidol and glycidol-d5, glycidol-d5 was synthesized from 3-MCPD-d5 in our laboratory according to the process described by the International Life Sciences Institute [4] with some modifications. Briefly, 40 µL of solution of 3-MCPD-d5 (IS) was dissolved in 5 mL of diethyl ether and 400 µL of potassium hydroxide (2 N) added. After a 10 min reaction, the reaction was stopped by adding 5 mL of acid bromide solution. At this point, 3-MCPD-d5 is quantitatively transformed into glycidol-d5, which is stabilized into 3-MBPD-d5. Finally, the organic phase was removed and 40 µL of 3-MCPD-d5 and 300 µL of PBA solution were added to continue with the normal sample treatment.

By measuring of this solution it can be quantified the concentration of glycidol-d5 obtained. Assuming that the transformation of 3-MCPD-d5 into glycidol-d5 under these conditions is quantitative, the found concentration of glycidol-d5 can be estimated and compared with the theoretical concentration. No differences were observed between concentrations.

2.4. GC-MS/MS conditions

Determination of 3-MCPD and glycidol derivatives was performed on a triple quadrupole GC-MS/MS system. In order to maximize the signal-noise ratio, the optimal transitions and collision energies were selected based on the product ion spectra for each compound. At the same time, and taking into account that the identity of the analytes is unequivocal, the energy of the electronic impact was also optimized. Usually, this energy is set at -70 eV allowing the use of libraries with spectra obtained at this energy value. When energy is set at -50 eV, the signal-to-noise ratio increases 70% (data not shown), while the spectra profile does not vary, whereby the optimized transitions do not have to be re-optimized. Table 1 shows the optimized instrumental conditions.

Table 1. *Instrumental parameters*

Chromatographic parameters					
Column (x2)	HP-5MS (15 m x 250 µm x 0.25 µm)				
Injector Temperature	250 °C				
Injection volume	2 µL				
Split mode	Pulsed-Splitless				
Constant flow	1 mL min^{-1}				
	°C/min	°C	time (min)		
		50	1		
Temperature gradient	40	140	0		
	2	160	0		
	40	300	5		
Transfer line Temperature	280 °C				
Backflush time	5 min				
Backflush pressure	50 psi				
MS parameters					
	Precursor ion (m/z)	Quantify ion (m/z)	CE (V) (quantify)	Qualify ion (m/z)	CE (V) (Qualify)
3-MBPD-d5	245	150	5	93	30
3-MBPD	240	147	5	91	30
3-MCPD-d5	201	150	5	93	30
3-MCPD	196	147	5	91	30
Filament energy	-50 eV (Delta EMV = 400)				
Solvent delay	6.5 min				
MS-Off	14 min				

CE: Collision energy

The use of GC-MS/MS instead of GC-MS, which is usually employed for the determination of 3-MCPD and glycidol derivatives, greatly improved the signal-to-noise ratio, while minimizing sample treatment and manipulation of samples as shown in figure 1.

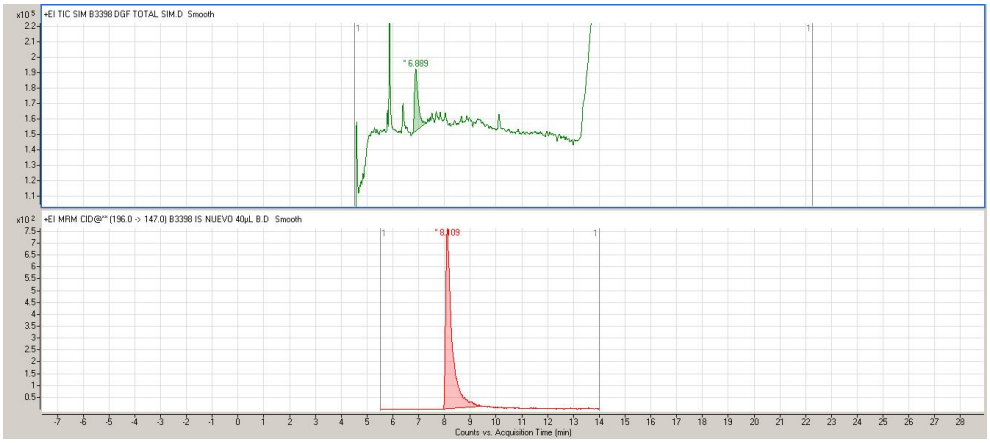


Figure 1. *Comparison of chromatograms obtained from a real contaminated oil sample. As example, 3-MCPD peak from extracted ion in SCAN mode (up) and by multiple reaction monitoring in MS/MS mode (down).*

2.5. Calculations and mathematical equations

As previously mentioned, different reference methods using different deuterated internal standards have been reported to monitor the conversion of 3-MCPD into glycidol. The use of internal standards is necessary to differentiate between native glycidol, originally present in the sample, and the glycidol formed in the alkaline hydrolysis process. When deuterated glycidol and deuterated 3-MCPD esters are used in the same assay as internal standards, the deuterated 3-MBPD from the conversion of 3-MCPD-d5 to glycidol and the added glycidol-d5 cannot be differentiated.

The single-step method proposed here uses only 3-MCPD-d5 as internal standard. To monitor the conversion yield, the quantity of glycidol-d5 generated from 3-MCPD-d5 (via 3-MBPD-d5) has to be determined and compared with the theoretical quantity added assuming that all 3-MCPD-d5 is transformed into glycidol-d5.

Decay of 3-MCPD is directly corrected by the internal standard, and any mathematical expression is needed. In order to minimize the cost of analysis per sample, we considered the possibility of using 3-MCPD-d5 instead of its ester as internal standard. However, this approach can lead to underestimation of the 3-MCPD content because the ester hydrolysis reaction is not monitored and therefore the 3-MCPD ester that does not react is not detected. However, in these experimental conditions, the ester yield is consistent and stable. In this work, we demonstrate that the recoveries obtained for an “in house” reference standard and proficiency testing data are above 90% and the inter-day precision is below 10% for 3-MCPD and below 15% for glycidol derivatives (see Results and Discussion section). Therefore, it can be assumed that 3-MCPD-d5 can be used for the quantitative analysis of total 3-MCPD content (ester and free).

To obtain the total glycidol concentration, two components need to be taken into consideration to correct the experimental glycidol concentration obtained. One is the contribution of degradation of the 3-MCPD present in the sample in alkaline media, and the other is the increase in concentration due to the decrease in the internal standard value (in the quantification of glycidol, 3-MCPD-d5 is also used as internal standard because both molecules have similar structures, therefore it can be assumed that their

extraction ratio from solvents is very close. Figure 2 shows the reactions that take place in the sample treatment:

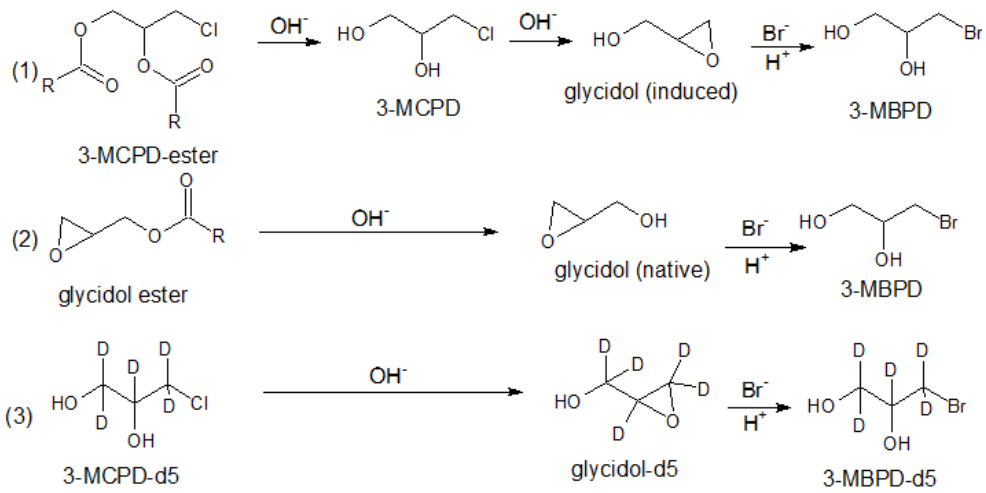


Figure 2. *Reactions that occur in the alkaline sample treatment.*

The sole source of glycidol-d5 in the reaction medium is the undesired conversion of 3-MCPD-d5. Therefore, it can be deduced that the conversion yield can be calculated as:

$$[\text{glycidol} - \text{d5}]\text{theoretical} \cdot \% = [\text{glycidol} - \text{d5}] \cdot (1 - \%)$$

And it can be deduced that:

$$\% = \frac{\dfrac{[\text{glycidol} - \text{d5}]}{[\text{glycidol} - \text{d5}]\text{theoretical}}}{1 + \dfrac{[\text{glicidol} - \text{d5}]}{[\text{glycidol} - \text{d5}]\text{theoretical}}}$$

where, [glycidol-d5] is the experimental concentration (µg kg^{-1}) of glycidol generated in the process from 3-MCPD-d5 (via 3-MBPD-d5) (induced glycidol) and [glycidol-d5] theoretical is the equivalent concentration of glycidol theoretically added assuming that all 3-MCPD-d5 (IS) is transformed into glycidol ([IS] x 0.67), where [IS] is the concentration of internal standard. This term can be considered as the maximum amount possible of induced glycidol-d5 that can be formed. Therefore, the expression for glycidol calculation would be:

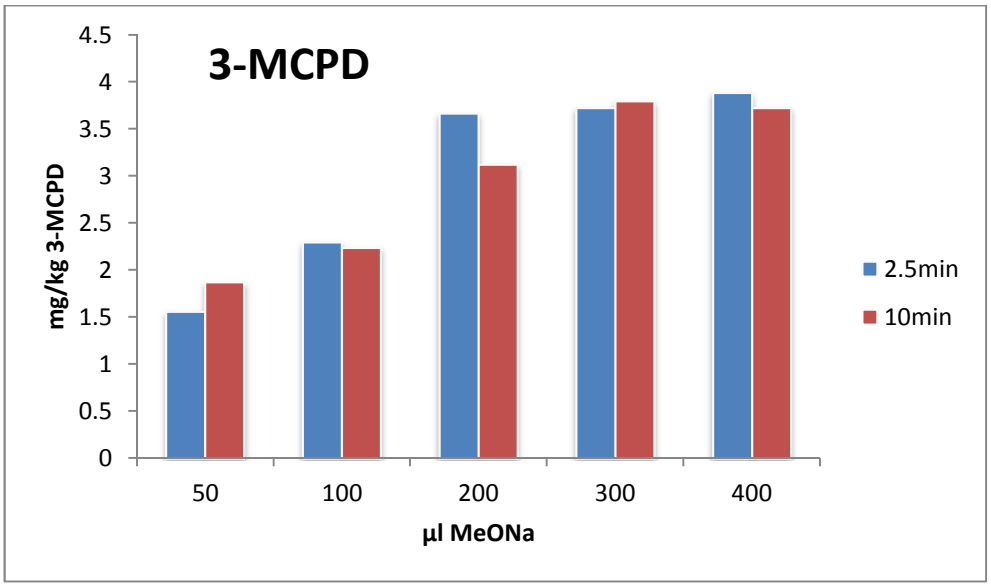
$$C_{glicidol} = \left([glycidol]_{vial} - (0.67 \cdot [3-MCPD] \cdot \%)\right) \cdot (1 - \%) \cdot \frac{100}{m_s \cdot 1000}$$

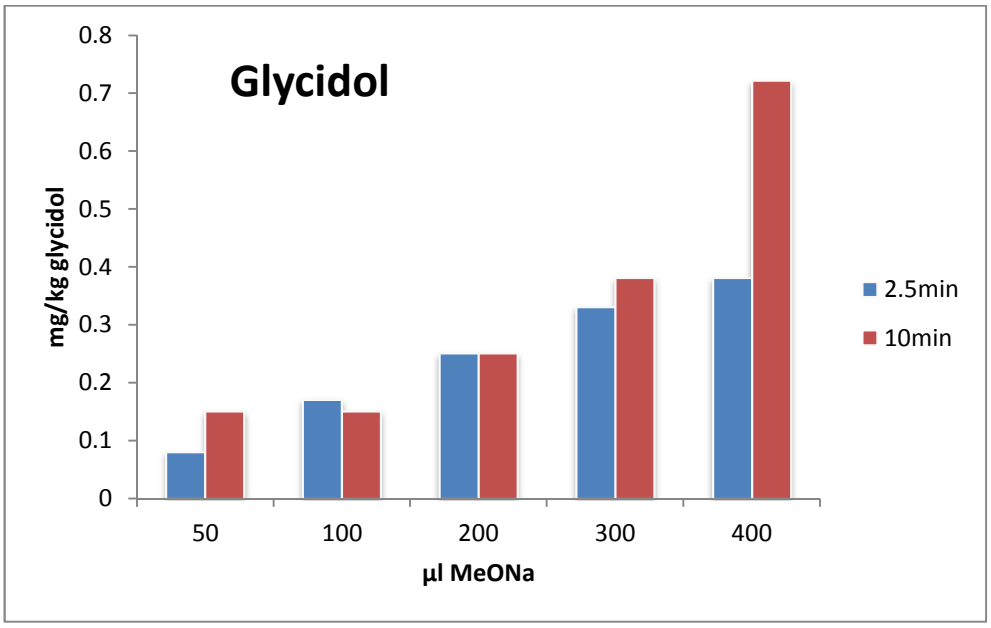
where $C_{glycidol}$ is the concentration of glycidol in samples expressed as mg kg^{-1}; [glycidol]vial is the total concentration of glycidol (sum of native and induced glycidol from 3-MCPD), this is the raw concentration obtained directly from the GC-MS (QqQ); [3-MCPD] is the concentration of 3-MCPD in vial expressed as µg L^{-1}; 0.67 is the ratio between the molecular weight of glycidol and 3-MCPD; m_s is the sample mass expressed as mg; % is the percentage of conversion yield of 3-MCPD into glycidol, and (1 - %) is a parameter used to compensate for the error induced by the decrease in internal standard due to using the same internal standard for both 3-MCPD and glycidol. The (glycidol) peak area/(3-MCPD-d5) peak area ratio will increase artificially if the denominator is decreased.

3. Results and discussion

3.1. Optimization of ester release conditions

In order to determinate the optimal conditions for the ester hydrolysis reaction, different quantities of methanolic sodium hydroxide and different reaction times were studied. It should be noted that a higher concentration of free 3-MCPD is expected with an increased in hydrolysis reaction yield, but this is associated with more undesired conversion into glycidol. Previous works [16] and reference methods ISO 18363-1:2015 and DGF Standard Method C VI 18 [21,22] carry out two analyses (presence and absence of chloride ions) so the difference between these two analyses is used as a measure of glycidol content in the sample. The assay where only 3-MCPD ester derivatives are generated, 2.5 min is enough for ester release, which is why 2.5 min was used as the minimum reaction time. Figure 3 shows the concentrations obtained of the studied compounds and the conversion ratios.


3-MCPD
mg/kg 3-MCPD
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
50
100
200
300
400
µl MeONa
2.5min
10min


Glycidol
mg/kg glycidol
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
50
100
200
300
400
µl MeONa
2.5min
10min

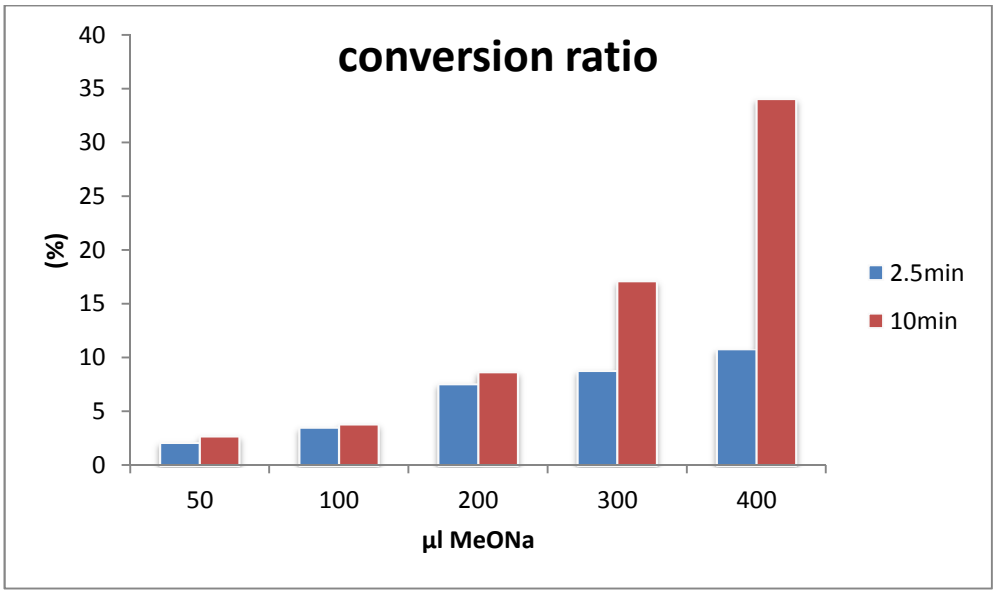


Figure 3. *(A) Variation of 3-MCPD concentration vs volume of NaOH (0.5 N) employed for ester hydrolysis. (B) Variation of glycidol concentration vs volume of NaOH (0.5 N) employed in the ester hydrolysis. (C) Variation of conversion yield vs volume of NaOH (0.5 N) employed in the ester release*

200 µL of 0.5 N methanolic NaOH (MeONa) was selected since the conversion yield is stable between 2.5 min and 10 min (7.5% and 8.6% respectively), improving the robustness of the sample treatment while keeping undesired conversion below 10%. Additionally, with 200 µL of MeONa, the glycidol concentration remains stable at the target value (0.37 mg kg^{-1}). With lower values the ester derivatives are not completely released; conversely, higher values lead to overestimation of the concentration due to the excessive decrease in internal standard IS concentration (conversion of IS into glycidol-d5). Additionally, 3-MCPD concentration and the ester reaction yield remain stable from 200 µL of MeONa.

3.2. Validation and application of method

A six-point matrix-matched calibration curve was obtained for each studied compound in the linear dynamic ranges that are shown in Table 2. Calibration curves were constructed using analyte/surrogate peak area ratio versus concentration of analyte.

Table 2. *Analytical and statistical parameters.*

Parameter	3-MCPD	Glycidol
n	12	12
b (kg mg^{-1})	0.443	0.339
s_b (kg mg^{-1})	0.006	0.008
a	0.054	-0.003
s_a	0.007	0.003
$s_{y/x}$	0.018	0.006
P_{lof} (%)	62.4	8.08
R^2 (%)	99.8	99.4
LOD (mg kg^{-1})	0.05	0.02
LOQ (mg kg^{-1})	0.2	0.07
LDR (mg kg^{-1})	0.2-3.5	0.07-0.80

b=slope; s_b = slope standard deviation, a= intercept; s_a= intercept standard deviation; $s_{y/x}$ = regression standard deviation; R^2= determination coefficient; LOD= limit of detection; LOQ = limit of quantification; LDR=linear dynamic range

The analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision). The quality parameters obtained for glycidol and 3-MCPD are summarized in Table 2.

Linearity. The determination coefficient (R^2) and the lack-of-fit test (Plof) were evaluated. The values obtained for R^2 for 3-MCPD and glycidol were 99.8% and 99.4%, respectively. Plof values were higher than 5% in all cases. Therefore, good linearity was observed within the concentration ranges.

Selectivity. This parameter was demonstrated by GC–MS/MS analysis of blanks. A blank sample and a spiked blank sample with the analytes were treated and their chromatograms were compared. No interferences were observed at the retention time of the analytes. These findings suggest that the spectrometric conditions ensure the high selectivity of the method.

Sensitivity. The limit of detection (LOD) and quantification (LOQ) were calculated by taking into account the standard deviation of the intercept (s_a) as an estimation of the standard deviation of the blank (s_o) and the slope (b) of the calibration graphs. The LOD was estimated as $3s_o$ and the LOQ was $10s_o$.

Accuracy (precision and trueness). Recovery assays were carried out to validate the method in terms of trueness. Recovery was determined by two different approaches: standard addition of 3-MCPD-palmitate and glicydol-stearate to a blank oil matrix, and comparison of results obtained from samples analysed by an accredited laboratory (SGS Germany GmbH) where reference method DGF C-VI 18 (10) A & B was used [22]. For the two

approaches, the concentrations of each compound were determined by interpolating from the standard calibration curve within the linear dynamic range and comparing with the known concentrations of analytes previously added to the blank fish oil and those obtained by the reference laboratory. Table 3 shows the results obtained from our accuracy study and Table 4 shows the comparison with the reference laboratory.

Table 3. *Accuracy of the method. Precision and trueness study of target compound based on standard addition of its ester derivatives (3-MCPD and glycidol respectively).*

Compound	Added Amount* (mg kg^{-1})	Trueness (Rec %)	Precision	
			RSD (%) intra-day	RSD (%) inter-day
3-MCPD-palmitate	0.125	102.0	2.3	5.3
	0.250	96.5	2.6	4.6
	0.625	99.5	4.1	6.2
	1.250	101.7	6.1	7.1
	3.750	99.3	4.2	5.1
	Average (%)	99.8	3.9	5.7
Glycidol-stearate	0.042	104	0.2	9.8
	0.084	97.5	0.5	5.1
	0.210	96.2	1.3	14.4
	0.420	97.7	3.8	6.0
	1.260	99.8	5.1	8.1
	Average (%)	99.0	2.2	8.7

* *Expressed as mg kg^{-1} of free ester released derivative. Rec: recovery; RSD: relative standard deviation*

***Table 4**. "In house" proficiency testing carried out between our laboratory vs SGS results.*

	Accredited Laboratory			Proposed Method				
Sample	3-MCPD (mg kg^{-1})	Δ3-MCPD (mg kg^{-1})	glycidol (mg kg^{-1})	3-MCPD (mg kg^{-1})	Δ3-MCPD (mg kg^{-1})	Glycidol (mg kg^{-1})	% Rec 3-MCPD	% Rec glycidol
B3395	3.27	0.37	0.25	3.04	0.42	0.28	93.0	113.5
B3396	3.25	0.45	0.30	3.12	0.53	0.36	96.0	117.8
B3397	3.40	0.39	0.26	3.23	0.48	0.32	95.0	123.1
B3398	3.26	0.57	0.38	3.32	0.49	0.33	101.8	86.0
B3399	3.36	0.39	0.26	3.60	0.39	0.26	107.1	100.0
B3400	3.25	0.55	0.37	3.21	0.50	0.34	98.8	90.9
DES 2	4.48	0.69	0.46	3.93	0.52	0.35	87.7	75.4
DES 3	3.57	0.44	0.29	3.03	0.36	0.24	84.9	81.8
DES 4	4.31	0.79	0.53	4.05	0.83	0.56	94.0	105.1
DES 5	2.10	0.88	0.59	1.60	0.95	0.64	76.2	108.0
H000421	2.32	0.61	0.41	2.15	0.53	0.36	92.7	86.9
						% Rec	93.4	98.9
						SD (%)	8.4	15.8

Rec: recovery; SD: standard deviation

As shown in Table 3 (standard addition) the recoveries obtained were very close to 100% (93.4% - 98.9% for 3-MCPD and glycidol, respectively), with average values 99.8 % for 3-MCPD and 99.0 % for glycidol.

Winterized and not winterized fish oils were sent to a reference laboratory (SGS Germany GmbH) in order to determine the 3-MCPD and glycidol content. The results obtained have been used as a reference to give additional evidences of the reliability of the proposed method. Table 4 (comparison with reference laboratory) shows that recoveries for all analysed samples are ranged from 87.7% to 107.1% for 3-MCPD and from 75.4% to 123.1% for glycidol. SGS results for glycidol are expressed as Δ3-MCPD. The results of glycidol have been calculated from SGS data by multiplication of Δ 3-MCPD by the 0.67 ratio.

The precision of the method in terms of inter-day variability was evaluated using spiked oil samples at five concentration levels (Table 3) for each compound. RSD (%) values obtained for 3-MCPD were between 0.2% and 6.1% and for glycidol were between 4.6% and 14.4%. Inter-day precision was lower than 15% in all cases. Therefore, all compounds were within the acceptable limits, which are considered which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20% [23]. Data indicate that the proposed method is reproducible. Additionally, precision (expressed as relative standard deviation, %RSD) was determined from the analysis of the internal quality control on two different days (day 1 and day 30). The values obtained are summarized in Table 5.

Table 5. Inter-day precision study for the same control sample analysis. Glycidol have been expressed as 3-MCPD difference in order to compare with the SGS reference result.

	3-MCPD (mg kg^{-1})	Glycidol (mg kg^{-1})		3-MCPD (mg kg^{-1})	glycidol* (mg kg^{-1})
Day 1	2.90	0.67	Day 30	3.00	0.66
Day 1	3.16	0.51	Day 30	2.80	0.52
Day 1	3.24	0.46	Day 30	3.10	0.57
Day 1	3.40	0.52	Day 30	3.10	0.52
		3-MCPD			Glycidol
Average (mg kg^{-1})		3.09			0.55
SD (mg kg^{-1})		0.19			0.07
RSD (%)		6.1			13.3
Reference value		3.27			0.57
Average Recovery (%)		94.4			97.2

** Expressed as 3-MCPD difference. SD: standard deviation; RSD: relative standard deviation*

The precision data obtained from the internal quality control sample for 3-MCPD and glycidol, are according with the data previously shown in the case of spiked samples (RSD <15%). The values of 3-MCPD and glycidol assigned to this quality control sample were established from the results of analysis obtained by the reference laboratory. The recovery values obtained were within the values established previously with spiked samples. Therefore, precision and trueness data indicate that the proposed method for the determination of 3-MCPD and glycidol in fish oil samples is accurate, and that the presence of co-extracted matrix components does not affect method performance.

4. Conclusions

We have improved a method for the simultaneous determination of 3-MCPD and glycidol derivatives in fish oil. The method involves triple quadrupole GC-MS/MS analyses and the use of a mathematical equation to compensate for the undesired reaction that takes place in alkaline media. The use of alkaline ester hydrolysis at room temperature was chosen for being fast and reproducible, making it an adequate routine method for quality control laboratory. In addition, the use of only 3-MCPD-d5 as internal standard significantly reduces the cost per sample, and the undesired conversion yield of 3-MCPD into glycidol can be monitored and compensated.

The proposed method has been accurately validated and our experimental data were compared to data from SGS Germany GmbH which uses the DGF C-VI 18 reference method. The results from the comparison demonstrate the reliability of the method and the fact that the method can be used for routine analysis of 3-MCPD and glycidol in fish oil with a significant reduction of the cost and analysis time while maintaining the reliability and robustness of the results. Finally, we propose a way to monitor the conversion of 3-MCPD into glycidol during ester release hydrolysis.

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2.3. Lactosa residual

2.3.1. Introducción

La lactosa es un disacárido formado por una molécula de glucosa unida a una molécula de galactosa mediante enlace entre el carbono 4 de la glucosa y el carbono 1 de la galactosa (β-D-galactopiranosil-(1→4)-D-glucopiranosa):

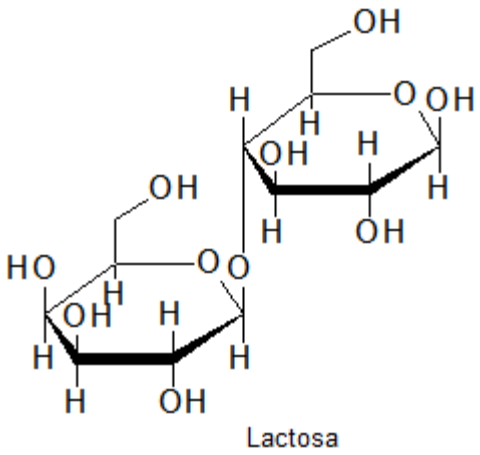

***Figura 4**. Estructura química de la lactosa.*

Se encuentra como azúcar principal de la leche en mamíferos, siendo su concentración en la leche de vaca de 4.5 g/100 g como valor promedio aproximado.

En el intestino delgado, este azúcar es hidrolizado gracias a la acción de una enzima, lactasa, que rompe el enlace β-glicosídico que se encuentra en la lactosa para generar una molécula de glucosa y otra de galactosa (figura 5):

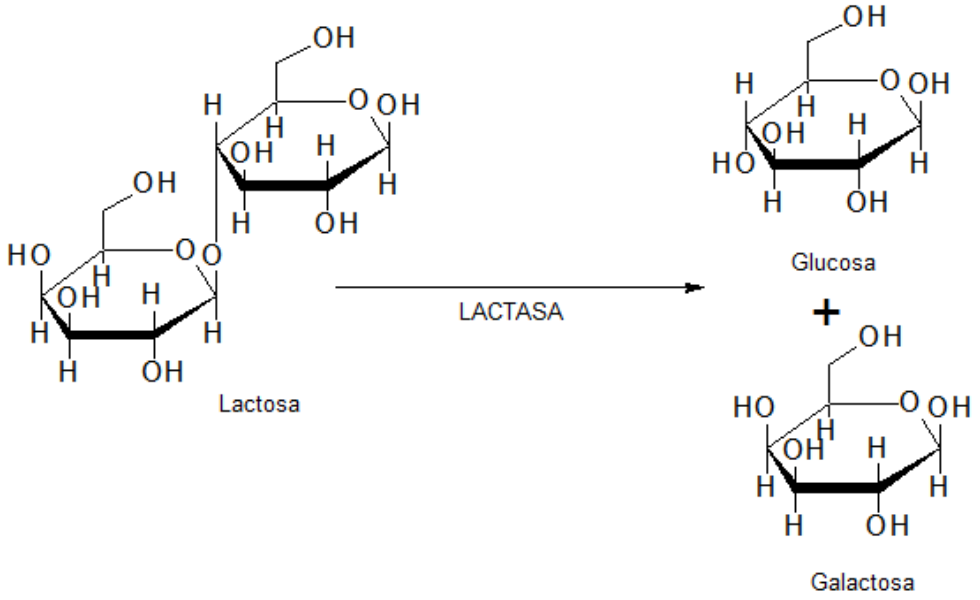

***Figura 5**. Hidrólisis de la lactosa.*

Esta enzima es generada en altas cantidades por los recién nacidos, que por tanto tienen una alta capacidad de digerir este azúcar. Esta capacidad disminuye a medida que se supera esta primera etapa de la vida, ya que la cantidad de lactasa generada se va reduciendo hasta alcanzar aproximadamente un 5-10% de la cantidad inicial. Esta disminución no es debida a ninguna enfermedad, sino que se trata de un patrón normal de la fisiología humana y es transmitido por un gen recesivo, y se conoce como *lactasa no persistente* (LNP). Sin embargo, aproximadamente un 25% de la

población sigue siendo capaz de generar esta enzima y por tanto de hidrolizar correctamente la lactosa a lo largo de su vida[62].

La intolerancia a la lactosa se puede definir como el desarrollo de síntomas gastrointestinales tras el consumo de lactosa. Estos síntomas se manifiestan siempre y cuando la cantidad de lactosa ingerida exceda a la capacidad del organismo para digerirla. Por tanto, la intolerancia a la lactosa se presentará dependiendo de la dosis de lactosa ingerida y de la capacidad del tracto gastrointestinal para responder ante esa cantidad.

La mayor parte de la población del norte de Europa, África Central y Oriente Medio tienen esta capacidad de digerir la lactosa, mientras que el resto (75% aproximadamente) de la población (asiáticos, nativos americanos, latinos y gran parte de africanos), sufren este tipo de intolerancia.

No se debe confundir esta intolerancia con la alergia a la leche. En este último caso, la reacción alérgica se debe a una respuesta inmune a ciertas proteínas de la misma. La intolerancia a la lactosa no tiene una base inmunológica, sino que únicamente se debe a una pérdida de la actividad intestinal de la lactasa, limitándose a la generación de síntomas gastrointestinales. Tampoco debe confundirse esta intolerancia con la *galactosemia*, la cual consiste en la incapacidad del organismo de metabolizar la galactosa en glucosa. En el tracto digestivo, la lactosa es hidrolizada en sus 2 componentes, glucosa y galactosa, los cuales pueden

[62] Suarez F., Shannon C., Hertzler S., Savaiano D. Encyclopedia of Food Sciences and Nutrition, 2nd Edition, Food Intolerance/Lactose Intolerance, Academic Press. 2003.

ser absorbidos por el intestino. Una vez en el torrente sanguíneo, esta galactosa es transformada en glucosa gracias a la acción de una serie de enzimas: galactosa mutarotasa (*transforma D-galactosa en α-D-galactosa*) – GALK (*transforma la α-D-galactosa en galactosa-1-phosphate*) - GALT (*transforma galactosa-1-phosphate más uridinadifosfato-glucosa en glucosa-1-fosfato y uridinadifosfato-galactosa*) - GALE (*transforma uridinadifosfato-galactosa en uridinadifosfato-glucosa*)[63]. La disminución de cualquiera de esta serie de enzimas provoca en el organismo una acumulación en sangre de galactosa la cual puede causar un deterioro fatal de la función hepática y renal, así como cataratas en recién nacidos o menores de corta edad. Estos síntomas pueden revertir si se elimina la galactosa de la dieta, sin embargo, a pesar del control de la dieta, hay un retraso en el crecimiento y desarrollo normal en la mayoría de pacientes, así como una insuficiencia ovárica en la mayoría de pacientes femeninas. Por tanto, aquellos alimentos en los cuales la lactosa ha sido hidrolizada, pero en los cuales no se ha eliminado la galactosa del alimento, no son aptos para el consumo por población afectada con galactosemia.

La lactosa no puede ser absorbida a través de la mucosa de la pared intestinal, sino que debe ser previamente hidrolizada para acceder al organismo. En aquellos casos donde la lactosa no es hidrolizada, esta pasa al intestino grueso, donde es fermentada por la flora intestinal transformándola en ácido láctico, ácidos grasos de cadena corta que son absorbidos por el colon, además de en gases como el hidrógeno, dióxido de

[63] EFSA, European Food Safety Authority. Scientific Opinion on lactose thresholds in lactose intolerance and galactosaemia. EFSA J. 8(9):1777 (2010) 1-29.

carbono o metano. Esta producción de gases es la responsable de la aparición de los diferentes síntomas gastrointestinales (dolor abdominal, flatulencia, nauseas, diarrea, etc.). Estudios concluyen que la dosis tolerable a partir de la cual se presentan este tipo de síntomas en población que sufre LNP, es de 12 g de lactosa[64].

Actualmente existen en el mercado diferentes productos derivados de la leche que han sido manufacturados específicamente para reducir su contenido en lactosa. Se etiquetan como *"bajos en lactosa"* o *"sin lactosa"*, dependiendo de la cantidad remanente que persista en el producto.

Actualmente no existe una armonización entre los países miembros para concretar un valor límite a partir del cual se deba emplear el término "*sin lactosa"*. En la *Directiva 2006/141/CE de la Comisión, de 22 de diciembre de 2006, relativa a los preparados para lactantes y preparados de continuación y por la que se modifica la Directiva 1999/21/CE Texto pertinente a efectos del EEE*, se establecen los niveles de lactosa permitidos para este tipo de preparados en base a valores determinados para población de corta edad afectados con galactosemia (10 mg/100 kcal), sin embargo, no se establecen valores para otro tipo de productos dirigidos a población sin ese tipo de afección.

[64] Wilt, T.J., Shaukat, A., Shamliyan, T., Taylor, B.C., MacDonald, R., Tacklind, J., Rutks, I., Schwarzenberg, S.J., Kane, R.L., Levitt, M. Lactose Intolerance and Health. Evidence Report/Technology Assessment No. 192 (2010) 1-399.

En la tabla 6 se recogen algunos de los valores establecidos por estados miembros para productos etiquetados como sin lactosa o bajos en lactosa:

***Tabla 6**. Threshold levels in some EU Member States for the use of the terms "lactose-free" and "low-lactose" in foods other than foods for particular nutritional uses. Fuente de la tabla.* [63]

Country	**"Lactose-free"**	**"Low lactose"**
Denmark	10 mg / 100 g	1 g / 100 g
Estonia	10 mg / 100 g	1 g / 100 g
Finland	10 mg / 100 g	1 g / 100 g
Norway	10 mg / 100 g	1 g / 100 g
Sweden	10 mg / 100 g	1 g / 100 g
Germany	100 mg / 100 g	NA
Slovenia	100 mg / 100 g	NA
Hungary	100 mg / 100g or mL	NA
Ireland	No lactose present No galactose present	1 g / 100 g

Según el Reglamento (UE) Nº 1169/2011 sobre etiquetado nutricional de los alimentos envasados que debe facilitarse al consumidor, se establece que entre la información que debe aparecer, debe incluirse aquellas sustancias susceptibles de provocar algún tipo de alergia o

intolerancia. De esta forma, aquellos consumidores con sensibilidades especiales, tales como aquellas que sufren intolerancia a la lactosa, puedan elegir aquellos productos que más se adecuen a sus necesidades con conocimiento de causa. Sin embargo, no se establece en este Reglamento menciones especiales por las que se puedan regir las denominaciones "*sin lactosa*" o "*bajo contenido en lactosa*" en el etiquetado de los alimentos.

Hasta que no se armonice una regla general a nivel de todos los países miembros, la Agencia Española de Consumo, Seguridad alimentaria y nutrición (AECOSAN) recomienda que en España se establezcan las siguientes denominaciones en función del contenido de lactosa residual que permanezca en el alimento[65]:

- 0.01% para alimentos considerados como "*sin lactosa*".
- 1% para alimentos considerados con "*bajo contenido en lactosa*".

El nivel de concentración de lactosa residual establecido para alimentos sin lactosa, tiene en cuenta la consideración de que este compuesto debe estar ausente en el alimento, y esta ausencia se acredita teniendo en cuenta que se siguen las técnicas analíticas más sensibles al estado actual de la ciencia. Por tanto, deben desarrollarse nuevas técnicas

[65] Agencia española de consumo, seguridad alimentaria y nutrición (AECOSAN). Subdirección General de Promoción de la Seguridad Alimentaria, Condiciones de Empleo de las Menciones: "Sin Lactosa" y "Bajo Contenido en Lactosa", Aprobada en Comisión Institucional de 30 de septiembre de 2015, Disponible en: http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/seguridad_alimentaria/interpretaciones/nutricionales/sin_lactosa.pdf (Visitada 18/10/2019)

analíticas que garanticen un contenido de lactosa lo más bajo posible en aquellos alimentos destinados a este grupo de población más sensible.

Existen disponibles diferentes tipos de métodos oficiales para la determinación de lactosa en leche: métodos polarimétricos (AOAC 896.01), gravimétricos (AOAC 930.28), espectrofotométricos en la zona de infrarrojo cercano (AOAC 972.16), que sin embargo no permiten diferenciar entre los distintos tipos de azúcares que conforman la fracción de carbohidratos de la leche. Por tanto, este tipo de metodologías no son adecuadas para la determinación específica de lactosa en aquellos productos que han sufrido hidrólisis de esta, ya que la cantidad en masa de azúcares de la muestra no varía.

Por otra parte, existen otro tipo de métodos analíticos oficiales para determinar lactosa (AOAC 2006.06), que se basan en la hidrólisis enzimática y posterior determinación de alguno de los monosacáridos resultantes. Normalmente, se determina la galactosa al estar presente la glucosa como componente adicional en numerosos alimentos. Una vez hidrolizada la lactosa, la galactosa resultante del proceso, es oxidada gracias a una enzima (galactosa deshidrogenasa) en la presencia de NAD+ para transformarse en ácido D-galactónico y reducir el NAD+ a NADH. Por tanto, la cantidad de NADH es directamente proporcional a la cantidad de galactosa, y así mismo, de lactosa presente inicialmente. Esta cantidad de NADH puede determinarse mediante absorbancia a una longitud de onda de 340 nm. La secuencia de reacciones que tienen lugar a lo largo de este proceso se resume esquemáticamente en la figura 6:

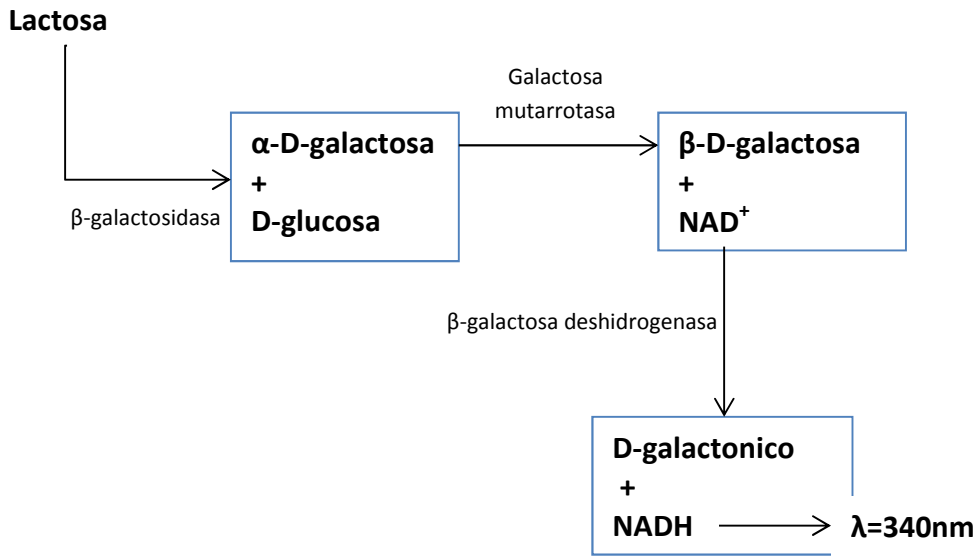

***Figura 6**. Resumen de las reacciones que tienen lugar en el proceso de determinación de lactosa mediante kit enzimático.*

Sin embargo, la β-galactosa deshidrogenasa actúa solamente sobre el anómero beta, siendo la cinética de conversión entre ambos anómeros muy lenta (del orden de 1 h). Este hecho hace que el proceso de determinación de lactosa mediante este tipo de procedimientos requiera un tiempo de incubación alto. Para evitar este fenómeno, se debe introducir una enzima adicional que catalice la transformación del anómero α en β de manera rápida (proceso patentado por la empresa "*Megazyme*").

Para la determinación de lactosa en productos sin lactosa o bajos en lactosa, este tipo de métodos presenta una desventaja debido a que estos productos han sido sometidos a procesos de hidrólisis, y por tanto, la cantidad presente de galactosa es muy elevada. Esto hace que previo a la determinación de la lactosa se debe eliminar la galactosa presente para no

interferir en la medición de la galactosa proveniente de la lactosa que se pretende determinar. Para ello, la muestra se debe someter a un proceso de reducción previo con borohidruro sódico en medio ácido, el cual reduce los azúcares presentes a sus correspondientes alcoholes (glucosa a sorbitol, galactosa a galactinol y lactosa en lactitol). Una vez neutralizado, la enzima hidroliza el lactitol en sorbitol más galactosa, la cual puede ser determinada mediante su reacción con β-galactosa deshidrogenasa y galactosa mutarrotasa en presencia de NAD^+ del mismo modo que se realiza para la determinación de lactosa en leche sin hidrolizar. Adicionalmente a la necesidad de tratamientos de muestra extra, este tipo de técnicas presentan la desventaja de que la determinación se realiza mediante medida de la absorbancia, por lo que para muestras coloreadas se necesita además un proceso previo de clarificación con polyivinilpirrolidona (PVP) que evite este tipo de interferencias.

La determinación cromatográfica de la lactosa, y en general de hidratos de carbono, presenta 2 grandes dificultades: la ausencia de grupos cromóforos y la elevada polaridad de la molécula.

La ausencia de grupos cromóforos hace que no puedan emplearse para su determinación detectores basados en la medida de la absorbancia o fluorescencia. Para poder emplearlos, se debe derivatizar previamente para incluir grupos funcionales que permitan el uso de este tipo de detectores. Para determinar lactosa sin recurrir a reacciones de derivatización, se utilizan normalmente detectores de *índice de refracción*. Sin embargo, este tipo de detectores adolece de la sensibilidad suficiente

como para poder detectar y cuantificar adecuadamente lactosa a nivel de trazas.

A continuación de enumeran alguno de los detectores empleados para la determinación de lactosa a nivel traza:

- Electroquímicos.
- Light scatering.
- Espectrometría de masas.

En el primer caso, el uso de detectores electroquímicos conlleva una serie inconvenientes como son: el alto pH necesario de la fase móvil (gradientes donde se emplea NaOH 50% junto con acetato sódico a alta concentración), falta de separación entre lactosa y compuestos interferentes en análisis de rutina, así como la alta presión que ofrece la columna catiónica necesaria (PA20 – Dionex). Respecto al detector por light scatering, el principal problema que presenta es que la respuesta ofrecida no es lineal, con lo que el modelo matemático que se debe emplear es más complejo, y este hecho dificulta el cálculo e interpretación del resultado final.

La otra gran dificultad a la hora de determinar cromatográficamente la lactosa, es la elevada polaridad de la molécula. Esta elevada polaridad hace que no puedan emplearse columnas con relleno hidrofóbico (fase inversa) debido a la poca interacción de la lactosa con este tipo de rellenos. En general, este tipo de moléculas apenas son retenidas y eluyen junto con el frente de fase móvil, obteniéndose una separación insuficiente que impide

su adecuada cuantificación. Para permitir que exista una interacción entre la fase estacionaria y la molécula de lactosa, se debe recurrir a columnas con rellenos altamente polares (fase normal). Sin embargo, este modo cromatográfico emplea fases móviles orgánicas apolares (hexano, acetato de etilo, etc.) las cuales son incompatibles con los medios acuosos donde es soluble la lactosa. Para solventar esta dificultad, se debe recurrir al modo HILIC (cromatografía líquida de interacción hidrofílica), la cual emplea columnas con relleno polar (sílice, -CN, $-NH_2$, etc.) aunque con fases móviles polares formadas normalmente mezclas de acetonitrilo y agua en un porcentaje inferior al 50% de agua. Este modo cromatográfico intermedio entre la fase reversa y normal, permite una buena separación de compuestos muy hidrofílicos ya que permite la interacción entre este tipo de moléculas con la fase estacionaria, al mismo tiempo que emplea fases móviles moderadamente polares que permiten una adecuada disolución de las mismas.

Señalar que en este modo, la fuerza elutrópica de la fase móvil aumenta al incrementar la proporción de agua en su composición, de manera contraria a lo que sucede en fase inversa, donde un alto contenido en agua provoca un aumento en la retención de los compuestos que se encuentra en el interior de la columna cromatográfica.

Uno de los rellenos polares más empleados para la determinación de azúcares en modo HILIC, es la sílice funcionalizada con grupos propilamino ($-NH_2$). Sin embargo, la lactosa tiene carácter reductor, ya que posee un grupo hidroxilo hemiacetal en el carbono 1 de la glucosa. Esto tiene relevancia analítica ya que puede reaccionar con grupos amino para formar

bases de Schiff. Debido precisamente al carácter reductor de la lactosa, esta reacciona con la fase estacionaria formando iminas:

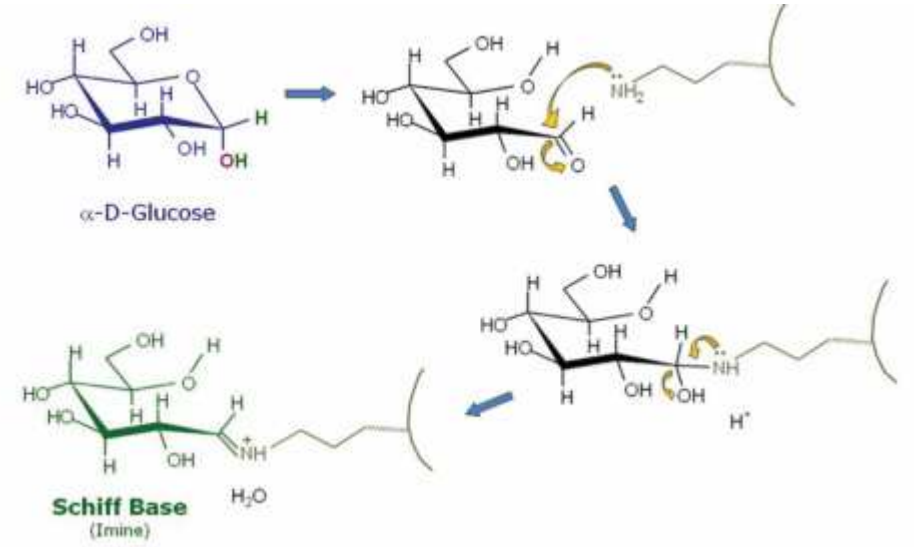

***Figura 7**. Reacción de la lactosa con la fase estacionaria tipo propilamino formando bases de Shiffs.*[66]

Por tanto, este tipo de relleno no es adecuado para la determinación cromatográfica de la lactosa, ya que provoca una deriva constante a lo largo de la vida útil de la columna en la que la respuesta del sistema cromatográfico va aumentando constantemente. La lactosa reacciona con la fase estacionaria y va pasivando los sitios activos a medida que se van inyectando muestras. La cantidad absoluta de lactosa capaz de atravesar la

[66] Jenkins K. HILIC separation of carbohydrates using BEH amide particle technology. Chromatography Today. November/December 2015. 14-18. Disponible en: https://www.chromatographytoday.com/article/bioanalytical/40/waters-corporation/hilic-separation-of-carbohydrates-using-beh-amide-particle-technology/1959 (Visitado 18/10/2019)

columna al comienzo de su vida útil, con muchos sitios activos intactos, será menor a la que es capaz de hacerlo tras varias tandas de muestras, ya que la cantidad de grupos propilamina habrá disminuido notablemente, observándose un aumento constante en la respuesta de sistema, al tiempo que disminuye la capacidad de separación de la columna, aumentando el factor de cola y disminuyendo la resolución entre picos.

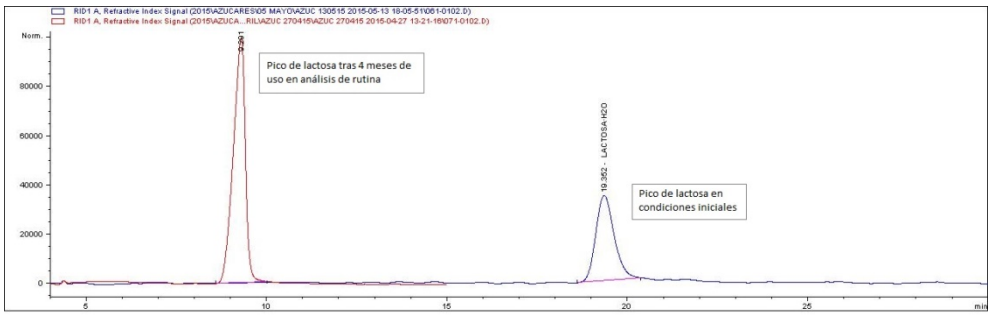

***Figura 8**.Cromatogramas obtenidos con una columna amino nueva y usada tras 3 meses de uso en rutina (aproximadamente 500 inyecciones).*

Para evitar estos inconvenientes, se ha empleado una columna funcionalizada con grupos ***amida*** en lugar de con grupos propilamina. De esta forma se consigue un relleno altamente polar y de comportamiento similar al relleno amino, pero con unas propiedades químicas diferentes, que hacen que no se puedan formar iminas y por tanto se evita la reacción entre lactosa y fase estacionaria. De esta forma, se obtienen buenas separaciones con resoluciones (superiores 1.5) y constantes a lo largo de la vida útil de la columna. Este relleno amida es además compatible con el empleo de detectores de masas debido a su bajo sangrado, lo cual lo hace adecuado para su uso en métodos de determinación de lactosa a bajas concentraciones.

Para emplear este tipo de relleno en la determinación de azúcares con carácter reductor, se debe adicionar un aditivo a la fase móvil que aumente el pH de la misma. Esto es debido a que este tipo de azúcares poseen 2 formas anoméricas: α y β, que desdoblan el pico cromatográfico a pH neutro y temperatura ambiente.

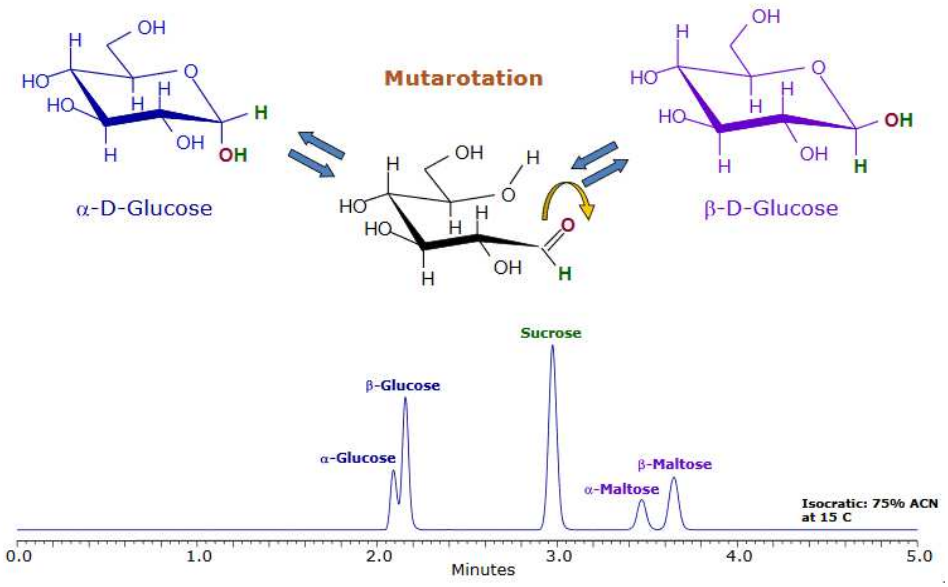

Figura 9. *Formas anoméricas de la glucosa y cromatograma correspondiente donde se puede observar el desdoblamiento de los picos cromatográficos debido a la separación de ambas formas.*[67]

[67] Ross E. Waters amide column technology for food analysis. Waters Corporation (2012) Disponible en: http://www.waters.com/webassets/cms/library/docs/local_seminar_presentations/Nordic_Amide_for_food_presentation_Euan.pdf (Visitada 18/10/2019)

Para evitar este fenómeno se debe adicionar NH_3 para aumentar el pH al tiempo que se debe aumentar la temperatura de la columna para colapsar ambas formas y obtener así un pico cromatográfico definido.

2.3.2. Objetivos

En este trabajo se ha desarrollado un método para la determinación de lactosa a nivel de trazas en leche y productos lácteos hidrolizados dirigidos a personas con intolerancia a este compuesto. Para ello, se ha empleado una columna con relleno polar (amida) en fase HILIC acoplado a detectores de espectrometría de masas triple cuadrupolo.

La finalidad del método es el análisis de rutina de un alto número de muestras con resultados de buenas características analíticas, esto es, un método de rutina validado. Para alcanzar este objetivo, se debe desarrollar un tratamiento de muestra rápido y con un bajo consumo de reactivos y disolventes, con el cual se obtenga un extracto limpio compatible con la determinación por espectrometría de masas para la adecuada cuantificación de lactosa a bajas concentraciones sin la existencia de interferentes.

Esta metodología analítica ha sido validada y aplicada a muestras reales, siendo la base para la implantación en el laboratorio de Servicios Analíticos de Biosearch S.A. de un método de rutina acreditado por ENAC según la norma UNE-EN ISO/IEC 17025.

2.3.3. Publicación V

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Determination of residual lactose in lactose-free cow milk by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry

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ABSTRACT Lactose is the major carbohydrate found in milk and dairy products. Lactose intolerance means the body cannot digest foods with this natural sugar in them. In this context, the lactose-free market has experienced a steep increase in recent years. A new method for the determination of residual lactose in lactose-free dairy products using liquid chromatography coupled to tandem mass spectrometry triple quadrupole (HPLC-MS/MS) has been developed. Hydrophilic interaction chromatography (HILIC) has been used for this purpose. An amide chromatographic column with an alkaline mobile phase were selected as

optimal. In addition, a fast, cost-effective and reliable sample treatment has been developed for routine analytical laboratory use. The method has been validated by using matrix-matched calibration standards and a recovery assay on a lactose-free milk sample obtained by lactase hydrolysis of regular milk. The limit of quantification (LOQ) was 15 mg L^{-1}, while the recovery was close to 100% with relative standard deviation lower than 9 % in all cases. The method was applied to several lactose-free products and the results showed that lactose values in these products are not always below the recommended maximum value of 100 mg L^{-1}.

Keywords: Food safety; Food Composition; Food Analysis; Lactose intolerance; Lactose-free milk products; HILIC-HPLC-MS/MS; Amide column; High accuracy; High throughput

1. Introduction

Lactose is a major component of milk from mammals and has important nutritional and prebiotic properties. Lactose concentration in human milk is relatively high (7.0%), while in cow milk is about 4.6% (Perati et al., 2016). In the small intestine, it is hydrolysed by the enzyme lactase (β-galactosidase) into glucose and galactose to allow absorption through the intestinal mucosa (van Scheppingen et al., 2017). Approximately 70% of the global adult population and 95% of Asian population have lactase deficiency and are unable to digest lactose (ADILAC, 2016; Schaafsma, 2008). This is known as lactose intolerance (LI). Newborn mammals subsist on milk over the first few months of life, and after weaning there is a genetically-programmed decrease in lactase expression. A large majority of

humans show this typical lactase decrease early in life, therefore, adults are unable to properly digest lactose. These individuals are lactose intolerant and are said to have the trait of lactase non-persistence (LNP). Congenital absence of lactase due to a mutation in the gene that is responsible for producing the enzyme is a very rare cause of lactase deficiency, and the symptoms of this type of lactase deficiency begin shortly after birth (Ingram et al., 2009). LI is bothersome but usually not serious and symptoms include abdominal pain, diarrhea, abdominal bloating and distension which reflect the osmotic effects of the unassimilated lactose in the intestinal lumen, plus the fermentation products (such as hydrogen and methane) generated by bacteria in the large intestine.

The demand for lactose-free products is driven by the high prevalence of LI and the worldwide increase in incidences of food intolerances. The dairy industry is continually launching new lactose-free food products. Although the European Food Safety Agency sets the limit of residual lactose content for products labelled as "lactose-free" to 1 g L^{-1}, many dairy companies have set a lower value (0.01%) as a quality feature (Spanish Agency for Consumer Affairs, Food Safety and Nutrition, 2017). In addition, some dairy foods are marketed using the claim ''low-lactose". In that case, the concentration of lactose is < 5 g L^{-1} (Trani et al., 2017). The resulting dairy products contain varying amounts of residual lactose.

In this context, there is a clear need for simple analytical methods to monitor the amount of residual lactose in these products in routine quality control analysis. Different methods have been traditionally applied for the

determination of residual lactose such as differential pH techniques, paper chromatography (Honer and Tuckey, 1953) and gas chromatography (Idda et al., 2016). The current methods recommended by the Association of Official Analytical Chemists (AOAC) are based on the enzymatic hydrolysis of lactose to glucose and galactose followed by different biochemical pathways. The detection of reaction products is done with uv-vis or fluorescence (Bankar et al., 2009; Essig and Kleyn, 1983; Lynch et al., 2007; Megazyme International Ireland, 2014; Trani et al, 2017). The main drawbacks of these methods are that they need large amounts of reagents and all them also require sample deproteinization because proteins are interfering compounds. Additionally, the presence of large amounts of glucose and galactose in hydrolysed milk may also result in interferences. An additional reduction with sodium borohydride is required to obtain non-interfering sugar alcohol derivatives (Megazyme International Ireland, 2014). To our knowledge, and due mainly to the interferences described above, these methods have been validated for regular milk and dairy products, but not for low-lactose or lactose-free products.

Normal-phase chromatography with refractive index detector has been commonly used for the determination of sugars (Chavez-Servin et al., 2004; Trani et al., 2017; Indyk et al., 2004). However, this detector lacks sensitivity and is not appropriate for low/free-lactose products. The use of ion pair in the mobile phase (Erich et al., 2012) or the derivatization for fluorescent or mass spectrometric detection have been also reported (Mopper, 1983; Zhang et al., 2010), but these procedures are tedious and expensive. More recently, a method based on the use of HILIC-HPLC

coupled with mass spectrometry has been reported for residual lactose determination with good results (Trani et al., 2017). This method uses an amine stationary phase, but the use of amine for routine analysis produces stability problems due to the reactivity of reducing the sugar with the stationary phase. Additionally, the LOQ of this method is below the EFSA recommendations (EFSA, 2010) (100 mg L^{-1} for products labelled lactose-free). High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is the most commonly used chromatographic technique (Trani et al., 2017; QCL, 2010; van Scheppingen et al., 2017). The main drawback in this case is that the laboratory needs specific instruments capable of managing highly alkaline mobile phases. Moreover, the resolution obtained in most cases is low due to the presence of interferences that makes adequate quantification in routine analysis difficult. This usually occurs when the column has analysed several sets of samples.

The aim of this work is to validate a simple, reliable, fast and cost-effective analytical method to determinate residual lactose in milk using HILIC-HPLC-MS/MS. The method has been applied to different commercial lactose-free milk samples, proving a useful tool for routine quality control analysis.

2. Materials and methods

2.1. Chemicals and reagents

Water (18.2 MΩ cm) was purified using a MilliQ system from Millipore (Molsheim, France). Analytical grade standards, lactose monohydrate and

melezitose were purchased from Sigma-Aldrich (St. Louis, MO, USA). A total of 100 mg of lactose (105 mg of lactose monohydrate) were accurately weighted and diluted in 100 mL of purified water in a volumetric flask. The melezitose solution, used as surrogate, was prepared in a volumetric flask by dissolving 40 mg of the solid substance in a final volume of 100 mL of purified water. Mass spectrometry grade acetonitrile, ammonium hydroxide, phosphotungstic acid · $4H_2O$, zinc acetate · $2H_2O$, and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lactase Ha5200 was purchased from CHR-HANSEN Holding A/S (Hoersholm, Denmark). Phosphotungstic acid solution for protein and fat precipitation was prepared in a 100 mL volumetric flask by dissolving of 9.1 g of zinc acetate and 5.46 g of phosphotungstic in 70 mL of water. After complete dissolution, 5.8 mL of glacial acetic acid were added and the flask was filled to the mark with water (International dairy federation, 1998). The solution was stored in a dark and cool place for a maximum of five days.

2.2. Instrumentation and software

HPLC-MS/MS analysis of lactose was performed using an Agilent Technologies 1200 series HPLC instrument coupled to a 6460 triple quadrupole mass spectrometer (Palo Alto, CA, USA) with electrospray ionization (ESI) source. The control of the instrument was carried out with MassHunter WorkStation software vs. B.08.00. For HPLC-MS analyses, four type of columns were used: two propylamine columns: Carbohydrate-NH_2 (5 µm, 250 x 4.6 mm) purchased from Teknokroma (Barcelona, Spain) and Luna-NH_2 (5 µm, 250 x 4.6 mm) from Phenomenex (Torrance, CA, USA); a

BEH Amide column (3.5 µm, 250 x 4.6 mm) from Waters (Milford, MA, USA); and a strongly acid cation exchange resin MCI®GEL CK08E (9.0 µm, 8 x 300 mm) from Mitsubishi Chemical (Tokyo, Japan), were tested as stationary phases. For HPAE-PAD analysis, assays were carried out with a chromatograph Dionex AMMS III-4 mm equipped with an AS50 autosampler, an AS50 column compartment, an ED50 electrochemical detector, and a GP50 gradient pump. The software that control of the instrument was Chromeleon (v. 6.40). A strong anion-exchange column Dionex PA20 (150 x 3 mm) provided with a guard column (30 x 3 mm) from Thermo Fisher Sci (Sunnyvale, CA, USA) was tested. Other laboratory equipment such as an Eppendorf microcentrifuge (Eppendorf, Hamburg, Germany) and a vortex mixer (Heidolph, Schwabach, Germany) were also used. For calibration and statistical analyses of data, Statgraphics Plus software vs 5.0 (Manugistics Inc., Rockville, MD, USA) was used.

2.3. Extraction procedure

Based on the procedure previously proposed by Zafra-Gomez et al. (2008) an aliquot of 500 µL of homogenized milk sample (raw, whole, semi-skimmed and skimmed milk) was introduced into a 1.5 mL Eppendorf tube and 100 µL of the melezitose internal standard solution (0.4 g L^{-1}) was added. After vortex homogenization for 10 seconds, 600 µL of pure acetonitrile was added and vortexed for 1 min. The mixture was centrifuged for 5 min at 14.500 rpm (14.100 x g) and the supernatant was filtered through a 0.22 µm cellulose regenerated filter. The final extract was directly injected into the HPLC-MS/MS system. The analysis was carried out in triplicate.

2.4. Preparation of calibration standard and spiked samples

For calibration purposes, different volumes of standard solutions (250 µL, 500 µL, 750 µL, 1000 µL, 1250 µL and 1500 µL of a 1000 mg L^{-1} lactose standard solution) were transferred to 10 mL volumetric flasks. After filling to the mark with water, 500 µL of each standard solution underwent the previously described sample treatment procedure. For standard addition studies and matrix-matched calibration, different volumes of the standard solutions (250 µL, 500 µL, 750 µL, 1000 µL, 1250 µL and 1500 µL of a 1000 mg L^{-1} lactose standard solution) were transferred to 10 mL volumetric flasks filled to the mark with lactose-free milk. Finally, 500 µL of each standard solution were treated according the previously described sample treatment. The lactose-free milk was obtained by adding 1.6 mL of lactase to 250 mL of UHT milk, which was allowed to hydrolyze for 10 hours at 25°C. The hydrolysed milk was heated to the boiling point in order to completely deactivate lactase.

2.5. HPLC-MS/MS conditions

Four of the most common stationary phases used for the determination of sugars by liquid chromatography were tested in order to obtain an adequate separation. Two propyl-amine columns, Carbohydrates NH_2 (5µm, 250 x 4.6 mm) and Luna NH_2 (5 µm, 250 x 4.6 mm); a BEH Amide (3.5 µm, 250 x 4.6 mm); a strong acid cation-exchange column MCI®GEL CK08E (9.0 µm, 8 x 300 mm), and a strong anion-exchange column Dionex PA20 (150 x 3 mm) were investigated. Except for the Dionex PA20 column, the columns were tested in the same HPLC-MS/MS instrument, with only

the BEH Amide column showing an amount of bleed low enough for mass spectrometry determination. Therefore, the chromatographic separation was carried out using a Waters BEH Amide column (250 x 4.6 mm, 3.5 µm). The mobile phase was a mixture of acetonitrile and deionized water (75:25, v/v) with 0.1% of NH_3 (flow of 1.0 mL min^{-1}); the column temperature was 45 ^{o}C, and the injection volume was 3 µL. The sample tray was maintained at 20 ^{o}C. Total run time was 22 min.

Electrospray ionization (ESI) in negative ion mode was used for MS analysis. The source employed in this method uses "jet stream" technology, which enhances the sensitivity by the inclusion of an additional stream of N_2 that focuses the spray. This additional hot nitrogen stream favours the desolvation of the spray, whereby the fogging pressure, flow and temperature of the drying gas may be lower. This is very important in this application, to prevent possible phenomena of polymerization of sugars at high temperatures at the interface. The tandem mass spectrometer was operated in the multiple reactions monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Table 1 summarizes the mass transitions for each compound studied. Other instrument parameters were: source gas temperature, 300 ^{o}C; source gas flow, 5 L min^{-1}; nebulizer pressure, 20 psi; sheath gas temperature, 275 ^{o}C, sheath gas flow, 7 L min^{-1}; capillary voltage, -3.5 kV; nozzle voltage, 0 V; collision gas flow, 0.15 mL min^{-1}, and nebulizer gas pressure, 20 psi. Nitrogen (99.995%) was used as desolvation gas and nitrogen (99.999%) as collision gas. Dwell time was set at 200 ms, and a solvent delay of 12.5 min

was set in order to avoid ion suppression. Table 1 also shows the collision energies (CE) and cell accelerator voltages optimized for each transition.

Table 1 Mass transitions for lactose and internal standard

	SRM1	CE	SRM2	CE
Melezitose	503→323	16	503→89.1	24
Lactose	341→161	0	341→101	0

[a] SRM1, transition used for quantification; SRM2, transition used for confirmation; [b] CE, collision energy (V)

2.6. Quality control. Quality assurance of the method

In order to ensure the validity of the analytical results, a quality control was performed by addition of blank samples. A hydrolysed milk sample with a lactose content below the limit of quantification was spiked alternatively at different days at three different levels (20, 100 or 150 mg L^{-1} of lactose) in each set of samples, so that ever three sample set, the whole linear range will be controlled. Additionally, a standard solution prepared in 50:50 (v/v) acetonitrile:water at the same concentration as the spiked samples was injected in duplicate for each set of samples.

3. Results and discussion

The BEH Amide column selected showed an amount of bleed low enough for mass spectrometry determination. The amine column reacts with reducing sugars such as lactose, forming a Schiff's base. This causes a constant signal increase of reducing sugars over the lifetime of the column, and a loss of resolution and a decrease in the retention time of the

compounds. As a result, the constant variation of the response factor needs to be taken into account to compensate for this effect, which can induce errors in reliability and precision of the results.

It is important to highlight that since the amide group has no basic character, an alkaline modifier should be added to the mobile phase in order to improve the separation. The reason is that α and β anomeric forms of sugars are resolved in neutral media, which can give chromatograms with two peaks making difficult an adequate integration. The more alkaline the medium is, the faster the equilibrium between the α and β anomeric forms is reached, resulting in a well-defined single peak in the chromatogram. This effect is enhanced by increasing the column temperature. Therefore, temperatures in the range 20 - 60 ^{o}C were investigated and 45 ^{o}C was selected as the optimal column temperature.

As mentioned before, the most commonly used chromatographic system for analysis of residual lactose involves the use of strong anion-exchange resins coupled to amperometric detection (HPAE-PAD). The chromatographic parameters used for this separation were the described by the manufacturer in the application note 248 for lactose free products (Perati, et al., 2016). However, some drawbacks of HPAE-PAD have been found when applied for routine analysis including: long run times (over 60 min per sample), presence of an unknown interfering compound that affect to an appropriate integration of lactose, and the need for using highly alkaline mobile phases, as shown in figure 1.

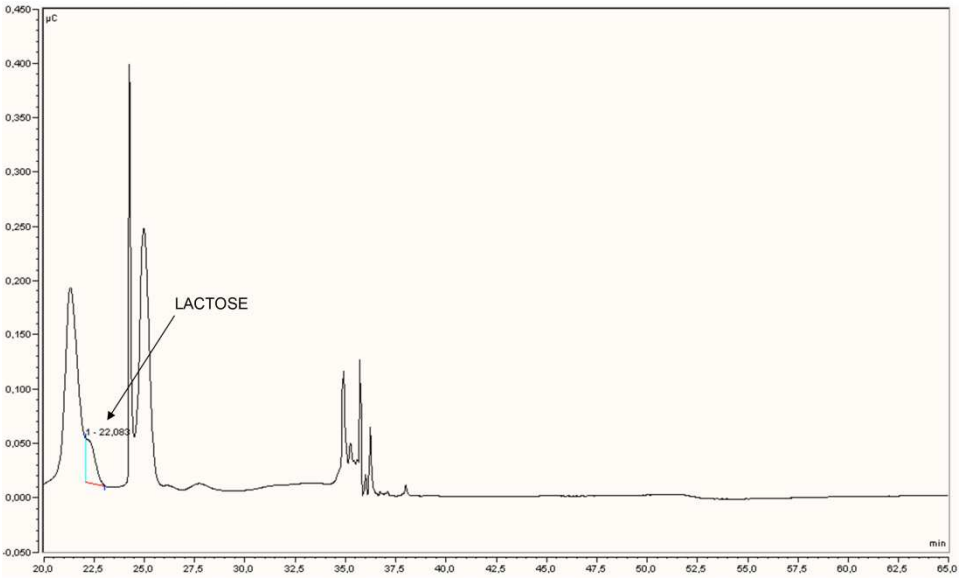

Fig. 1 *Chromatogram obtained using HPAE-PAD.*
The chromatogram corresponds to a commercial sample of lactose free milk.

In contrast, when using HPLC-MS/MS there are not interfering substances allowing for an appropriate integration and quantification of lactose, as shown in figure 2.

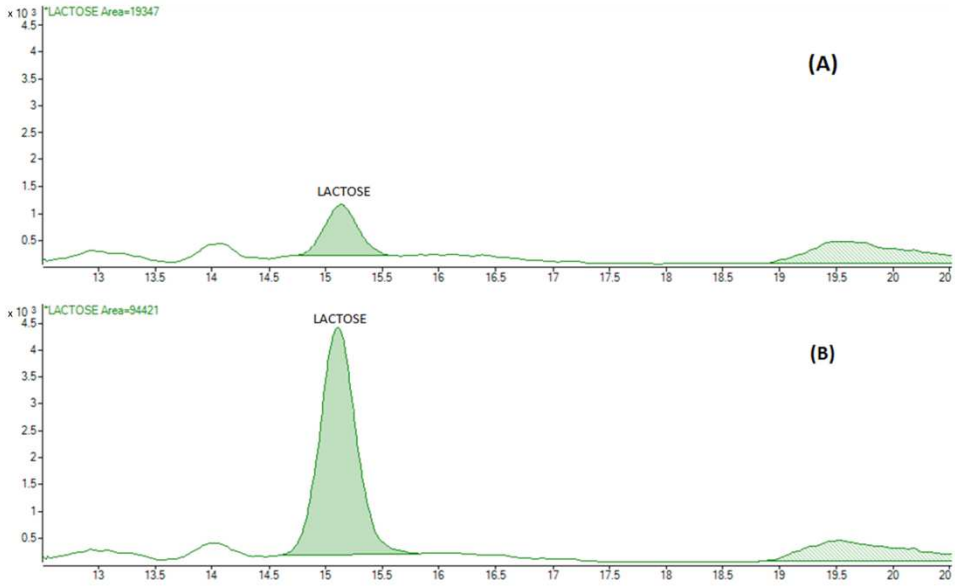

Fig. 2 *(A) Chromatogram of a sample with 35 mg L^{-1} of added lactose; (B) Chromatogram of a sample with 150 mg L^{-1} of added lactose.*

3.2. Sample treatment strategy

Once the chromatographic system was selected and optimized, the most adequate sample treatment strategy was investigated in order to achieve high throughput, reliability and robustness in the application of the method. Lactose is a highly polar compound that is very soluble in water. The sample treatment should be able to separate milk sugars from milk proteins (mainly caseins) and fats. Different methods based on isoelectric precipitation of caseins (Perati et al., 2016; Trani et al., 2017; Zafra-Gómez et al., 2006) and on the addition of organic solvents (Zafra-Gómez et al., 2008) have been described in the literature. In this work, these two

strategies have been tested in order to optimize the sample treatment in terms of high throughput and robustness, with high accuracy (trueness and precision) of the analytical method.

The method based on isoelectric precipitation involves adjusting the pH to the value at which casein is least soluble (the isoelectric point) by addition of 1 mL of a phosphotungstic acid solution to 5 mL of milk. After shaking for 30 seconds and reacting for 15 min, the mixture was centrifuged at 4500 rpm (1920 x g) for 5 min, and 500 µL of the supernatant were introduced in a chromatographic vial containing 100 µL of internal standard solution (0.4 g L^{-1}) and 400 µL of milli-Q water.

Casein precipitation by organic solvent addition was done according to the procedure described in Section 2.3. The extracts obtained with both treatments were directly injected in the HPLC-MS/MS system. The recovery was calculated from the initial concentrations of spiked lactose. The results obtained are shown in table 2.

Table 2 Comparison between sample treatments: pH adjustment and organic solvent addition

	pH adjustment method		Organic solvent addition method	
Spiked concentration (mg L^{-1})	Concentration found (mg L^{-1})	Rec (%)	Concentration found (mg L^{-1})	Rec (%)
24.9	19.7	79	25.1	101
	16.1	65	25.8	104
49.8	37.3	75	50.3	102
	28.4	57	49.2	99
74.7	52.7	71	74.5	100
	41.2	55	73.9	99
	Average Rec (%)	67	Average Rec (%)	101
	RSD (%)	9.7	RSD (%)	1.8

Rec: Recovery; RSD: Relative Standard Deviation

The results show that the addition of organic solvent sample treatment results in better recoveries, precision data and cleaner extract. This may be due to the dirt accumulated in the ion source of the electrospray probe when the extract obtained after pH adjustment is injected in the HPLC system.

3.2. Validation of the method

A six-point curve was obtained for lactose in the linear dynamic range from the LOQ to 150 mg L^{-1}. The calibration curve was constructed using the analyte/surrogate peak area ratio versus concentration of analyte. First, the matrix effect was estimated using two calibration curves for each compound: one in the initial mobile phase and the second one in milk. The statistical comparison of the slopes of the calibration curves was carried out with a Student's t-test. The t calculated was compared with a tabulated t value (t_{tab}) for the appropriate number of degrees of freedom and the appropriate level of confidence P (%). Since the p-value obtained for slopes comparison (0.0054) is lower than 0.01, it can be concluded that there are statistically significant differences between slopes for a 99% of probability, and consequently, the matrix-matched calibration was used for quantification. The quality parameters obtained for the calibration curve of lactose are summarized in table 3.

Table 3 Analytical and statistical parameters.

Calibration in milk			
Parameter		Parameter	
b (L mg^{-1})	$2.04 \cdot 10^{-2}$	P_{lof} (%)	13.95
S_b (L mg^{-1})	$3.37 \cdot 10^{-4}$	R^2 (%)	99.8
a	$7.86 \cdot 10^{-3}$	LOD (mg L^{-1})	5
S_a	$2.65 \cdot 10^{-2}$	LOQ (mg L^{-1})	15
$S_{y/x}$	$2.06 \cdot 10^{-2}$	LDR (mg L^{-1})	15 – 150
Calibration in aqueous solution			
Parameter		Parameter	
b (L mg^{-1})	$1.89 \cdot 10^{-2}$	P_{lof} (%)	27.7
S_b (L mg^{-1})	$2.52 \cdot 10^{-4}$	R^2 (%)	99.9
a	$7.85 \cdot 10^{-3}$	LOD (mg L^{-1})	5
S_a	$1.98 \cdot 10^{-2}$	LOQ (mg L^{-1})	15
$S_{y/x}$	$1.77 \cdot 10^{-2}$	LDR (mg L^{-1})	15 – 150

b = slope; S_b = slope standard deviation, a = intercept; S_a = intercept standard deviation; $S_{y/x}$ = regression standard deviation; R^2 = determination coefficient; LOD = limit of detection; LOQ = limit of quantification; LDR = linear dynamic range

According to the guidelines for Bioanalytical Method Validation (FDA, 2001), the analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision).

Linearity. The determination coefficient (R^2) and the lack-of-fit test (P_{lof}) were evaluated. The value obtained for R^2 was 99.8%. P_{lof} value was

higher than 5%. Therefore, good linearity was observed within the concentration ranges. The residue analysis graph has also been included as supplementary material (Figure S2.)

Selectivity. This parameter was studied by HPLC-MS/MS analysis of blanks. A blank sample obtained by complete lactase hydrolysis of UHT milk (20 hours at 37^{o}C) and a sample obtained by partial hydrolysis (5 hours at 37^{o}C) containing 20 mg L^{-1} of residual lactose were treated and the corresponding chromatograms compared (Figure 3).

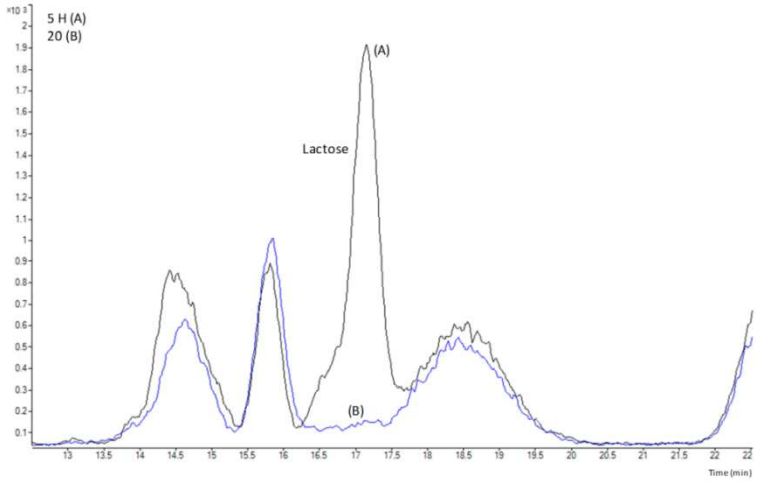

Fig. 3 *Comparison of chromatograms of UHT milk partially (A) and totally (B) hydrolysed by lactase.*

No interferences were observed at the analyte retention time (17 min). These findings suggest that the spectrometric conditions ensure a high selectivity of the method.

Sensitivity. The limit of detection (LOD) and quantification (LOQ) were calculated by taking into account the standard deviation of the intercept (s_a) as an estimation of the standard deviation of the blank (s_o) and the slope (b) of the calibration curve. The LOD was estimated as $3 \cdot s_o$ and the LOQ was $10 \cdot s_o$. Table 3 shows the results obtained.

Accuracy (precision and trueness). A recovery assay was carried out to validate the method in terms of trueness. Recoveries were determined by standard addition of lactose to a blank free-lactose milk matrix obtained by hydrolysis with lactase as described above, and by comparison of the results obtained from a proficiency test material (German reference office for food proficiency testing and reference materials, DRRR) to semi-skimmed and whole UHT milk samples. The concentration of lactose was determined by interpolation in the standard calibration curve within the linear dynamic range and compared to the amount of analytes previously added to the blank lactose-free milk and those obtained from the proficiency test material. Table 4 shows the results obtained.

Table 4 Accuracy study. Trueness and precision of the method.

Standard addition study							
	Day 1		Day 2		Day 3		
Added (mg L^{-1})	Found (mg L^{-1})	Rec (%)	Found (mg L^{-1})	Rec (%)	Found (mg L^{-1})	Rec (%)	RSD (%)
23	22	94	21	95	21	95	2.5
	24	102	22	92	23	96	4.1
	24	101	22	92	22	92	3.8
73	66	91	62	94	72	109	7.6
	68	93	64	94	69	102	4.0
	69	95	68	99	76	110	6.4
140	139	99	136	98	158	114	8.4
	145	104	143	99	164	113	7.8
	146	104	143	98	161	110	6.5
Proficiency test results (mg L^{-1})							
Lactose-free whole UHT milk				20			
Lactose-free semi-skimmed UHT milk				25			

Rec: Recovery; RSD: Relative Standard Deviation

In the recovery study, the values were close to 100% in all cases (91-114%). The data from the proficiency test did not offer evaluable information because of the heterogeneity of the data provided by the participants. They used very different analytical methods (infrared, enzymatic, IC HPLC and HPLC-MS/MS), and the LOQs obtained were very different. This did not allow the calculation of the z-score, but the organizers of the proficiency test concluded that the results obtained by our laboratory were highly satisfactory because were found to be fewer than 100 mg L^{-1} in both cases.

The precision of the method in terms of intra- and inter-day variability was evaluated using spiked lactose-free milk samples at three concentration levels. Precision (expressed as relative standard deviation, %RSD) was determined from triplicate spiked samples during the same day and in three different days. The values obtained are also summarized in Table 4. RSD values were between 2.5% and 8.4%, being the inter-day precision lower than 10%. Therefore, all compounds were within the acceptable limits, which are considered <15% of the actual value (FDA, 2001). The data demonstrate the repeatability of the method.

Precision and trueness data indicate that the method to determine the target compound in milk samples is accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the method performance.

3.3. Application of the method

The method was applied to 12 samples of whole, semi-skimmed and skimmed milk collected from different supermarkets located in the city of Granada (South-East of Spain). The samples were collected from milk labeled as “lactose free” from well-known brands and from brands with the largest market share in Spain. Brands were selected based on the lactose levels recommended by the Spanish legislation (lactose <0.01g/100g). Table 5 summarizes the results obtained. The results demonstrate that many of the milks labelled “lactose-free” had undergone incomplete hydrolysis as lactose concentration was above 100 mg L^{-1}. This is probably due to the application of different industrial technologies for the hydrolysis processes, where different yield ratios are reached (Jelen and Tossavainen, 2003).

Table 5 *Lactose content found in samples from different UHT lactose free liquid milk products, labelled as <0.01g/100g in the Spanish market*

Product		Lactose (mg L^{-1})
Brand A	skimmed milk	71 ± 11
	semi-skimmed milk	76 ± 12
Brand B	skimmed milk	108 ± 17
	semi-skimmed milk	103 ± 16
	skimmed milk	105 ± 16
	semi-skimmed milk	106 ± 16
Brand C	semi-skimmed milk	44 ± 7
	whole milk	57 ± 9
Brand D	skimmed milk	80 ± 13
	semi-skimmed milk	39 ± 6
	semi-skimmed milk	145 ± 23
	whole milk	135 ± 21

The milk hydrolysis can be done before or after the heat treatment. When lactase is added after the heat treatment, hydrolysis takes place during inside the package over time, during the storage and distribution of the processed milk and the residual lactose level is usually much lower. However, this process requires the use of aseptic enzyme, which is more expensive than the non-aseptic form. On the other hand, when the enzyme is added before the heat treatment, the non-aseptic enzyme is denatured, and the hydrolysis process should be completed by batch wise, therefore, higher quantities of enzyme and longer times are required to ensure an

adequate hydrolysis yield (Harju et al., 2012). However, incomplete hydrolysis can be caused by insufficient reaction times or enzyme dosage, resulting in higher levels of residual lactose.

4. Conclusions

A simple novel method for the determination of lactose in lactose-free milk has been developed and validated using HPLC coupled to tandem mass spectrometry triple quadrupole detector. The method employs the trisaccharide melezitose as internal standard (surrogate) and a simple procedure for sample treatment. Milk blank was obtained by lactase hydrolysis of UHT milk. The method has demonstrated its reliability, sensitivity, repeatability and high throughput in comparison to other methods previously proposed in the literature, where the presence of interferences (HPAE-PAD) or tedious sample preparation and irreproducibility (derivatization, ELISA kits, etc.), make difficult the adequate quantification of lactose in lactose-free milk at trace levels. The selection of the proper column and the optimization of the ionization conditions for the mass spectrometer proved to be key to develop a reliable method for routine analysis. The method has been validated and is currently being used by the company Biosearch S.A. for routine analysis. The validated method is in the process of being certified by the ENAC (the Spanish Entity for Accreditation).

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2.4. Hidrocarburos aromáticos policíclicos en extractos vegetales

2.4.1. Introducción

Los hidrocarburos aromáticos policíclicos (PAHs) son una familia de compuestos semivolátiles que están formados por condensación de 2 o más anillos aromáticos los cuales carecen de heteroátomos o grupos funcionales[68]. Los PAHs que contienen 5 o más anillos aromáticos son más estables y tóxicos que aquellos con menos de 5 anillos[69]. Son compuestos muy estables y químicamente inertes que provienen de la combustión incompleta de materia orgánica. Estos compuestos se distribuyen vía aérea y son introducidos en diferentes procesos ambientales a través de su depósito en medios acuosos, suelos o plantas. Como consecuencia, aquellas plantas desarrolladas en suelos contaminados, absorberán este tipo de contaminantes. Además de por contaminación ambiental, los PAHs pueden ser introducidos en la cadena trófica por los procesos industriales a los cuales son sometidos los alimentos (secado, ahumado, calentamientos, etc.). En este caso, los PAHs son formados por la degradación térmica de los componentes orgánicos del alimento (triglicéridos, aminoácidos, etc.) que pueden sufrir reordenamientos tipo Diels-Alder. La mayoría de la

[68] Kataoka, H., Ishizaki, A. Handbook of Polycyclic Aromatic Hydrocarbons. Chapter 2 Polycyclic aromatic hydrocarbons in foods and herbal medicines. Analysis and occurrence. School of Pharmacy, Shujitsu University, Nishigawara, Okayama, Japan, Nova Science Publishers, 2013.

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población está expuesta a este tipo de contaminantes debido a fuentes alimentarias (salvo el caso de fumadores o por causas laborales)[70].

La mayor preocupación respecto a la ingesta de PAHs es el riesgo de aparición de diferentes tipos de cáncer, ya que este tipo de contaminantes son considerados como altamente carcinogénicos y mutagénicos, estando implicados en la aparición, entre otros, de cáncer de mama, pulmón y colon en humanos[71-77]. Los PAHs son metabolizados a través de diferentes vías en los tejidos, resultando en productos intermedios como epóxidos,

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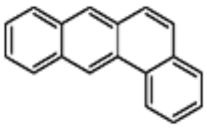
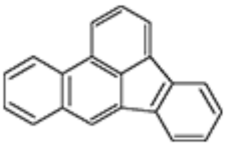
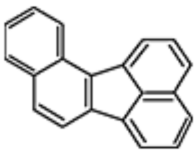
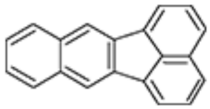
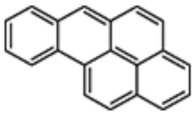
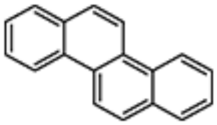
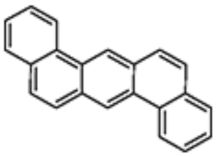
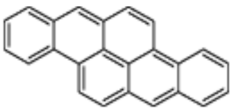
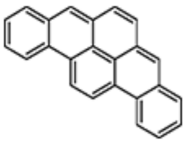
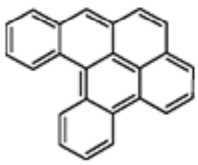
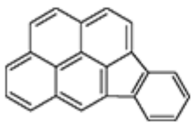
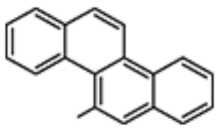
dihidrodioles, fenoles, quinonas y sus posibles combinaciones[78]. Estos metabolitos (principalmente epóxidos) se unen covalentemente a las cadenas de ADN y a otras macromoléculas como proteínas, y presumiblemente son los responsables de iniciar los procesos de mutagégesis y carcinogénesis celular. La "International Agency for Research on Cancer" (IARC) clasificó a los PAHs como carcinogénicos (Grupo 1) o probables (Grupo 2A y Grupo 2B) cancerígenos humanos de acuerdo a la siguiente tabla[79]:

Tabla 7. *Clasificación de la IARC de los 12 PAHs más relevantes.*

Compuesto	Estructura química	Grupo
Venzo(a)antraceno		2B
Venzo(b)fluorantrene		2B
Venzo(j)fluorantrene		2B

[78] U.S. Department of health and human services, Public Health Service Agency for Toxic Substances and Disease Registry. Toxicological profile for polycyclic aromatic hydrocarbons (PAHs) (1995) 77-78

[79] AECOSAN agencia española de consumo, seguridad alimetaria y nutrición. Subdirección General de Promoción de la Seguridad Alimentaria. Ministerio de Sanidad Hidrocarburos Aromáticos Policíclicos (HAPs). Servicios Sociales e Iguadad.

Compuesto	Estructura química	Grupo
Benzo(k)fluorantrene		2B
Benzo(a)pireno		1
Cruceño		2B
Dibenzo(a,b)antraceno		2A
Dibenzo(a,b)pireno		2B
Disenso(a,b)piren		2B
Dibenzo(a)pireno		2A
Indeno(1,2,3-cd)pireno		2B
5-metilcriseno		2B

Por tanto, la exposición de la población en general a este tipo de compuestos contaminantes, representa un riesgo real para la salud pública.

Como marcador de la presencia de PAHs se estableció en 2002 el benzo(a)pireno (B(a)P). Sin embargo, en 2008 la EFSA emitió un documento[80] donde se establece que el B(a)P por sí sólo no constituía un buen indicador de la presencia y efectos de los PAHs en los alimentos, recomendando los siguientes 3 grupos de compuestos:

- **PAH2**: benzo(a)pireno + criseno
- **PAH4**: benzo(a)antraceno + benzo(b)fluoranteno + benzo(a)pireno + criseno.
- **PAH8**: benzo(a)antraceno + benzo(b)fluoranteno + benzo(k)fluoranteno + benzo(g,h,i)perileno + benzo(a)pireno + criseno + dibenzo(a,h)antraceno + indeno(1,2,3-cd)pireno.

Finalmente, el Panel de Contaminantes de la EFSA concluyó que los marcadores que mejor definían la presencia y efecto de este tipo de contaminantes eran los grupos de sustancias definidos en PAH4 y PAH8, y que a su vez, el grupo de sustancias PAH8 no representaba una mejora significativa respecto a PAH4. Por este motivo, la legislación europea emplea como marcador de referencia el grupo PAH4, manteniéndose el valor máximo independiente para el benzo(a)pireno con el fin de garantizar

[80] Scientific Opinion of the Panel on Contaminants in the Food Chain. Polycyclic Aromatic Hydrocarbons in food (Question N° EFSA-Q-2007-136) adopted on 9 June 2008. EFSA J. 724 (2008) 1-114.

la trazabilidad y compatibilidad de los resultados ya obtenidos anteriormente y los futuros.

Esta opinión de la EFSA dio lugar por tanto a una modificación del Reglamento 1881/2006 a través del Reglamento 835/2011, donde se definen los nuevos criterios para el establecimiento de los valores máximos permitidos. Este reglamento entró en vigor en 2012, y permite mantener bajo control aquellos alimentos en los que no se detecte B(a)P, pero que puedan contener otros PAHs.

Por otro lado, se ha demostrado que bajo ciertas condiciones de fabricación, los niveles de PAHs pueden variar, por lo que aunque en muchos casos la concentración de PAHs detectada en alimentos puede provenir de contaminaciones ambientales, existen otros casos en los que para asegurar unas buenas prácticas de fabricación, los niveles presentes deben encontrarse por debajo de los límites establecidos en este Reglamento, ya que unas malas prácticas en el proceso de secado puede revertir en un aumento de la concentración presente de este tipo de sustancias en el alimento. Estos aspectos son recogidos en sucesivos Reglamentos (Reglamento 1327/2014, Reglamento 1933/2015 y Reglamento 1125/2015) los cuales han modificado igualmente al Reglamento 1881/2006 y han incorporado y modificado nuevos límites en diversas matrices alimentarias.

En particular, el Reglamento 1933/2015 se refiere a los límites para este tipo de sustancias en preparados botánicos a base de especies herbales definidas como aquellos "*… preparados que se obtienen de*

productos vegetales (por ejemplo, plantas enteras, partes de plantas, plantas fragmentadas o cortadas) mediante diversos procesos (por ejemplo, prensado, extracción, fraccionamiento, destilación, concentración, secado o fermentación). Esta definición incluye las plantas trituradas o en polvo, las partes de plantas, las algas, los hongos, los líquenes, las tinturas, los extractos, los aceites esenciales (excepto los aceites vegetales contempladas en el punto 6.1.1), los zumos exprimidos y los exudados tratados", de este modo, la legislación europea atiende específicamente a los requerimientos en relación a complementos alimenticios a base de extractos vegetales. Por tanto, en el punto 2 del Anexo del Reglamento 1933/2015 se recoge específicamente la modificación que se incorpora al Reglamento general 1881/2006 y que afecta a los niveles de PAHs permitidos en extractos vegetales. A continuación se transcribe esta modificación en la siguiente tabla:

***Tabla 8**. Captura del reglamento 1933/2015 donde se recoge la tabla en la que se especifican los niveles permisibles de PAHs en hierbas secas y complementos alimenticios.*

2) Se añaden los puntos 6.1.11, 6.1.12, 6.1.13, 6.1.14 y 6.1.15 siguientes:

«6.1.11	Fibra de cacao y productos derivados de la fibra de cacao destinados a ser utilizados como ingredientes en alimentos	3,0	15,0
6.1.12	Chips de plátano	2,0	20,0
6.1.13	Complementos alimenticios que contengan sustancias botánicas y sus preparados ([39]) (*) (**) Complementos alimenticios que contengan propóleo, jalea real o espirulina y sus preparados ([39])	10,0	50,0
6.1.14	Hierbas secas	10,0	50,0
6.1.15	Especias secas, excepto el cardamomo y el *Capsicum* spp. ahumado	10,0	50,0

(*) Los preparados botánicos son preparados que se obtienen de productos vegetales (por ejemplo, plantas enteras, partes de plantas, plantas fragmentadas o cortadas) mediante diversos procesos (por ejemplo, prensado, extracción, fraccionamiento, destilación, concentración, secado o fermentación). Esta definición incluye las plantas trituradas o en polvo, las partes de plantas, las algas, los hongos, los líquenes, las tinturas, los extractos, los aceites esenciales (excepto los aceites vegetales contempladas en el punto 6.1.1), los zumos exprimidos y los exudados tratados.

(**) El contenido máximo no se aplica a los complementos alimenticios que contengan aceites vegetales. Los aceites vegetales utilizados como ingredientes en los complementos alimenticios deben respetar el contenido máximo establecido en el punto 6.1.1.».

En este punto, es de destacar la evolución de la industria de los complementos alimenticios en los últimos años. La tendencia a nivel mundial es a un aumento de la obesidad, con el consiguiente riesgo para la salud cardiovascular. En este contexto, la preocupación de la población en general por una mejora de la salud, ha desencadenado un aumento por el interés en dichos complementos.

En las 2 últimas décadas, el volumen de ventas de los complementos alimenticios ha aumentado desde los 49.1 mil millones de € en 1999, hasta

los 127.8 mil millones de € en 2017[81], con un crecimiento sostenido anual de entre 5% al 7%. Estas cifras dan una idea de la importancia que este tipo de productos está teniendo en el mercado mundial, y por tanto, deben establecerse las medidas de seguridad que aseguren un alto nivel de confianza por parte de la población general en este tipo de productos. Entre estas medidas, se encuentra el aumento del control analítico sobre ellas, con el fin de asegurar que estos productos sean seguros y que los niveles de concentración de los posibles contaminantes se encuentran por debajo de los límites establecidos.

Entre los complementos alimenticios se encuentran los extractos vegetales (*dried vegetal extract*), los cuales están despertando un interés creciente entre la población debido a sus efectos beneficiosos sobre gran variedad de aspectos de la salud. Desde un punto de vista farmacológico, se ha podido demostrar la actividad de determinados constituyentes de ciertas plantas usadas desde hace siglos según la medicina tradicional. Este uso fitoterapéutico de las plantas se ha basado tradicionalmente en la experiencia, siendo en los últimos años cuando se ha desarrollado un enfoque clínico que pueda confirmar empíricamente la acción farmacológica de ciertos extractos vegetales, teniendo en cuenta la

[81] Asociación de las Empresas de Dietéticos y Complementos Alimenticios. La evolución de la industria de los complementos alimenticios de la década de los 90 hasta nuestros días. Londres, IADSA, 2018.

actuación sinérgica de varios de los componentes que conforman el extracto vegetal[82].

En este contexto surge una importante necesidad analítica para controlar la seguridad de este tipo de productos, y detectarlos a nivel traza (µg/kg) en matrices con altos contenidos en ácidos orgánicos, clorofilas, polifenoles, etc., que requieren un tratamiento de muestra optimizado previo para asegurar la ausencia de interferentes que dificulten o imposibiliten una adecuada determinación.

Para el caso concreto de PAHs, se han empleado una gran variedad de tratamientos de muestra. Los primeros métodos oficiales fueron establecidos por la AOAC en 1972 y adoptados por la IUPAC en 1975, y consistían en una saponificación etanólica, seguida de una extracción líquido-líquido con disolventes orgánicos (iso-octano) seguido de una SPE con Florisil[83]. Posteriormente, métodos oficiales aplicados en grasas animales y vegetales y basados en la determinación mediante HPLC-FLD en fase inversa previo *clean-up* con alúmina (ISO 15302), fueron estandarizados por ISO. Sin embargo, el empleo de detectores de fluorescencia tiene una serie de desventajas en el caso de este tipo de contaminantes, como son: imposibilidad de empleo de patrones internos

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82 Mendoza Mendoza, N., Diaz-Ropero, M.P., Aragon, M., Maldonado, V., Llaneza, P., Lorente, J., Mendoza-Tesarik, R., Maldonado-Lobon, J., Olivares, M., Fonolla, J. Comparison of the effect of two combinations of myo-inositol and D-chiro-inositol in women with polycystic ovary syndrome undergoing ICSI: a randomized controlled trial. Gynecol. Endocrinol. 35 (2019) 695-700.

83 Zelinkova, Z., Wenzl, T. The occurrence of 16 EPA PAHs in food - a review. Polycycl. Aromat. Comp. 35 (2015) 248-284.

marcados isotópicamente y falta de sensibilidad en ciertos compuestos de esta familia de contaminantes[84]. Por esta razón, la cromatografía de gases acoplada a detectores de espectrometría de masas se ha ido convirtiendo en la técnica instrumental más empleada para este tipo de sustancias.

Para el caso concreto de extractos vegetales, la Unión Europea no ha establecido metodologías oficiales que prescriban un procedimiento a seguir, aunque sí que se establecen los criterios que deben cumplir los métodos de análisis desarrollados a nivel interno para que demuestren de manera objetiva su funcionamiento. Estos criterios vienen descritos en el Reglamento 333/2007, y para el caso concreto de los PAHs, pueden resumirse en la siguiente tabla:

Tabla 9. *Captura del Reglamento 333/2007 donde se especifican los criterios de funcionamiento que deben cumplir los métodos de análisis de ciertas sustancias contaminantes.*

Cuadro 7

Criterios de funcionamiento aplicables a los métodos de análisis para el benzo(a)pireno

Parámetro	Valor/comentario
Aplicabilidad	Alimentos especificados en el Reglamento (CE) nº 1881/2006
LOD	Menos de 0,3 µg/kg
LOQ	Menos de 0,9 µg/kg
Precisión	Valores $HORRAT_r$ o $HORRAT_R$ inferiores a 2
Recuperación	50-120 %
Especificidad	Libre de interferencias de la matriz o del espectro, verificación de detección positiva

[84] Wise, S.A., Hilpert, L.R., Byrd, G.D., May, W.E. Comparison of liquid chromatography with fluorescence detection and gas chromatography/mass spectrometry for the determination of polycyclic aromatic hydrocarbons in environmental samples. Polycycl. Aromat. Comp. 1 (1990) 81-98.

Cuando se pretenden analizar muestras complejas de origen vegetal, en el proceso de extracción y purificación de los PAHs, inevitablemente se co-extraen una gran cantidad de compuestos procedentes de la matriz, tales como ácidos orgánicos, carotenoides, polifenoles, clorofilas, etc. Para el caso de extractos vegetales, esta dificultad se acentúa debido a que consisten en materiales que han sido tratados con mezclas hidroalcohólicas en caliente para favorecer la extracción de los diferentes principios activos que confieren funcionalidad a este tipo de productos. El material bruto de planta medicinal se somete a varias etapas de extracción/maceración y el líquido resultante se seca mediante atomización a alta temperatura. Se trata por tanto de materiales donde se ha concentrado el material de partida en ratios que van desde 1:2 a 1:50, es decir, por cada kilogramo de extracto vegetal obtenido en el proceso extractivo, se necesitan entre 2 y 50 kg de producto de partida (planta medicinal). Por tanto, la concentración de compuestos potencialmente interferentes en este tipo de productos se encuentra, además, incrementada.

Para la extracción eficaz de PAHs de estas matrices complejas, se han desarrollado numerosos tratamientos de muestra y se han empleado numerosas técnicas instrumentales. Un pre-requisito de estos tratamientos de muestra es que deben conseguir separaciones efectivas de los interferentes sin que se afecte adversamente el rendimiento de extracción de los analitos de interés. Además de esto, se debe tener en cuenta la capacidad de procesamiento de muestras, el cual debe ser un criterio importante a la hora de desarrollar metodologías implementables en laboratorios con un alto volumen de trabajo. Este tratamiento de muestra

depende así mismo de la técnica instrumental, ya que a medida que se incremente la capacidad de detección y selectividad de la técnica instrumental, menor será el tratamiento de muestra necesario. Tradicionalmente se han empleado como técnicas instrumentales HPLC-FLD y GC-MS, siendo esta última la más extendida por las razones descritas anteriormente (gran capacidad de detección a bajos niveles de concentración de la mayor parte de PAHs, independientemente de la fluorescencia nativa del compuesto, mayor capacidad de resolución cromatográfica, etc.).

Para el tratamiento de muestra, tradicionalmente se ha empleado la saponificación y extracción líquido-líquido[85], Soxhlet[86], extracciones asistidas por presión[87] o microondas[88], GPC[89] o SPE, SPME[90] y diferentes combinaciones de estas. Recientemente se ha introducido el empleo de los

[85] Kumari, R., Chaturvedi, P., Ansari, N.G., Murthy, R.C., Patel, D.K. Optimization and validation of an extraction method for the analysis of polycyclic aromatic hydrocarbons in chocolate candies. J. Food Sci. 77 (2012) 34-T40.

[86] Suchanova, M., Hajslova, J., Tomaniova, M., Kocourek, V., Babicka, L. Polycyclic aromatic hydrocarbons in smoked cheese. J. Sci. of Food Agric. 88 (2008) 1307-1317.

[87] Ramalhosa, M.J., Paiga, P., Morais, S., Ramos, S., Delerue-Matos, C., Oliveira, M.B. Polycyclic aromatic hydrocarbon levels in three pelagic fish species from atlantic ocean: inter-specific and inter-season comparisons and assessment of potential public health risks. Food Chem. Toxicol. 50 (2012) 162-167.

[88] Purcaro, G., Moret, S., Conte, L.S. Optimisation of microwave assisted extraction (MAE) for polycyclic aromatic hydrocarbon (PAH) determination in smoked meat. Meat Sci. 81 (2009) 275-280.

[89] Yu, L., Cao, Y., Zhang, J., Cui, Z., Sun, H. Isotope dilution-GC-MS/MS analysis of 16 polycyclic aromatic hydrocarbons in selected medicinal herbs used as health food additives. Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 29 (2012) 1800-1809.

[90] Risticevic, S., Niri, V.H., Vuckovic, D., Pawliszyn, J. Recent developments in solid-phase microextraction. Anal. Bioanal. Chem. 393 (2009) 781-795.

QuEChERS para la extracción de este tipo de compuestos en una gran variedad de matrices. En particular, para el caso de extractos herbales se ha aplicado esta metodología con gran éxito[91-93]. El proceso se basa en la extracción en un solo paso de los diferentes analitos de interés, empleando para ello ACN u otros disolventes orgánicos, seguido de la adición de sales, tales como cloruro de sodio, sulfato de magnesio, acetato de sodio, etc., dependiendo del método y la aplicación concreta, que inducen la separación de fases entre disolvente orgánico y agua. A continuación se añade algún tipo de adsorbente de los habitualmente empleados en SPE dispersiva (PSA, C18, alúmina, etc.), que elimina componentes mayoritarios tales como ácidos orgánicos, clorofilas y carotenos, que interfieren en la determinación instrumental.

Esta técnica facilita el procesado masivo de muestras ya que se trata de un proceso rápido y sencillo que además tiene un consumo reducido de disolvente y genera una cantidad relativamente reducida de residuos, obteniéndose igualmente métodos con características de funcionamiento analíticas adecuadas en comparación con métodos clásicos más tediosos como extracciones líquido-liquido o Soxhlet.

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91 González-Curbelo, M.A., Socas-Rodríguez, B., Herrera-Herrera, A.V., González-Sálamo, J., Hernández-Borges, J., Rodríguez-Delgado, M.A. Evolution and applications of the QuEChERS method. TRAC-Trend Anal. Chem. 71 (2015) 169-185.

92 Escarrone, A.L., Caldas, S.S., Furlong, E.B., Meneghetti, V.L., Fagundes, C.A., Arias, J.L., Primel, E.G. Polycyclic aromatic hydrocarbons in rice grain dried by different processes: Evaluation of a quick, easy, cheap, effective, rugged and safe extraction method. Food Chem. 146 (2014) 597-602.

93 Pincemaille, J., Schummer, C., Heinen, E., Moris, G. Determination of polycyclic aromatic hydrocarbons in smoked and non-smoked black teas and tea infusions. Food Chem. 145 (2014) 807-813.

Sin embargo, existen ciertas matrices de extractos vegetales donde el empleo de QuEChERS no es suficiente para eliminar los diferentes interferentes que son co-extraídos en el proceso. Estos interferentes afectan negativamente de manera significativa en la determinación de los PAHs.

Existe una variante de SPE (MIP-SPE) en la que el adsorbente empleado es un polímero que presenta una cavidad en su diseño capaz de retener selectivamente a un compuesto específico o familia de ellos. Este tipo de SPE se ha aplicado con éxito en la determinación de PAHs en aceites, ofreciendo una alta selectividad, y siendo capaz de retener de forma específica compuestos a muy baja concentración incluso cuando estos están acompañados de un gran número de interferentes[94].

La combinación de ambas técnicas ha posibilitado el desarrollo de una metodología capaz de permitir la cuantificación de diferentes PAHs en ciertos extractos vegetales con un alto efecto matriz.

[94] Application Note 192 (Sigma-Aldrich). Extraction & Analysis of PAHs in Olive Oil Using SupelMIP™ SPE – PAHs and GC-MS.

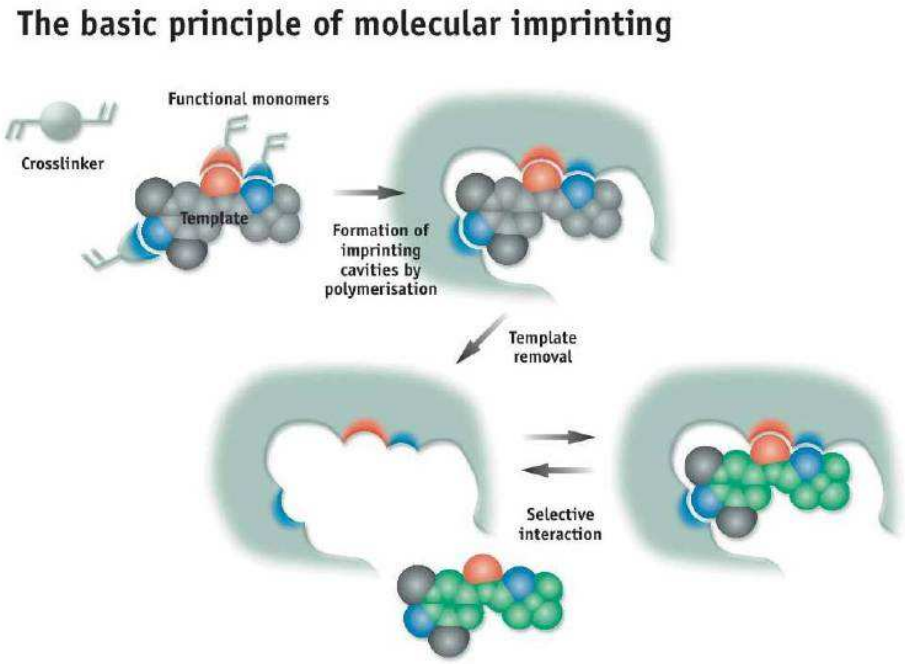

Figura 10. *Representación esquemática del proceso de fabricación y funcionamiento de MIP SPE*[95]

2.4.2. Objetivos

El objetivo de este trabajo es el desarrollo de una metodología de buenas características analíticas (de acuerdo con el Reglamento 333/2007 por el que se establecen los criterios de calidad mínimos que deben cumplir los métodos analíticos propuestos para el análisis de ciertos

[95] Biotage, AFFINILUTE™ MIP Columns. https://www.biotage.com/product-page/affinilute-mip-columns (Visitado 20/10/2019)

contaminantes, entre los que se encuentran los PAHs, en alimentos), capaz de cuantificar concentraciones de PAHs de acuerdo a la legislación europea vigente en una serie de extractos vegetales en los que se ha observado un elevado efecto matriz.

Se ha implementado un tratamiento de muestra novedoso, simple y eficaz que combina la extracción en fase sólida dispersiva (QuEChERS) con el empleo de columnas de extracción en fase sólida de impronta molecular (MIP-SPE), específicas para esta familia de compuestos definidas en el grupo PAH4 a los niveles de concentración requeridos por la legislación Europea en este tipo de matrices.

Para la determinación analítica se ha usado la cromatografía de gases acoplada a la espectrometría de masas triple cuadrupolo como técnica de detección. Esta metodología ha permitido el análisis de muestras de extractos vegetales con un alto contenido de interferentes, ya que se evitan de este modo problemas de supresión iónica en el detector espectrométrico, deriva de la línea base cromatográfica o disminución del factor de simetría de pico por la deformación de los picos cromatográficos. Esta deformación en los picos cromatográficos puede ser debida a la interacción inespecífica de los compuestos de interés con interferentes, que a su vez provoca que una cierta población de moléculas no interactúen adecuadamente con la fase estacionaria y como consecuencia se provoque la aparición de hombros y colas en los picos cromatográficos.

2.4.3. Publicación VI

Microchemical Journal

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Use of QuEChERS and MIP-SPE followed by gas chromatography with tandem mass spectrometry for the quantitative analysis of polycyclic aromatic hydrocarbons in complex dry extracts

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ABSTRACT

A sensitive and selective method based in a combination of QuEChERS and Molecularly Imprinted Polymer technology is validated for the analysis of polycyclic aromatic hydrocarbons in complex dry extracts of *Eleutherococcus senticosus, Salvia officinalis, Camellia sinensis, Zingiber officinale, Uncaria tomentosa, Humulus lupulus, Pinus sylvestris L., Spirulina maxima,* propolis and royal jelly. The method has been optimized using gas chromatography coupled to tandem mass spectrometry. An additional sample treatment of the dry extracts, based on the combined use of MIP-

SPE and QuEChERS, was required because of the strong matrix effect observed related to interferences affecting analyte quantification. Estimation of the method detection limit and quantification limit was carried out for validation. The quantification limits were found to be 0.2 ng g^{-1} for benzo[a]antracene to 0.4 ng g^{-1} for benzo[b]fluorantene and chrysene; and the detection limits were found to be range from 0.07 ng g^{-1} to 0.1 ng g^{-1}. Recoveries are close to 100 % (88 % to 114) % and % RSD was < 14% in all cases. The method was applied in routine analysis to a wide variety of dry extracts from EU and non-EU manufacturers.

Keywords: Polycyclic aromatic hydrocarbons; Dry extracts; Dispersive solid phase extraction (QuEChERS); Molecularly imprinted polymer (MIP); GC-MS/MS

1. Introduction

PAHs (polycyclic aromatic hydrocarbons) are a large group of hydrocarbons that contain two or more aromatic rings fused to one another. PAHs are ubiquitous compounds produced by natural processes (e.g., volcanic emissions, naturally occurring forest fires) or anthropogenic activity (oil spills, motor vehicles, incinerators, and in general any type of industrial processes involving the combustion of fuels such as oil, gas, and coal). Human exposure to PAHs occurs primarily through food contaminated from environmental pollution and from industrial food processing and domestic cooking practices (grilling, drying, smoking or frying). The interest in these contaminants is related to their potential to

produce mutations and cancer confirmed by the International Agency for Research on Cancer [1-3].

Food supplements are a wide group of concentrated nutrients or other substances used for their potential effect on the prevention and treatment of human diseases. In addition to vitamins and minerals, other substances such as fish oils, bee products and plant extracts are used in the manufacturing of food supplements. The global food supplement market has grown steadily between 5% and 7% per year over the last two decades, with global sales of €49.1 billion in 1999 to €127.8 billion in 2017 [4]. Dry extracts are obtained by industrial processes that usually involve heating and drying treatments. The use of high temperatures in these industrial processes may result in the formation of PAHs. In addition, environmental pollution in the area of collection of the plant material is another source of contamination.

In 2002, benzo[a]pyrene (B[a]P) was considered a marker of the occurrence and concentration of PAHs in foodstuffs. However, the document issued in 2008 by the Panel on Contaminants in the Food Chain from the European Food Safety Authority (EFSA) [5] recommended the use of a group of PAHs rather than the individual compound. This group consisted of PAH2 (chrysene + benzo[a]pyrene), PAH4 (benzo[a]anthracene + chrysene + benzo[b]fluoranthene + benzo[a]pyrene), and PAH8 (benzo[a]anthracene + chrysene + benzo[b]fluoranthene + benzo[k]fluoranthene + benzo[a]pyrene + benzo[g,h,i]perylene + dibenzo[a,h]anthracene + indene[1,2,3-cd]pyrene). Finally, the Panel concluded that the best indicators of the occurrence and effect of PAHs in

food were groups PAH4 and PAH8, but the determination of the compounds included in group PAH8 does not add significant value over the determination of those included in PAH4. For this reason, the European legislation establishes the use of PAH4 as a reference marker and maintains the maximum content of benzo[a]pyrene to ensure traceability and comparison of previous and future data.

The European Commission through its Commission Regulation No 1881/2006 (subsequently amended by Commission Regulation No 835/2011) established the maximum levels of PAHs in foodstuffs and specifically in cocoa fibre, banana chips, dried herbs, dried spices, and food supplements containing spirulina, botanicals, propolis, and royal jelly [6]. This Regulation defines botanicals as preparations obtained from whole or plant parts and fragmented or cut plants by different processes such as pressing, squeezing, extraction, fractionation, distillation, concentration, drying and fermentation. As the PAH content in food supplements depends on the specific product and the production process, further collection of data and analysis seems warranted. Manufacturers must also ensure that this content is below the established limits during the Quality Control assessment of these products by control the manufacture conditions in order to safeguard the health of the human population.

PAHs have been detected and determined in a large variety of food matrices such as meat (red meat, chicken, duck and ham), seafood (mussels, oysters, cockles, shrimps and sea urchin), vegetables (tea and rice grains) and milk using HPLC with fluorescence or mass spectrometric detection and GC coupled to mass spectrometry [7-9]. In addition, a wide

range of methods have been validated for the determination of PAHs in herbal preparations used in traditional Chinese medicine. Most of these methods are based on the use of QuEChERS [10-13], accelerated solvent extraction [14], solvent extraction and GPC [15], SPME [16-18] and stir bar sorptive extraction [19] for sample treatment followed by GC-MS. Cloud point extraction followed by HPLC-FLD has also been investigated [20]. However, some dry extracts have a high matrix effect that prevents an adequate chromatographic determination and therefore an adequate quantification.

Several methods based on the use of SPE have been developed to determinate PAHs in oils [21] and herbal medicines [22], where a combination of different SPE sorbents is used depending on the type and part of the plant analysed (root, leaves, stem, or flowers). The main advantages of this type of extraction method are the low solvent consumption and the easy operational conditions. However, the use of several SPE columns per sample makes the process costly and time consuming which makes it unsuitable for routine lab analysis. Molecularly Imprinted Polymer (MIP) is a very selective SPE technology that uses a cross linked polymer in which cavities have been engineering with binding sites to retain a single molecule or a group of related molecules [23]. This allows selective retention and extraction of analytes at very low concentrations even in complex analytical matrices with high amounts of potential interferences. MIP has been mainly applied to the analysis of PAHs in oils [24, 25].

The high levels of PAHs found in dietary supplements containing dry extracts makes the determination of these compounds necessary to protect public health. The present paper describes a new analytical method which combines the well-known QuEChERS technique with the MIP-SPE technology prior to GC-MS/MS analysis. This combination has allowed the determination of PAHs in complex matrices of *Eleutherococcus senticosus, Salvia officinalis, Camellia sinensis, Zingiber officinale, Uncaria tomentosa, Humulus lupulus, Pinus sylvestris L*, *Spirulina maxima,* propolis, and royal jelly dry extracts, at very low concentrations with analytical characteristics that comply with European regulations.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade reagents were used. For water purification (18.2 MΩ cm) a Milli-Q system from Merck Millipore (Bedford, MA, USA). Benzo[a]anthracene, B[a]A; chrysene, CHR; benzo[b]fluoranthene, B[b]F; benzo[k]fluoranthene, B[k]F; benzo[a]pyrene, B[a]P; were from Sigma-Aldrich (Madrid, Spain) as a mix solution in methylene chloride:benzene (1:1, v/v). Deuterated benzo[a]pyreno, B[a]P-D_{12}; was used as internal standard. Stock solutions of the commercial mix of each compound, at a concentration of 2 mg L^{-1}, were prepared in hexane and stored at -20 ºC until use. Working solutions (120 µg L^{-1}) were obtained by diluting the stock standard solutions in acetonitrile. For internal standard, a solution of 40 µg L^{-1} used as internal standard was prepared in ethyl acetate. These solutions

were stored at -20ºC and prepared fresh every week. Dark glass bottles were used to store the solutions. Commercial QuEChERS extract pouches (EN method) and dispersive SPE 15 mL tubes (EN method for fruit and vegetables) were purchased from Agilent Technologies (USA, CA, Palo Alto). SupelMIP SPE-PAHs columns (50 mg/3 mL) (Supelco), LC–MS grade hexane, cyclohexane and acetonitrile, were supplied by Sigma-Aldrich.

2.2. Instrumentation and software

The gas chromatographic determination was carried out on an Agilent Technologies 7890 GC system coupled to a mass spectrometer triple quadrupole 7000B series. A "back flush" system was employed to connect a DB-EUPAH column (20 m x 180 µm x 0.14 µm id) and a HP-5MS column (15 m x 250 µm x 0.25 µm i.d.), both from Agilent Technologies (USA, CA, Palo Alto), in serial mode. This back flush system enables a faster cleaning of the highly hydrophobic compounds retained in the column connected to the injector. The dry extracts studied were obtained from different EU and non-EU providers as well as from the Spanish manufacturer Biosearch S.A. (Talayuela, Cáceres, Spain). A vortex-mixer (IKA, Staufen, Germany), a centrifuge Spectrafuge™ 24D (Labnet International, Inc., New Jersey, USA), a bath of ultrasounds (J.P. Selecta S.A.u, Barcelona, Spain), and a sample concentrator (Stuart, Staffordshire, UK) were also used. Statgraphics Plus software 5.1 (Statpoint Technologies Inc., Virginia, USA) was used for statistical analysis.

2.3. Basic procedure

2.3.1. Preparation of spiked samples

The unavailability of references made necessary the preparation of spiked samples for method validation and calibration. Calibration standards were obtained by spiking samples (5 g) of hops and green tea at different concentration levels from 0.6 to 12 ng g^{-1} (see table 3), depending of the accurate concentration of each component in the standard mix, for quality control and method validation. Homogenization of the samples was carried out by carefully chopping and stirring of spiking samples. The spiked samples were shaken for 10 before treatment.

2.3.2. Sample treatment

An aliquot of 5 g of dry extract was placed into a 50 mL polypropylene tube and 10 mL of MilliQ water was added. The resulting mixture was shaken vigorously for 5 min in a vortex-mixer. Subsequently, 10 mL of acetonitrile was added and the mixture vortexed for 1 min. At this point, the content of the QuEChERS pouch containing 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, and 0.5 g disodium citrate sesquihydrate was added to the extraction tube and shaken for 5 min. The phases were separated by centrifugation for 5 min at 2,500 *x g*, and subsequently 6 mL of the supernatant was put into a dispersive SPE tube containing 150 mg primary secondary amine (PSA) and 900 mg $MgSO_4$ and, after shaking for 5 min in vortex, the mixture was centrifuged for 5 min at 2500 *x g*. The supernatant (2 mL) was evaporated to dryness under a N_2 stream at 40 °C. The resulting solid residue was dissolved with 2 mL of cyclohexane and passed through

the SupelMIP SPE-PAHs columns previously washed with 1 mL of fresh cyclohexane and dried for conditioning. The content was eluted from the column using 3 mL ethyl acetate added sequentially in 1 mL fractions. Finally, the solvent was completely removed and the solid residue dissolved with 1 mL internal standard solution in ethyl acetate. The solution was transferred to a chromatographic vial and directly injected into the GC-MS/MS system.

2.3.3. Gas chromatography–tandem mass spectrometry analysis

For separation of compounds a DB-EUPAH column and a HP-5MS column were coupled in series using a backflush system to prevent cross contamination of the DB-EUPAH column. The compounds were separated using the temperature gradient program shown in Table 1. This table also shows the chromatographic and spectrometric conditions optimized.

Table 1. Chromatographic-spectrometric parameters for GC-MS/MS analysis

Chromatographic Parameters					
Column 1	DB-EUPAH (20 m *x* 180 µm *x* 0.14 µm)				
Column 2	HP-5MS (15 m *x* 250 µm *x* 0.25 µm)				
Injector temp.	320 °C				
Injection vol.	2 µL				
Split mode	Splitless				
Constant flow column 1	1.3 mL/min				
Constant flow column 2	1.5 mL/min				
Purge flow	100 mL/min at 1.2 min				
Temp. ramp	Ramp °C/min	Temperature (°C)	Hold (min)		
		150	1.4		
	10	270	0.5		
	5	310	3.6		
Backflush	Pressure	70 psi			
	Temperature	315 °C			
	Time	5 min			
Transfer line temp.	280 °C				
Run time	25.5 min + 5 min backflush				
Mass Spectrometer Parameters					
	Ion precursor	Ion Quantify	CE Quantify	Ion Qualify	CE Qualify
B[a]A	228	226	40	202	35
CHR	228	226	40	202	35
B[b]F	252	250	45	226	35
B[k]F	252	250	45	226	35
B[a]P	252	250	45	226	35
B[a]P-D_{12}	264	260	40	234	40
Energy filament	-70 eV (Delta EMV = 400)				
Source Temp.	300 °C				
Solvent delay	4 min				

CE: collision energy

2.3.4. Method validation

Validation of the proposed method was performed following the recommendations of the ICH Harmonised Tripartite Guideline for bioanalytical assay validation in terms of linearity, selectivity, accuracy (trueness and precision), and sensitivity [26].

Residual plots and determination coefficients (%, R^2) were used for checking the linearity of the calibration graphs. In order to verify the selectivity, the chromatogram of the analytical blank was compared with the corresponding spiked dry extract. Additionally, and due to the unavailability of certified reference compounds, the trueness and reproducibility of the method were verified by performing an experiment with spiked samples (see table 3). Participation of our laboratory in two external proficiency programs carried out by FAPAS® in two consecutive years, 2017 and 2018, ensured the performance of the method. Intra- and inter-day precision of the analytical method was determined by the evaluation of three replicates of the spiked samples (six days). On the other hand, trueness was verified by the proficiency test performed for two consecutive years and a by analyte recovery. Finally, the limits of detection (LODs) and limits of quantification (LOQs) were determined by considering the SD of intercept (s_a), of the calibration graphs as a SD of the response at low concentration levels in accordance with Q2 (R1) Validation of Analytical Procedures [26]. The LOD was $3.3 \cdot s_a$ and the LOQ was $10 \cdot s_a$.

2.3.5. Quality assurance and quality control

In order to ensure the quality of the method (QA/QC) a large number of controls were also addressed. Procedural blanks prepared following the method described above for the samples before injection into the GC–MS/MS system were analyzed. The analysis revealed no detectable concentrations amounts of target compounds. Additionally, blank samples were spiked with the analytes at 0 and 1.6 ng g^{-1} and injected in duplicates into to GC system to rule out contamination and to assess the analytical variability of the instruments.

3. Results and discussion

3.1. Gas chromatographic-mass spectrometric analysis

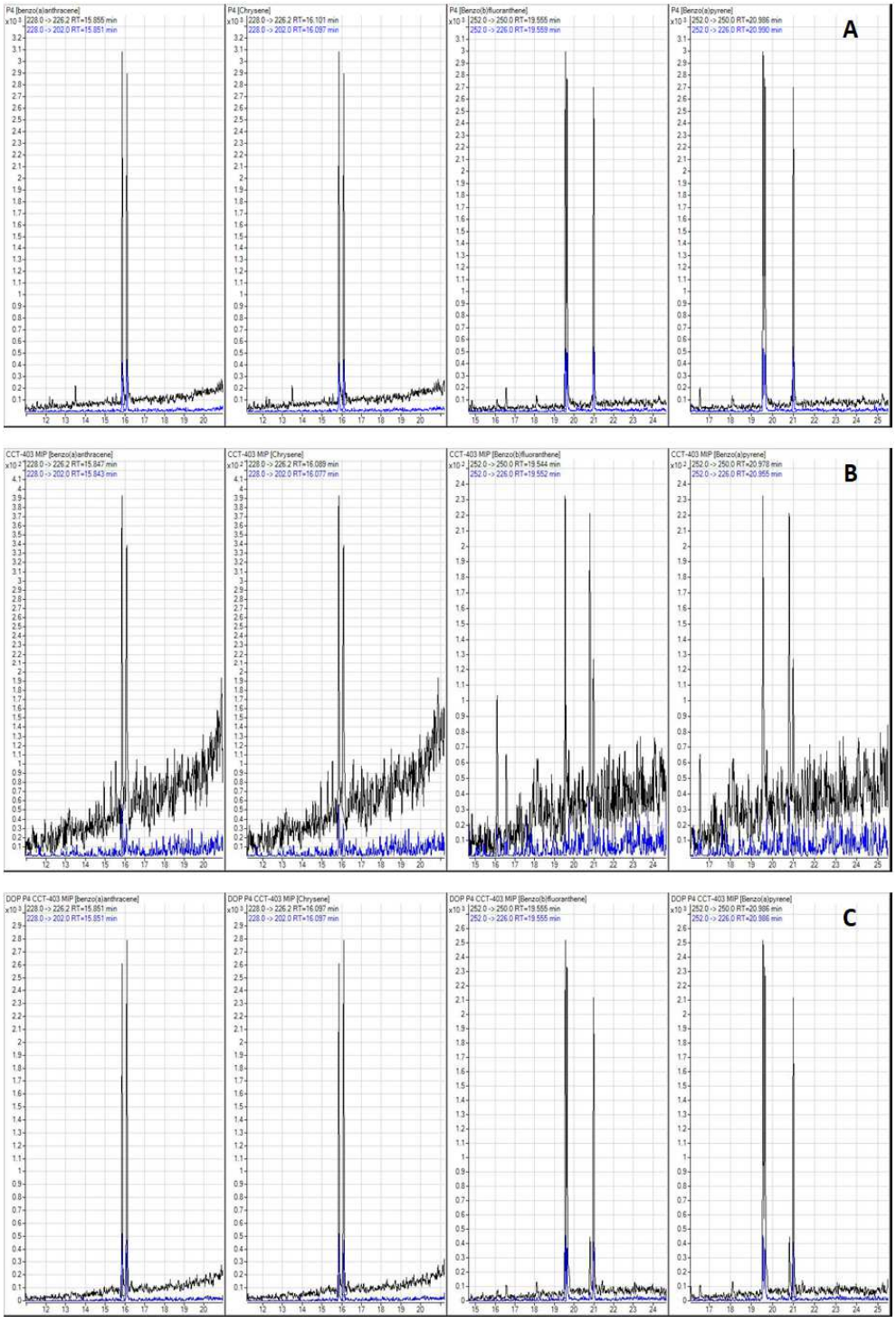
For PAHs determination (5%-phenyl)methylpolysiloxane columns are usually selected [27]. However, some columns are specifically designed for separation of PAHs, including some critical isomers that are usually difficult to separate chromatographically (benzo[b,k,j]fluoranthenes). J&W DB-EUPAH was chosen as a complementary tool for the (5%-phenyl)methylpolysiloxane column. The serial coupling of both columns provides higher chromatographic resolution besides facilitating the change of the analytical column for other applications. This method improves the analytical efficiency by drastically reducing the time required for system conditioning, since venting of the mass spectrometer is not required. This

fact is important for routine laboratories, where different families of compounds are analyzed in the same system daily.

Backflushing also prevents contamination with compounds strongly retained in the primary column by reversing a continuous flow of the carrier gas through the column. Thereby, hydrophobic compounds are eluted through the inlet vent system and do not pass through the second column to reach the MS detector.

3.2. Optimization of the extraction procedure

The extraction procedure was based on the use of QuEChERS technique [28] with some modifications. First, in order to maximize the extraction yield, the optimum solvent for analyte isolation was optimized. Acetonitrile and different mixtures of acetone:hexane (30:70, 50:50, 70:30, v/v) were tested. Acetonitrile provided the highest recoveries and cleanest extracts for all the PAHs, and therefore it was selected. A second modification of the QuEChERS method, involving the addition of a MIP-SPE step, was performed and compared with the results of using QuEChERS alone. The presence of potential strong interferences makes difficult an adequate quantification because the chromatograms show poor peak shape and unstable baseline pattern, as well as the presence of ion suppression effect. In order to remove this effect, aliquots of different dry extract samples spiked with the studied compounds at a concentration level of 1.6 ng g^{-1} were used. Figure 1 shows the chromatograms of a PAH standard mix and those corresponding to spiked dry extract of hops (*Humulus lupulus*) treated with QuEChERS and with QuEChERS+MIP-SPE.


A
B
C

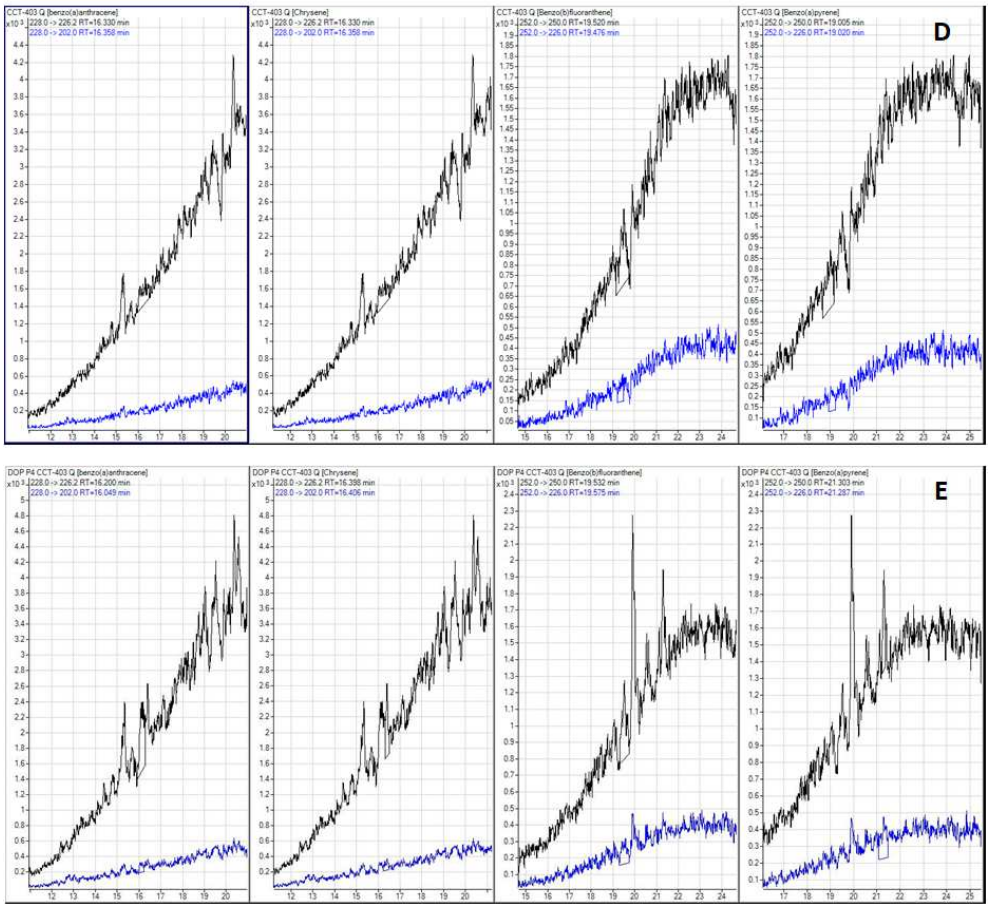


Fig. 1. Chromatograms. (A) PAH standard mix of B[a]A, CHR, B[b]F and B[a]P (1.6 ng g^{-1}). (B) Hops dry extract with native PAH content treated with QuEChERS+MIP-SPE. (C) Hops dry extract spiked with 1.6 ng g^{-1} of B[a]A, CHR, B[b]F and B[a]P and treated with QuEChERS+MIP-SPE. (D) Hops dry extract with native PAH content treated with QuEChERS alone. (E) Hops dry extract spiked with 1.6 ng g^{-1} of B[a]A, CHR, B[b]F and B[a]P and treated with QuEChERS.

Figure 2 also shows how the use of QuEChERS or QuEChERS+MIP-SPE sample treatments affects the peak shape of PAHs in an extract of Siberian ginseng (*Eleutherococcus senticosus*).

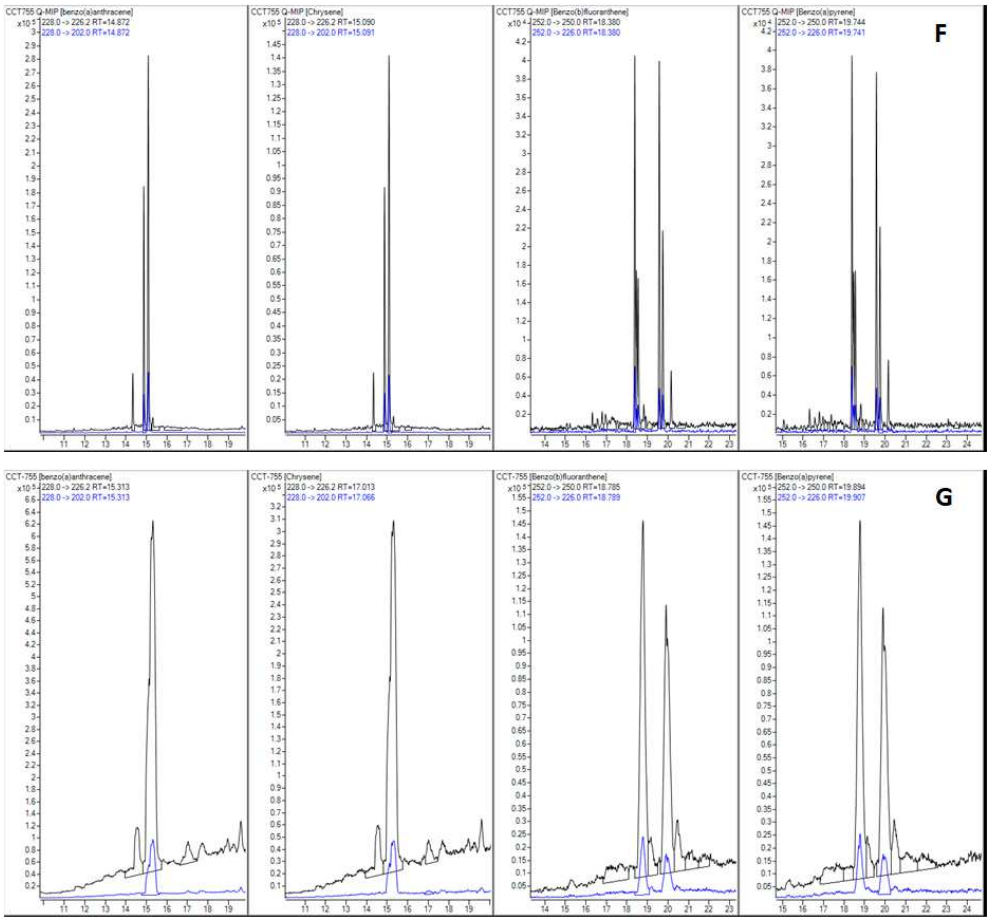


Fig. 2. Chromatograms. (F) Dry extract of Siberian ginseng treated with QuEChERS+MIP-SPE; (G) Siberian ginseng extract treated with QuEChERS alone.

These chromatograms reveal the presence of strong matrix effects when no additional treatment with SPE column is applied to the dry extracts. The presence of interferences leads to an increased instrumental noise and ionic suppression, which complicates the integration of the peaks in the baseline and, consequently, the quantification is strongly affected. Additionally, it is observed that the magnitude of the baseline when QuEChERS is used alone is very high compared to the one obtained with the additional clean-up step with MIP-SPE.

3.3. Analytical performance

For calibration of the selected compound, a calibration curve was prepared using next concentrations: 0.6, 1.5, 3.0, 6.0, 9.0 and 12.0 ng g^{-1}. The calibration standards were prepared by adding the corresponding volume (50 to 1000 µL) to 5 g blank dry extract. Each level of concentration was prepared in triplicate. The samples were vortexed and the methanol was removed until original weight was achieved. The samples were treated according to the previously described protocol and after making the corresponding measurements, the analyte concentration was plotted versus the area ratio of the analyte to that of the internal standard to prepare the calibration curves. B[a]P-D_{12} (40 ng g^{-1}) was used as internal standard. Calibration curves were prepared using MRM mode. Table 2 summarizes the results obtained and analytical parameters evaluated.

Table 2. Analytical parameters of the method

	B[a]A	CHR	B[b]F	B[a]P
Slope (g ng^{-1})	$3.82 \cdot 10^{-2}$	$1.99 \cdot 10^{-2}$	$1.49 \cdot 10^{-2}$	$1.50 \cdot 10^{-2}$
s_{slope} (g ng^{-1})	$1.68 \cdot 10^{-4}$	$1.63 \cdot 10^{-4}$	$1.18 \cdot 10^{-4}$	$0.89 \cdot 10^{-4}$
intercept	$4.20 \cdot 10^{-3}$	$0.91 \cdot 10^{-3}$	$1.58 \cdot 10^{-3}$	$1.25 \cdot 10^{-3}$
$s_{intercept}$	$0.87 \cdot 10^{-3}$	$0.85 \cdot 10^{-3}$	$0.61 \cdot 10^{-3}$	$0.46 \cdot 10^{-3}$
$s_{y/x}$	$1.80 \cdot 10^{-3}$	$3.77 \cdot 10^{-3}$	$1.19 \cdot 10^{-3}$	$0.9 \cdot 10^{-3}$
R^2	0.9993	0.9999	0.9996	0.9998
LOD (ng g^{-1})	0.07	0.1	0.1	0.09
LOQ (ng g^{-1})	0.2	0.4	0.4	0.3

3.4. Method validation

Method validation was performed using hops (*Humulus lupulus*) and green tea (*Camelia sinensis*) extracts, which are considered to have strong matrix effect. The validation parameters were:

Linearity. The concentration range selected was from the LOQ that is the smallest analyte content that can be measured, to 12 ng g^{-1}. R^2 values were close to 99.9% and the residual values were lower than 10% in all cases. These values indicate good linearity within the established ranges.

Selectivity. The absence of significant interference from endogenous compounds at the retention time of the analytes confirms the high selectivity of the GC–MS/MS method. Figure 3 shows the chromatograms, in SRM mode, of the blank sample extract.

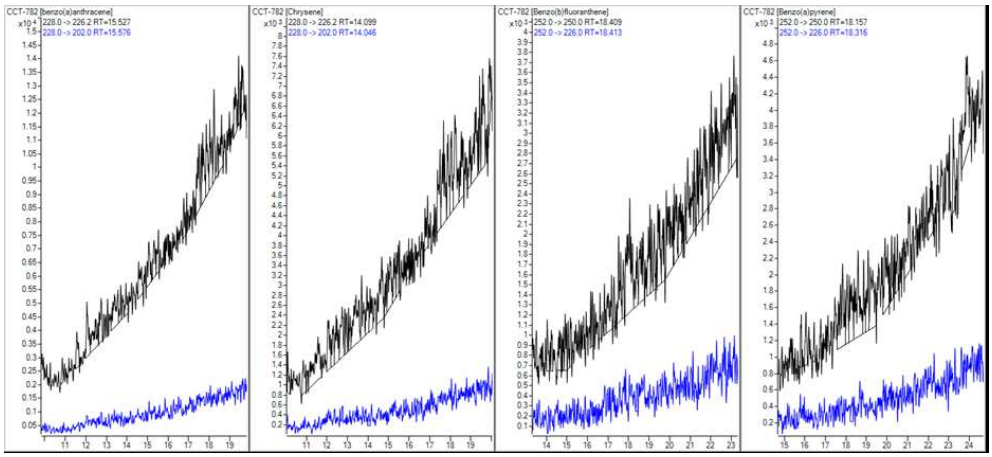


Fig. 3. SRM Chromatogram of a blank.

Accuracy, precision and trueness. Values obtained are summarized in table 3.

Table 3. Recovery and precision of QuEChERS and QuEChERS+MIP-SPE in the extraction of PAHs from hops and green tea dry extracts.

Hops dry extract					
	Added (ng g^{-1})	Background (ng g^{-1})	Found (ng g^{-1})	Rec (%)	RSD (%)
QuEChERS					
B[a]A	0.43		2.10	488	38
	1.70	< LD	8.63	508	21
	8.50		23.40	275	15
CHR	0.39		1.80	461	65
	1.56	< LD	4.07	261	26
	7.80		12.80	164	19
B[b]F	0.54		3.40	629	48
	2.17	< LD	5.23	241	35
	10.90		22.80	209	62
B[a]P	0.50		2.18	436	32
	2.00	< LD	2.69	134	16
	10.00		17.60	176	48
QuEChERS-MIP					
B[a]A	0.43		0.63	114	14
	1.70	0.14	1.54	90.6	7.1
	8.50		8.43	99.2	5.3
CHR	0.39		0.62	97.4	12.5
	1.56	0.24	1.69	108	9.2
	7.80		7.91	101	8.3
B[b]F	0.54		0.83	104	14
	2.17	0.27	1.92	88.5	12
	10.90		11.00	101	6.3
B[a]P	0.50		0.48	96.0	10
	2.00	< LD	1.79	89.5	6.7
	10.00		10.30	103	9.7

Table 3 cont. Recovery and precision of QuEChERS and QuEChERS+MIP-SPE in the extraction of PAHs from hops and green tea dry extracts.

Green tea dry extract					
	Added (ng g^{-1})	Background (ng g^{-1})	Found (ng g^{-1})	Rec (%)	RSD (%)
QuEChERS					
B[a]A	0.43		NQ	NQ	-
	1.70	NQ	NQ	NQ	-
	8.50		NQ	NQ	-
CHR	0.39		NQ	NQ	-
	1.56	NQ	NQ	NQ	-
	7.80		NQ	NQ	-
B[b]F	0.54		NQ	NQ	-
	2.17	NQ	NQ	NQ	-
	10.90		NQ	NQ	-
B[a]P	0.50		NQ	NQ	-
	2.00	NQ	NQ	NQ	-
	10.00		NQ	NQ	-
QuEChERS-MIP					
B[a]A	0.43		0.39	90.7	8.4
	1.70	< LD	1.63	95.9	5.8
	8.50		7.53	88.6	4.7
CHR	0.39		0.41	105	10
	1.56	< LD	1.47	94.2	5.7
	7.80		6.93	88.9	3.8
B[b]F	0.54		0.50	92.6	12
	2.17	< LD	1.97	90.8	7.9
	10.90		10.20	93.6	6.3
B[a]P	0.50		0.46	92.0	8.3
	2.00	< LD	1.87	93.5	4.6
	10.00		9.73	97.3	6.8

Rec: Recovery; RSD: relative standard deviation (inter-day precision). NQ: not quantifiable

Data shown in Table 3 demonstrate the importance of the treatment with MIP-SPE columns in the experimental procedure since the recoveries obtained without this treatment exceeded 200% in almost all cases. The presence of strong matrix effect is evident. When the procedure is complete, the recoveries were between 88 and 114% in all cases and the inter-day precision was lower than 14% (RSD). Therefore, all compounds were within the acceptance limits (< 15% of the actual value, and < 20% at the LOQ), and therefore the extraction procedure for the target PAHs is reproducible. Additionally, the precision and trueness data obtained reveals the accuracy of the method. It can be stated that the concomitants that accompany the analytes do not significantly affect the analytical characteristics of the method, as no significant ionic suppression was observed.

Additionally, the results of the two proficiency programs in which our laboratory participated with the proposed method are shown in Table 4.

Table 4. Results from the two Proficiency Tests (FAPAS) performed on Spirulina powder in 2017 and 2018.

FAPAS PT 2017 (0673)			
	Assigned value (ng g^{-1})	Concentration found (ng g^{-1})	z-score
B[a]A	50.8	53.4	0.20
CHR	79.4	95.2	0.90
B[b]F	53.1	43.9	-0.80
B[a]P	59.5	67.7	0.60
PAH4	255.0	304.0	1.00
FAPAS PT 2018 (0678)			
	Assigned value (ng g^{-1})	Concentration found (ng g^{-1})	z-score
B[a]A	32.5	32.5	-0.70
CHR	51.9	50.5	-0.10
B[b]F	35.8	26.1	-1.20
B[a]P	36.7	25.1	-1.40
PAH4	157.0	129.0	-0.90

The data provided in Table 4 show the reliability of the proposed method, since the z-scores obtained in all cases are below ± 2, and therefore the method can be considered as fit-for-purpose.

Sensitivity. The method is able to achieve LODs from 0.07 ng g^{-1} for benzo[a]anthracene, to 0.1 ng g^{-1} for chrysene. The results obtained for these validation parameters are shown in table 2.

3.5. Method application

Once validated, the method was applied for the determination of PAHs in several dry extracts obtained from different manufacturers. The concentrations found expressed as mean of six determinations are summarized in Table 5.

Table 5. Concentration of PAHs in the dry plant samples analyzed.

Dry extract name	B[a]A (ng g^{-1})	CHR (ng g^{-1})	B[b]F (ng g^{-1})	B[a]P (ng g^{-1})	Sum PAH 4 (ng g^{-1})
Ginkgo	< LOD	< LOD	< LOD	< LOD	< LOD
Propolis	9.1	23.5	8.37	7.41	48.4
Ginkgo	2.4	3.47	1.5	1.01	8.34
Hops	2.9	6.11	4.38	3.46	16.8
Hops	3.9	7.57	4.44	2.76	18.7
Siberian ginseng-Root	63.5	134	32.9	12.9	243
Hops	16.4	19.7	8.4	5.59	50.1
Hops	4.5	14.7	7.58	3.13	29.9
Hops	8.9	20.8	10.3	7.48	47.5
Hops	4.1	9.26	3.24	2.07	18.7
Hops	7.7	14.7	5.26	2.94	30.6
Hops	D	D	D	D	D
Hops	2.0	4.2	1.7	1.0	9.0
Hops	8.2	14.8	6.2	3.8	33.0
Propolis	1.3	4.8	1.5	D	7.7
Propolis	1.5	8.7	3.6	1.7	15.4
Hops	6.8	13.6	6.5	5.6	32.5
Siberian ginseng-Root	12.7	22.7	7.1	4.8	47.3
Propolis	13.1	10.0	3.6	2.1	28.8
Propolis	3.7	13.0	5.2	4.1	26.0

D: Detected (LOD < value < LOQ)

As shown in Table 5, PAHs were found in 90% of the analyzed samples but their levels were below the limits established by the European Regulations for B[a]P and PAH4 in 95% of the samples. Only the sample of Siberian Gingsen root exceeded the limit for both parameters. The results obtained are in accordance with those reported for supplements containing botanicals in studies performed in 2008 and 2009 [29] (before the entry into force of the Commission Regulation (EU) No 835/2011 in September 2012), where an average of 90% of samples were below the B[a]P limit (10 ng g^{-1}) and the mean level for PAH4 was below the established limit (50 ng g^{1}). However, the same study reported that 27% of samples exceeded the B[a]P limit and the average PAH4 content was 99.7 ng g^{-1} for propolis, pollen and royal jelly. Our findings differ from these data because none of our samples were above the limit for PAH4. This indicates the necessity for monitoring the PAHs content in those food supplements whose manufacturing processes involve the formation or concentration of this family of carcinogenic contaminants. Some manufacturers, such as Biosearch S.A., have developed specific manufacturing processes for PAH removal or minimization in its products, being very important the monitoring of PAH content in the final product to ensure its quality and the compliance with European Regulations.

4. Conclusions

A new analytical method based in MIP technology has been developed and validated for the determination of PAH4 in dry extract samples. The

main advantage of the method is that it allows us to analyze samples in complex matrices with fewer steps for sample treatment. The method can be applied to any type of dry extract and is especially useful in complex matrices for its selectivity, robustness, environmental friendly and low cost compared to traditional methods. The method has been applied to several batches of dry extracts manufactured in different EU and non-EU countries. A total of 5% of the analyzed samples were contaminated with PAHs at concentrations exceeding the limit established by European Regulations, and 95% of samples were found to be contaminated with levels below the allowed limits. Only 10% of samples, which were subjected to specific manufacture processes, were found to be free of PAHs.

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Capítulo 3. Conclusiones

Como resultado de la investigación realizada en la presente Tesis Doctoral, se pueden extraer las siguientes conclusiones:

1. Se han desarrollado metodologías analíticas novedosas para el control de calidad de compuestos funcionales (vitaminas hidrosolubles e isoflavonas de soja) en matrices alimentarias complejas tales como leche, de manera que ofrecen una alta capacidad de procesamiento de muestras ofreciendo resultados analíticos precisos y exactos.

2. Los métodos desarrollados han mejorado de manera notable la capacidad de procesamiento de muestras en análisis de rutina, ya que requieren de un tratamiento de muestra con un bajo número de etapas y donde se minimiza además la cantidad de reactivos y disolventes necesarios respecto a métodos de análisis oficiales o tradicionales.

3. Para esta reducción en el número de etapas en el tratamiento de muestra, se han optimizado los parámetros de separación cromatográfica de forma que se obtienen resoluciones entre los compuestos de interés e interferentes que permiten la adecuada validación y obtención de resultados analíticos de buenas características.

4. Se han desarrollado metodologías analíticas novedosas para el control de la seguridad alimentaria en relación a compuestos contaminantes que pueden aparecer en los alimentos tales como quinolonas, ésteres de cloropropanol y glicidil ésteres, lactosa residual e hidrocarburos policíclicos aromáticos en diferentes matrices alimentarias (lácteos, aceites y extractos vegetales) que ofrecen resultados veraces y precisos con un tratamiento de muestra simple y aplicable en un

laboratorio analítico de rutina y que han demostrado su validez y aplicabilidad en matrices de muestras reales de diversa naturaleza.

5. La simplificación en los tratamientos de muestra se ha conseguido gracias al empleo de técnicas instrumentales con alta capacidad de selectividad, tales como la cromatografía tanto de líquidos como de gases acoplada a espectrometría de masas. Se han optimizado los parámetros instrumentales, permiténdose de este modo, por una parte un alto grado de selectividad gracias a su capacidad de aislamiento de los iones de los compuestos de interés, y por otra, un alto grado de confianza gracias al registro de 2 transiciones específicas de cada compuesto, minimizándose de este modo los posibles errores por falso positivo.

6. Como resultado de esta investigación, se han conseguido metodologías que actualmente son empleadas en laboratorios de Control de Calidad, y que permiten el control analítico de manera rápida y exacta de diferentes productos alimentarios, reduciendo costes y permitiendo por tanto el control de un mayor número de lotes fabricados, lo cual redunda en una mayor seguridad y confianza del cliente final en los productos que consume o comercializa.

7. Así mismo, actualmente se ha implantado y acreditado bajo los requerimientos de la Norma iInternacional UNE/EN ISO-IES 17025 dos de los trabajos desarrollados, concretamente la determinación de 3-MCPD, 2-MCPD y glicidil ésteres, y la determinación de lactosa residual en leche y derivados lácteos, siendo empleados satisfactoriamente de manera rutinaria en nuestro laboratorio de análisis químico.

Anexo I. Espectrometría de masas

En este anexo se describirán las técnicas analíticas espectrométricas empleadas para la consecución de los diferentes objetivos presentados en este trabajo de Tesis.

Como detector acoplado al sistema separativo, tanto en cromatografía de gases como de líquidos, en este trabajo de investigación se ha utilizado principalmente la espectrometría de masas. Por este motivo, y debido a la gran relevancia que ha tenido en esta serie de trabajos, se describirán brevemente los principios físicos y matemáticos en los que se fundamenta esta técnica.

En esta sección se dará una visión general los componentes que conforman de este tipo de detectores. Principalmente y de modo resumido fuente de ionización, analizador de masas y detector.

Fuente de ionización

Para que un espectrómetro de masas pueda ejercer su función, deben generarse partículas cargadas, ya que este tipo de instrumentos basan su funcionamiento en la interacción de dichas partículas con campos eléctricos o magnéticos. En esta primera parte del espectrómetro de masas es donde se lleva a cabo dicha ionización de los compuestos y donde se generan por tanto las partículas cargadas.

En el caso del acoplamiento a cromatografía de líquidos, existen diferentes tipos de fuentes de ionización, dependiendo básicamente de la naturaleza de los compuestos de interés. En la figura 11 se representan esquemáticamente las fuentes de ionización recomendadas en función de la polaridad y peso molecular de los compuestos de interés:

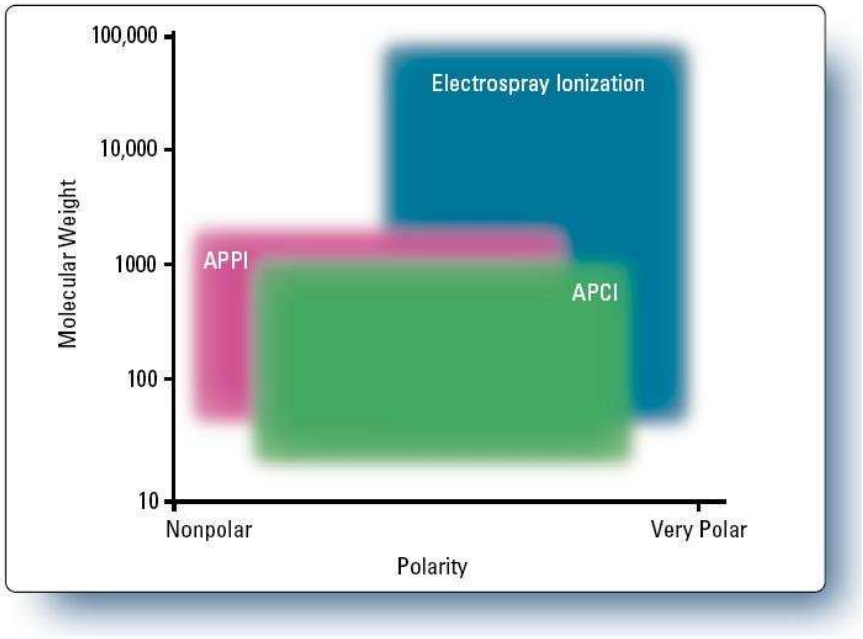

***Figura 11**. Representación de las diferentes fuentes de ionización recomendadas en función de la polaridad y peso molecular de los compuestos en estudio: APCI, Electrospray y APPI. (https://www.researchgate.net/figure/Figura-2-Aplicaciones-de-varias-técnicas-de-ionizacion-en-LC-MS-tomado-de-Agilent_fig8_280757548)*

Las fuentes de ionización más comunes son: electrospray (ESI) e ionización química a presión atmosférica (APCI), siendo la fotoionización química a presión atmosférica (APPI) menos empleada.

El proceso de ionización en cromatografía de líquidos presentó en sus inicios una serie de problemáticas a nivel de desarrollo debido a que se debían conseguir compuestos en estado gaseoso susceptibles de ser ionizados posteriormente, a partir de una fase móvil líquida. Por esta razón, la comercialización de este tipo de acoplamientos entre cromatografía de líquidos y espectrometría de masas fue posterior al correspondiente con cromatografía de gases. En cromatografía de gases, el proceso de ionización presenta menos dificultad, debido a que partículas se encuentran directamente en estado gaseoso. De esta forma, gracias a

un filamento incandescente que emite una corriente de electrones a una energía dada (normalmente a 70eV), los compuestos son fragmentados e ionizados ya a alto vacío y son introducidos en el analizador de masas para su posterior determinación.

Por su relevancia y extensión de uso, se va a describir a continuación de manera resumida los principios de funcionamiento de las fuentes más empleadas en cromatografía de líquidos.

ELECTROSPRAY: En este tipo de fuente, muy comúnmente usada para compuestos polares, la fase móvil procedente del cromatógrafo se introduce directamente a presión atmosférica a través del nebulizador, que consiste en un capilar alrededor del cual se encuentra una corriente de gas inerte (N_2) sometido a altas temperaturas (200°C – 400°C). En la figura 12 se muestra la configuración habitual de estos componentes:

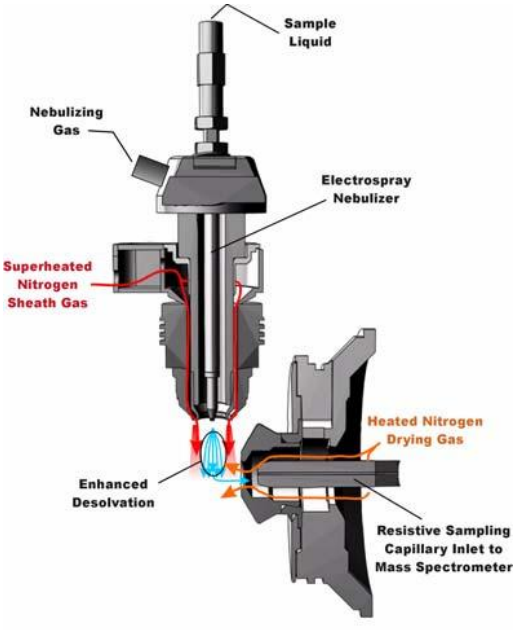

Figura 12. *Representación de una fuente de ionización ESI con sistema "JetStream" (Agilent) la cual emplea una corriente de nitrógeno sobrecalentado que desolvata y confina el spray, estrechando el haz y mejorando la entrada de iones al espectrómetro.*[96]

El flujo de fase móvil junto con los analitos disueltos y separados en el cromatógrafo de líquidos, es dispersado en forma de aerosol gracias a la corriente de gas inerte. Estas gotas formadas son evaporadas debido a la acción de este gas inerte a altas temperaturas. De este modo, se generan gotas cada vez más pequeñas donde los analitos disueltos en la fase móvil son concentrados progresivamente hasta que se llega a un punto donde estas gotas cada vez menores colapsan y los compuestos son ionizados por transferencia de carga desde la fase móvil (figura 13). Por esta razón la composición de la fase móvil debe incluir algún tipo de aditivo volátil (ácido fórmico, amoniaco, ácido acético o sus sales derivadas, etc.) que facilite la

[96] Mordehai, A., Fjeldsted J., Agilent Jet Stream Thermal Gradient Focusing Technology. 5990-3494EN, February 12, 2009.

ionización para este tipo de sonda. Este aditivo debe ser volátil ya que de lo contrario precipitaría en el capilar y colapsaría el espectrómetro de masas.

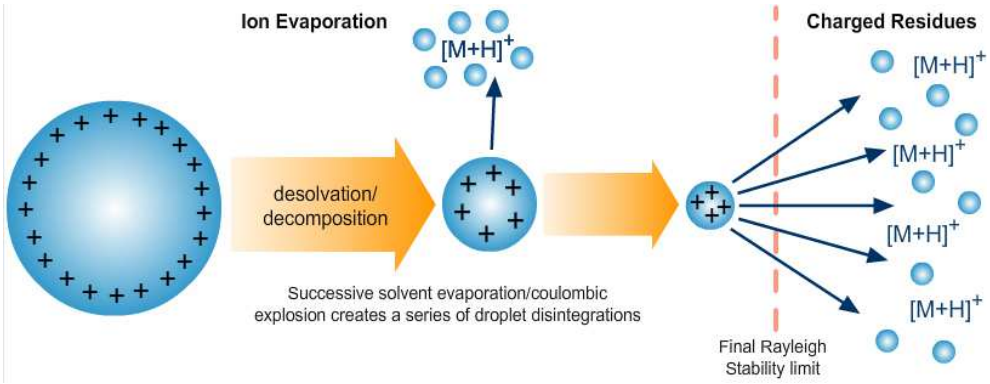

***Figura 13**. Representación del proceso de desolvatación e ionización en una fuente de ionización ESI.*[97]

APCI: Para el caso de compuestos con baja polaridad o con ausencia de grupos funcionales susceptibles de ser protonados, se emplea este tipo de fuente de ionización. En este caso la ionización se consigue gracias a la presencia de una aguja que se encuentra cargada y sometida a un elevado potencial de ionización (generalmente de entre 1 kV y 5 kV). Cuando el flujo de fase móvil es evaporado gracias a la acción de una corriente de gas inerte, este se encuentra con la punta de la aguja cargada, la cual le transfiere una carga a los analitos que entran en contacto con ella directamente o ioniza al disolvente de la fase móvil que a su vez transfiere su carga a los compuestos de interés (figura 14). De esta forma se consigue la ionización de compuestos poco polares sin necesidad de adicionar a la fase móvil agentes que faciliten la protonación o desprotonación de los

[97] Taylor T., Schug K. The CHROMacademy Essential Guide to Electrospray Ionization (ESI) for LC-MS (Part 1).

analitos como ocurre en el caso de la fuente por electrospray, ya que esta función es desempeñada por la aguja de descarga.

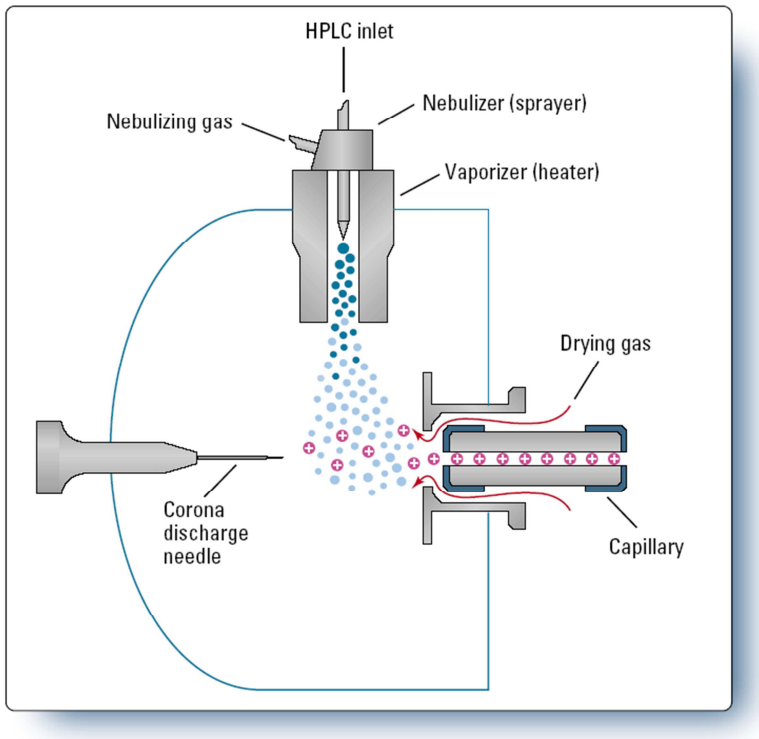

***Figura 14**. Esquema representativo de la fuente de ionización APCI. (https://www.researchgate.net/figure/Figura-5-Fuente-APCI-Agilent-Technologies-2001-APCI-es-aplicable-a-un-gran-numero-de_fig1_280757548)*

Este tipo de fuente de ionización se pueden emplear en sistemas cromatográficos en *fase normal* ya que al no ser necesaria la adición de estos agentes a la fase móvil, normalmente sales derivadas de compuestos volátiles, se pueden usar disolventes apolares en la composición de la fase móvil.

Analizadores de masas

Existen en el mercado diferentes tipos de analizadores de masas que son capaces de determinar la relación m/z de átomos o moléculas cargadas eléctricamente utilizando ciertas propiedades fundamentales de la materia. Tanto por su relevancia a nivel analítico en general, como por su aplicación en esta serie de trabajos en particular, se van a describir de manera resumida los fundamentos físicos y matemáticos en los que se basa el funcionamiento del ***cuadrupolo*** como analizador de masas.

El cuadruplo es el analizador de masas más empleado habitualmente en metodologías analíticas debido a una serie de ventajas que posee respecto a otros tipos de analizadores tales como de sector magnético o tiempo de vuelo (TOF). En la práctica, los analizadores de sector magnético presentan problemas de estabilidad, ya que es difícil obtener un campo magnético uniforme y estable con imanes permanentes. Esto provoca que existan pérdidas tanto en la resolución espectral como en la exactitud de la relación m/z medida.[98] Del mismo modo, para el caso de los analizadores de tiempo de vuelo (TOF), en la práctica presentan una serie de desventajas cuando son usados desde el punto de vista de la química analítica, como son:

- Elevado coste de inversión y mantenimiento.
- Es necesario la introducción constante de un agente calibrante que corrija las desviaciones en la exactitud de la relación m/z medida por el equipo. Esto es debido a que pequeñas variaciones de la

[98] Advanced Lab, Jan. 2008; Disponible en: http://instructor.physics.lsa.umich.edu/adv-labs/Mass_Spectrometer/MassSpecQMS.pdf (Visitado: 28/10/2019)

temperatura ambiente provocan elongaciones del tubo de vuelo que hacen que la distancia recorrida por los iones cambie aleatoriamente. Estos cambios en la distancia recorrida provocan a su vez que la relación m/z medida por el equipo en función de su tiempo de vuelo cambie contantemente a lo largo de la duración del análisis. Por esta razón es recomendable la introducción constante de un compuesto cuyos fragmentos tengan una relación m/z perfectamente conocida, que permita efectuar una corrección constante de las m/z de los analitos medidos.

- La ventaja de los analizadores de tiempo de vuelo sobre los cuadrupolos es que son capaces de ofrecer unas resoluciones espectrales muy superiores (es capaz de ofrecer una exactitud en la medida de m/z de 4 decimales para iones con una m/z de 1000, esto es, una resolución espectral del orden de las ppm), lo que permite conocer la distribución isotópica de los compuestos y de esta forma, a través de algoritmos donde se tiene en cuenta la abundancia isotópica de los diferentes átomos en la naturaleza, se puede conocer la fórmula empírica de los diferentes fragmentos ionizados. Sin embargo, esta ventaja respecto al cuadrupolo, provoca que el número de datos generados a la hora de efectuar un espectro de masas sea muy alto, con la consiguiente dificultad a la hora de tratar estadísticamente los datos obtenidos. El procesado informático de esa cantidad de datos resulta menos ágil y más tediosa que para el caso del cuadrupolo, con una resolución espectral mucho menor (del orden de 1 unidad de m/z).

La acción de filtrado de masas de un cuadrupolo se obtiene mediante la aplicación de un potencial eléctrico continuo (DC) en combinación con

uno dependiente del tiempo (AC) en el rango de las radio frecuencias (MHz) sobre 2 pares de electrodos (idealmente hiperbólicos) conectados entre sí dos a dos (figura 15) y se encuentran separados entre sí una distancia (r_0).

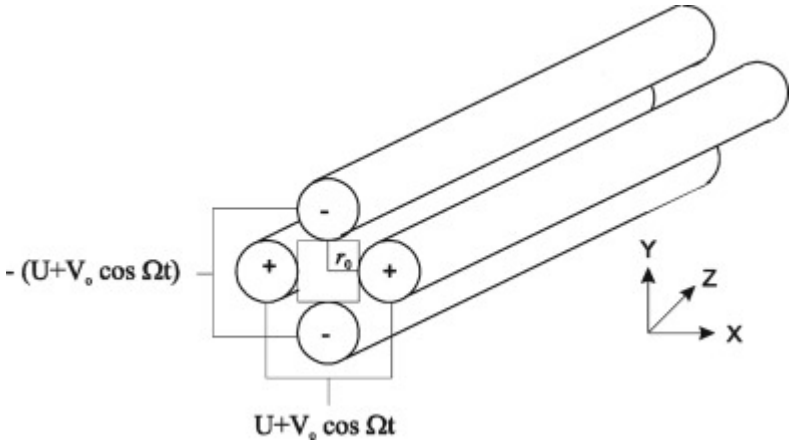

Figura 15. *Esquema de un cuadrupolo simple donde se indican tanto las direcciones de los ejes que se han considerado en este desarrollo, como las expresiones que rigen los potenciales aplicados a cada par de electrodos. (R.E. March, J.F.J. Todd, International Journal of Mass Spectrometry 377 (2015) 316–328).*

En primer lugar se dará una descripción cualitativa cómo se ven afectadas las partículas cargadas dentro de la estructura del cuadrupolo. A continuación se ofrecerá una justificación matemática de las fuerzas que interactúan en dicha estructura y que describen las trayectorias de dichas partículas cargadas.

Descripción cualitativa: De manera muy simplificada, y para entender mejor el comportamiento de un ion dentro del espacio contenido entre los electrodos, se considerará la trayectoria de un ion ***positivo*** con una carga z=1 respecto al eje Y-Z y X-Z por separado respectivamente.

El par de electrodos del plano X-Z está sometido a un potencial *positivo continuo*, además de a uno alterno. Cuando la partícula cargada *positiva* entra en la zona de los electrodos, será repelida por ambos elecrodos y tenderá a permanecer en el eje de la estructura. Sin embargo, el potencial alterno aplicado hace que la partícula oscile, de manera que en el ciclo negativo, si la partícula es lo suficientemente "ligera" (m/z baja), el ion colisionará con alguno de los electrodos. Por tanto, este par de electrodos actuará como un filtro de masas bajas, ya que aquellas partículas por debajo de cierta m/z serán eliminadas, y por el contrario, aquellas más "pesadas" (siempre y cuando asumamos que el valor de z es contante en este ejemplo), se verán menos afectadas por el potencial instantáneo negativo del campo alterno y serán capaces de atravesar la estructura de los electrodos.

Para el caso del eje Y-Z, el par de electrodos está sometido a un potencial *continuo negativo* y al mismo tiempo a un potencial alterno. En este caso, la partícula *positiva* al entrar en la zona de influencia, es atraída por los electrodos negativos, sin embargo, el potencial alterno aplicado tiende a estabilizar la trayectoria, de forma que en el ciclo positivo aplica impulsos que repelen y aceleran la partícula hacia el centro del eje Z. De este modo, si la partícula tiene una relación m/z alta ("pesada"), se verá poco afectada por los cambios instantáneos de polaridad del potencial alterno, siendo afectada en mayor medida a lo largo de su recorrido por el potencial neto *negativo* de los electrodos. Las partículas "pesadas" por tanto, tenderán a desviar su trayectoria hasta que finalmente colisionan con los electrodos y son eliminadas. Por el contrario, aquellas partículas con una relación m/z bajas (”ligeras”) se verán más afectadas por la acción estabilizadora del potencial alterno y serán capaces de atravesar la estructura de los electrodos. Por tanto, este par de electrodos con

potencial continuo negativo actuará como un filtro de masas altas, ya que aquellas partículas a partir de un cierto valor de m/z tendrán una trayectoria inestable y serán eliminadas.

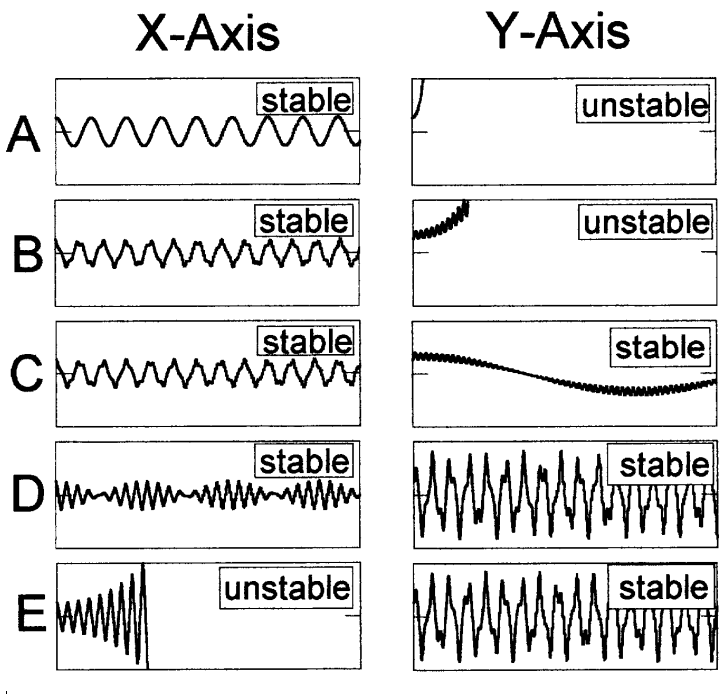

***Figura 16**. Representación de la trayectoria a través del eje XZ e YZ de un ion al que se somete a un potencial continuo fijo y donde se incrementa progresivamente la amplitud del potencial alterno. A y B: amplitud de voltaje alterno baja con trayectoria inestable en el eje YZ. C y D: amplitud de voltaje alterno moderada conde la trayectoria en ambos ejes es estable. E: amplitud de voltaje alterno alto con trayectoria en el eye XZ inestable.*[99]

Para que un ion pueda atravesar la estructura que conforman los dos pares de electrodos XZ e YZ, el ion debe ser capaz de tener una trayectoria estable respecto a ambos planos, y una relación m/z lo suficientemente baja como para que le permita atravesar el filtro de masas bajas y lo

[99] Steel, C., Henchman, M. Understanding the quadrupole mass filter through computer simulation. J. Chem. Edu. 75 (1998) 1049-1054.

suficientemente alta como para no ser eliminado por el filtro de iones de alta relación m/z (figura 17). En resumen, los iones pueden ser filtrados en función de su relación m/z.[100]

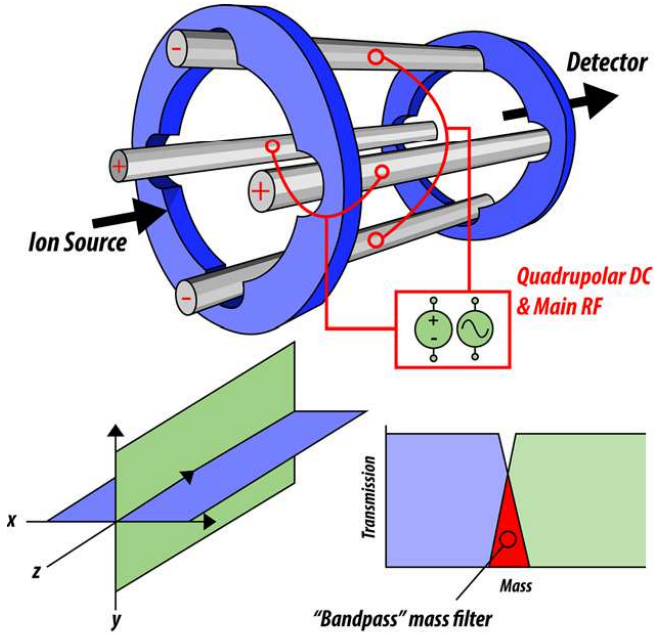

***Figura 17**. Representación de la población de iones que son capaces de atravesar el cuadrupolo y son transmitidos hacia el detector en ambos planos. Notar que la polaridad indicada en los electrodos está erróneamente designada. (John Paul Savaryn, Timothy K. Toby, Neil L. Kelleher. A researcher's guide to mass spectrometry-based proteomics. Proteomics. 2016 Sep; 16(18):2435-43)*

Una representación más ilustrativa se recoge en la figura 18, donde se representa una trayectoria estable de una partícula cargada a través del cuadrupolo, consecuencia de la combinación de movimientos ejercida por los 2 pares de electrodos en los 2 planos del cuadrupolo. Así mismo, en la figura 19 se representa una trayectoria inestable donde la partícula

[100] Miller, P.E., Bonner Denton, M. The quadrupole mass filter: basic operating concepts. J. Chem. Edu. 7 63 (1986) 617-622.

cargada colisiona con algún electrodo y es por tanto descargada y eliminada.

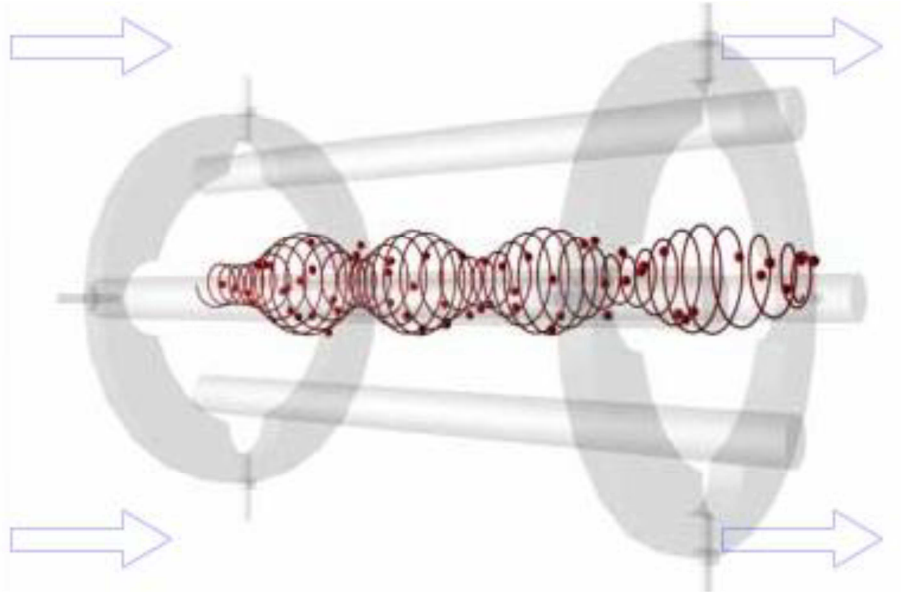

Figura 18. *Representación simplificada de una trayectoria estable a través de la estructura de los electrodos.*
(http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf)

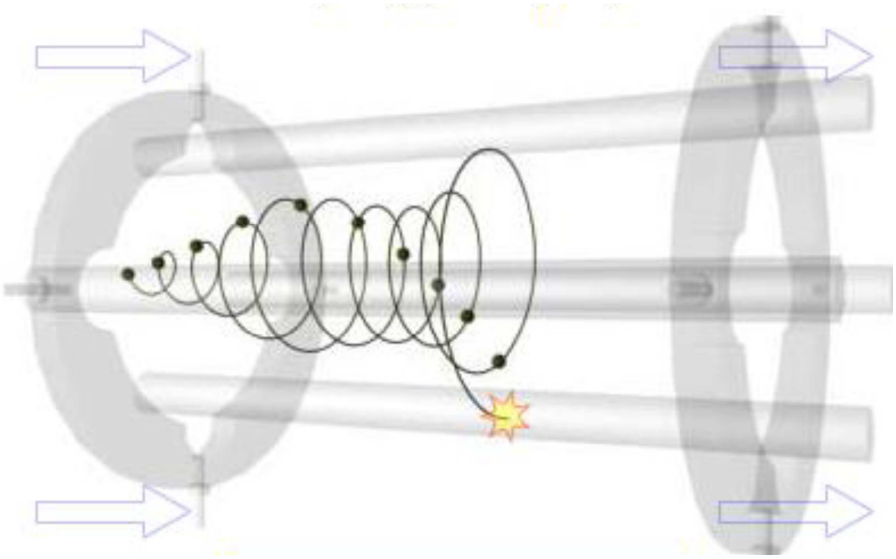

Figura 19. *Representación simplificada de una trayectoria inestable a través de la estructura de los electrodos.*
(http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf)

A continuación se expondrá una descripción detallada de la trayectoria de dichas partículas cargadas sometidas a amplitudes de voltaje de radiofrecuendias moderadas (estabilizantes) y altas (desestabilizantes):

Se debe tener en cuenta que la fuerza con que los electrodos atraen o repelen a las partículas cargadas es proporcional a su posición, de manera que cuanto más alejadas estén de estos electrodos, menor será la influencia y por tanto la fuerza que ejercen los electrodos sobre dichas partículas.

Teniendo esto en cuenta, vemos cómo se afecta una partícula positiva en el **plano YZ** con potencial continuo negativo sometido a amplitudes de corriente alterna moderadas (estabilizantes):

En la figura 20 se ve descrita la trayectoria de una partícula a lo largo de la distancia total de los electrodos respecto al plano YZ. Se ha ampliado la fracción inicial de su recorrido (los primeros 1.5 µs).

En línea de puntos se representa la fuerza que actúa en cada instante sobre la partícula, así como el vector que representa la fuerza que actúa en cada impulso. La línea continua representa la trayectoria real que describe dicha partícula. En la parte inferior de la figura se representa el potencial alterno que actúa en cada momento.

A t_0 la partícula positiva se encuentra en una posición aleatoria de la parte superior del instrumento. En ese instante, al ser el potencial de los electrodos negativo, la partícula es atraída. Inmediatamente después, en el intervalo t_1-t_2, el ion es repelido al haber invertido los electrodos su polaridad, de esta forma la partícula es dirigida hacia el centro del eje Z (impulso estabilizante). Durante el siguiente intervalo, t_2-t_3, el impulso cambia de dirección hacia los electrodos al cambiar de nuevo la polaridad y generar un potencial negativo (impulso desestabilizante). Sin embargo, al estar la partícula en este instante más cerca del centro del eje que durante el intervalo anterior, la fuerza con que es atraída en este segundo intervalo es menor al estar más separada de los electrodos (ya que como se ha comentado anteriormente, la fuerza es dependiente de la posición de la partícula respecto a los electrodos). De esta forma, la partícula es sometida alternativamente a impulsos estabilizantes y desestabilizantes, pero en donde cada impulso desestabilizante es menor que el impulso estabilizante anterior.

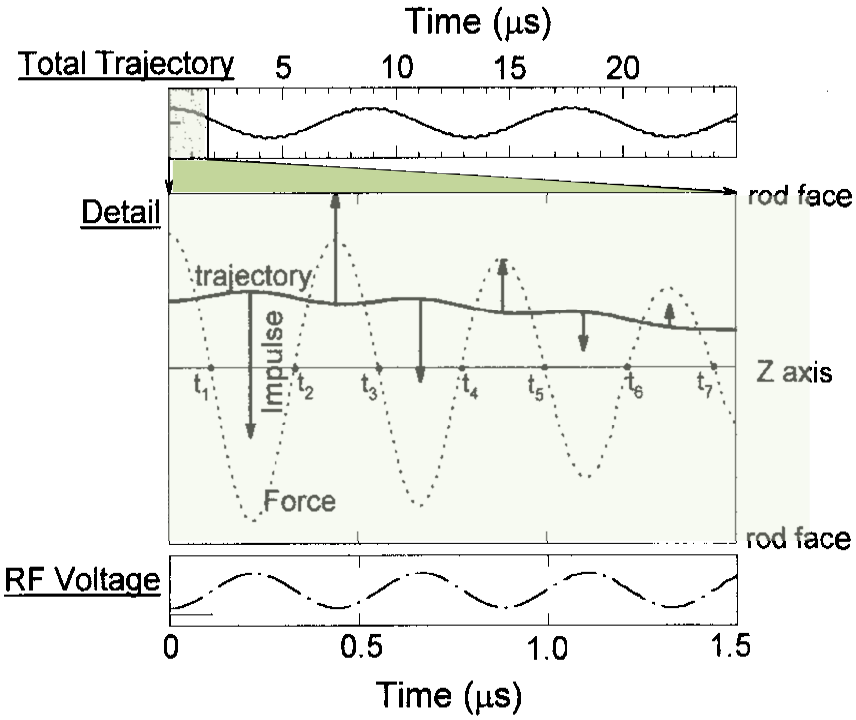

***Figura 20**. Descripción de la trayectoria en el eje YZ de una partícula cargada positivamente. En la parte media se representa una ampliación de los primeros 1.5μs del recorrido total de la partícula. (Colin Steel and Michael Henchman. Understanding the Quadrupole Mass Filter through Computer Simulation. Journal of Chemical Education 1049-1054).*

De esta forma se obtendría una trayectoria estable a lo largo del plano YZ del cuadrupolo.

Del mismo modo, se estudiará a continuación la trayectoria de la partícula a lo largo del **eje XZ** (figura 21) de una partícula positiva sometida a amplitudes de corriente alterna altas (desestabilizantes).

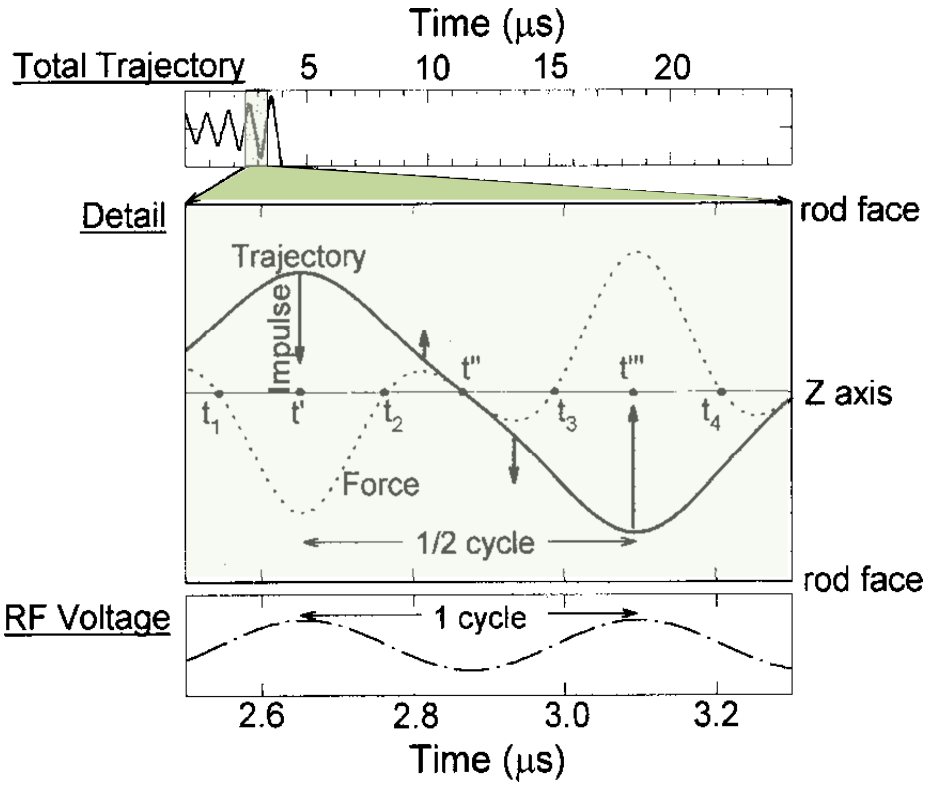

***Figura 21**. Descripción de la trayectoria en el eje XZ de una partícula cargada positivamente. En la parte media se representa una ampliación de la parte inicial de la trayectoria. (Colin Steel and Michael Henchman. Understanding the Quadrupole Mass Filter through Computer Simulation. Journal of Chemical Education, 75 (1998) 1049-1054).*

En este caso, en un instante t_1, la partícula es atraída hacia el electrodo al ser el potencial neto negativo y durante el intervalo t_1-t_2, el ion invierte su dirección debido al impulso que sufre al invertirse el potencial de los electrodos dirigiendo la partícula hacia el centro del eje central. Sin embargo, en esta ocasión, la amplitud del potencial alterno V_0 es lo suficientemente alta que, durante el siguiente intervalo t_2-t'', la partícula es dirigida cerca del eje central. Como en este punto la posición x es pequeña, la fuerza que actúa sobre ella también lo es (y también el impulso), con lo que no es capaz en esta ocasión de revertir la dirección del

ion. De hecho, en t'' la fuerza es cero ya que la partícula se encuentra justo en el eje central. Aunque los impulsos en los intervalos t_2-t'' y t''-t_3 son pequeños, ambos juegan un papel crucial, ya que si la magnitud del impulso durante t''-t_3 es mayor que durante el intervalo previo t_2-t'', el ion será dirigido más cerca del electrodo en el punto de retorno t''' que en el punto de retorno previo t', lo que se traduce en que la amplitud de la trayectoria se hace cada vez mayor y consecuentemente la hará inestable.

Por el contrario, si la posición en el instante t'' es desplazada a la derecha, mediante una disminución de la amplitud V_0, la amplitud del impulso de fuerza en t' será menor, y por tanto, la magnitud en el intervalo t_2-t'' comenzaría a ser mayor al estar la partícula más cerca de los electrodos que la correspondiente al intervalo t''-t_3, con lo que la posición de la partícula en el punto de retorno t''' estaría más lejos de los electrodos que el punto t'. En este caso por tanto la amplitud de la trayectoria comenzaría a disminuir y la trayectoria sería estable.

Descripción matemática: Para poder controlar el paso de partículas cargadas a través del cuadrupolo, se deben calcular matemáticamente cuáles son los voltajes que deben ser aplicados a los pares de electrodos.

Las expresiones que definen las fuerzas que actúan sobre la partícula cargada, tienen la forma de una ecuación diferencial de Mathieu, cuyo nombre proviene del matemático francés Emile Leonard Mathieu (1835 – 1890) quien desarrolló las soluciones a dichas ecuaciones que describen las vibraciones permitidas en un sistema oscilatorio con límites elípticos

acotados[101] (por ejemplo, las que se pueden dar en una membrana de un instrumento de percusión).

Las ecuaciones de Mathieu no tienen soluciones analíticas discretas y deben ser resueltas numéricamente. Una característica importante de estas soluciones es que solamente son estables en un cierto rango de valores de magnitud, en este caso que nos aplica, de campo eléctrico continuo y alterno. Es decir, solamente para cierto rango de valores de U y V_0, las partículas cargadas tendrán una trayectoria estable y no colisionarán con los bordes de los electrodos del cuadrupolo. Estas regiones estables se pueden representar mediante el *diagrama general de estabilidad* (figura 22), donde se observan las regiones que definen los rangos de voltajes aplicados a los electrodos en los cuales se establecen trayectorias estables dentro del plano YZ y XZ respectivamente:

[101] Mathieu, E. Mémoire sur le mouvement vibratoire d'une membrane de forme elliptique. J. Math. Pures Appl. Ser. 2 13 (1868) 137-203.

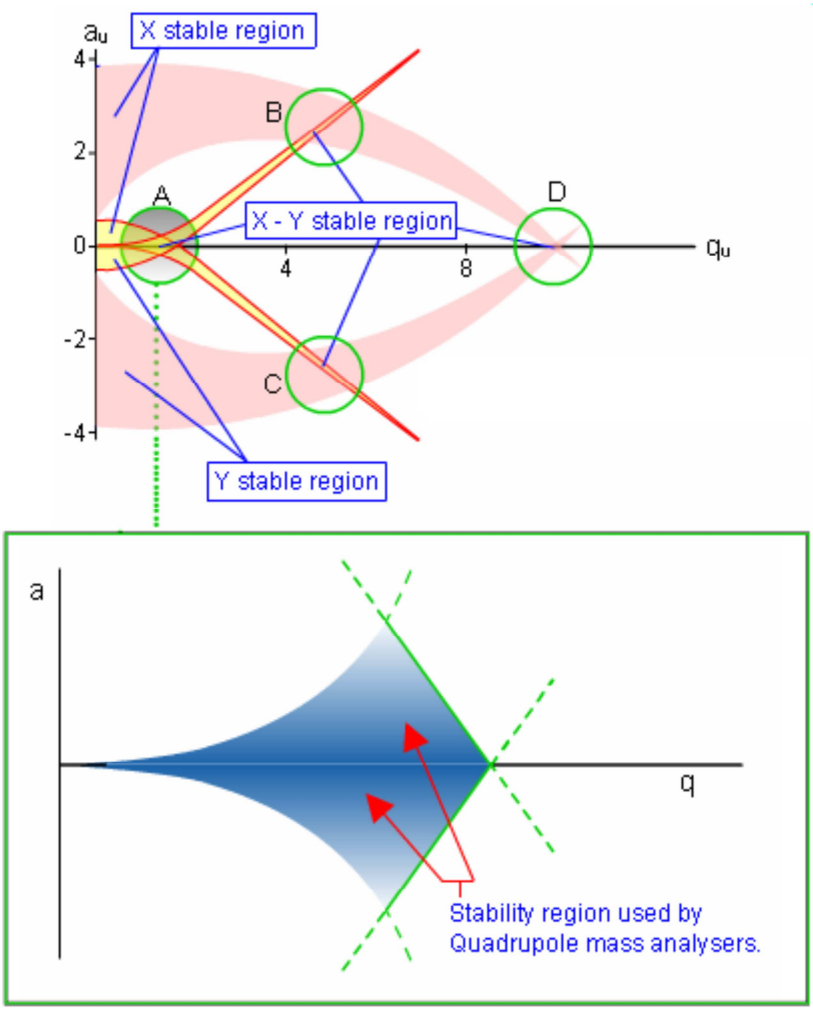

***Figura 22**. Soluciones de la ecuación de Mathieu para las cuales se obtienen trayectorias estables a través de ambos planos YZ y XZ. Las zonas donde se superponen ambas zonas, determinan los voltajes a los cuales una partícula describirá trayectorias estables a través de ambos planos. Normalmente se emplea la zona "A" en los instrumentos analíticos.*
(http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf)

Las zonas donde se superponen las regiones estables del plano YZ y XZ respectivamente definen aquellas condiciones donde la partícula cargada es capaz de atravesar el conjunto de electrodos (zonas marcadas como A,

B, C y D en la figura 12). Normalmente se emplea a zona "A" para trabajar y definir las condiciones instrumentales. Esta zona se conoce como "*diagrama de estabilidad*".

Para calcular el diagrama de estabilidad de una masa en concreto, basta con tener en cuenta dicha masa en los cálculos y determinar el área de estabilidad correspondiente a esa masa. Se obtienen por tanto áreas proporcionales para cada masa (figura 23):

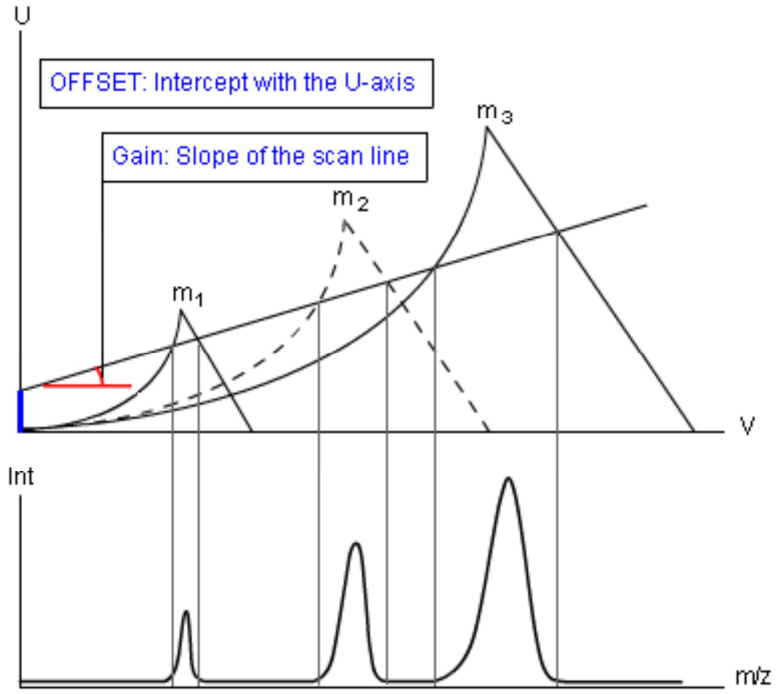

Figura 23. *Areas de estabilidad para iones con diferentes masas (m1<m2<m3).* *(http://www.ecs.umass.edu/eve/background/methods/chemical/Openlit/Chromacademy%20LCMS%20Intro.pdf)*

La relación entre el voltaje continuo aplicado y la amplitud del alterno (U/V_0) es constante y viene definida en este tipo de diagramas como una recta denominada "*línea de scan*". A la relación entre potenciales continuo y alterno que se aplica a los electrodos se le puede considerar como la

pendiente de la línea de scan del diagrama de estabilidad (GAIN), y al voltaje continuo aplicado como la ordenada en el origen de esta línea (offset).

Con la ayuda de este diagrama (figura 24), se puede ver cómo ajustando ambos parámetros se modifica tanto la anchura de banda del rango de masas que puede atravesar el cuadrupolo, esto es, la resolución del analizador de masas, como la intensidad de la señal analítica:

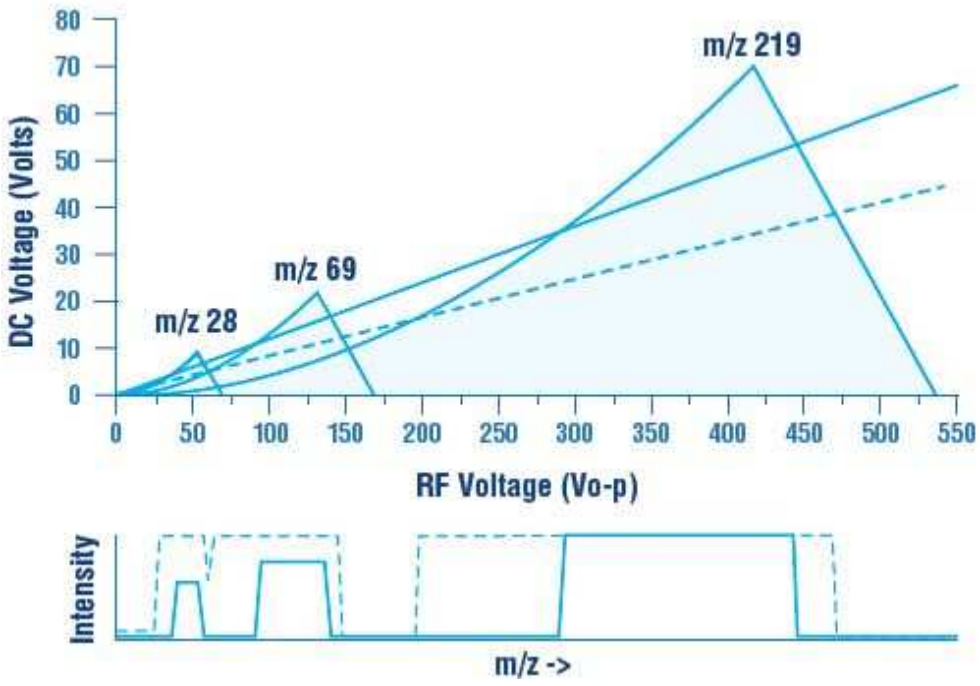

***Figura 24**. Comparación de la variación de la anchura de banda de varios iones al aumentar la ganancia. (https://www.azom.com/article.aspx?ArticleID=10996)*

En esta serie de diagramas de estabilidad se puede observar como aumentando la pendiente de la línea de scan (gain) y manteniendo la ordenada en el origen (offset), aumenta la resolución del espectro de masas y se disminuye la intensidad de la señal (línea sólida). Se puede ver también como al aumentar la ganancia se ven afectadas en mayor medida

las masas altas, sin embargo, cuando se aumenta el offset, se ven afectadas todas las masas de igual forma. Normalmente los analizadores de masas de cuadrupolo deben ser ajustados para optimizar tanto respuesta del equipo como la resolución espectral. Estos cambios son debidos al desgaste de los componentes electrónicos que conforman las placas base, cambios de condiciones ambientales, sustitución de fungibles, etc., que hacen que el equipo deba ser re-ajustado después de cada intervención de mantenimiento, rotura de vacío, etc.

Este ajuste consiste, entre otros, en obtener los valores de "offset" y "gain" óptimos para alcanzar los parámetros de sensibilidad (relación señal/ruido) y resolución espectral estipulados en las especificaciones del fabricante para cada equipo.

Una evolución de este tipo de instrumentos es el espectrómetro de **triple cuadrupolo**, el cual confiere una serie de ventajas adicionales a las vistas en el espectrómetro de cuadrupolo simple. Se trata de una concatenación de cuadrupolos en los cuales se lleva a cabo una función específica. En el primer cuadrupolo se lleva a cabo una selección de los iones precursores, de este modo se eliminan todos los demás iones que pueden interferir en la determinación del compuesto objetivo. Gracias a la alta velocidad de barrido que permiten los cuadrupolos, esta selección de iones precursores se realiza en el orden de los µs, con lo cual se pueden seleccionar un alto número de iones de manera independiente y sin interferir recíprocamente. A continuación, este ion precursor seleccionado entra al siguiente cuadrupolo, denominada *celda de colisión*. Normalmente se trata de un octapolo en lugar de un cuadrupolo debido a la mayor capacidad de transmisión que ofrecen los octapolos respecto a los cuadrupolos. En esta sección lo que se produce es una ruptura de los iones

precursores que genera sus correspondientes iones producto siguiendo un patrón de fragmentación propia de la naturaleza de cada compuesto. Gracias a la presencia de un gas inerte a baja presión y con una energía dada, los iones precursores son fragmentados mediante transferencia de energía por colisión contra estas moléculas de gas inerte. En esta sección no es necesario un filtrado o discriminación de iones, sino que lo que se persigue es que haya una menor pérdida de iones, razón por la cual se necesita una analizador de masas con alta capacidad de transmisión y baja capacidad de filtrado. Este es el caso del octapolo.

Finalmente, estos iones producto acceden al tercer cuadrupolo, donde nuevamente se realiza un filtrado y donde se seleccionan los fragmentos específicos del compuesto a determinar. Al proceso en el cual un ion precursor es seleccionado, posteriormente fragmentado y finalmente seleccionado un ion producto, se denomina *transición*. Normalmente se deben seleccionar un mínimo de 2 transiciones para asegurar la identidad del compuesto que se está determinando.

La premisa es que la relación entre la cantidad de fragmentos que se producen en una colisión bajo las mismas condiciones instrumentales (voltajes aplicados, temperaturas, flujo de gases, composición de fase móvil, etc.) debe ser constante y exclusiva de cada compuesto. Por tanto, para un compuesto, la relación de áreas entre los picos cromatográficos obtenidos registrando las diferentes transiciones de un mismo compuesto, deben ser fijas e invariantes dentro de unos márgenes establecidos. Esta herramienta junto con el tiempo de retención del pico cromatográfico, son los elementos que permiten asegurar la identidad del compuesto objetivo, evitándose de este modo posibles riesgos de falsos positivos o negativos.

Por tanto, el espectrómetro de masas de triple cuadrupolo respecto al de cuadrupolo simple es capaz de aumentar drásticamente la selectividad de la determinación, aumentándose por consiguiente la relación señal/ruido y por tanto la sensibilidad del equipo.

Existen en el mercado otras variaciones de este concepto donde se sustituye el tercer cuadrupolo por otro tipo de analizador de masas con otras características, por ejemplo un analizador de tiempo de vuelo (Q-TOF), una trampa de iones (Q-Trap), etc., dando como resultado instrumentos con capacidad adicional de ofrecer información estructural de los diferentes fragmentos producidos.

A continuación se describe esquemáticamente la estructura interna de un espectrómetro de triple cuadrupolo (figura 25):

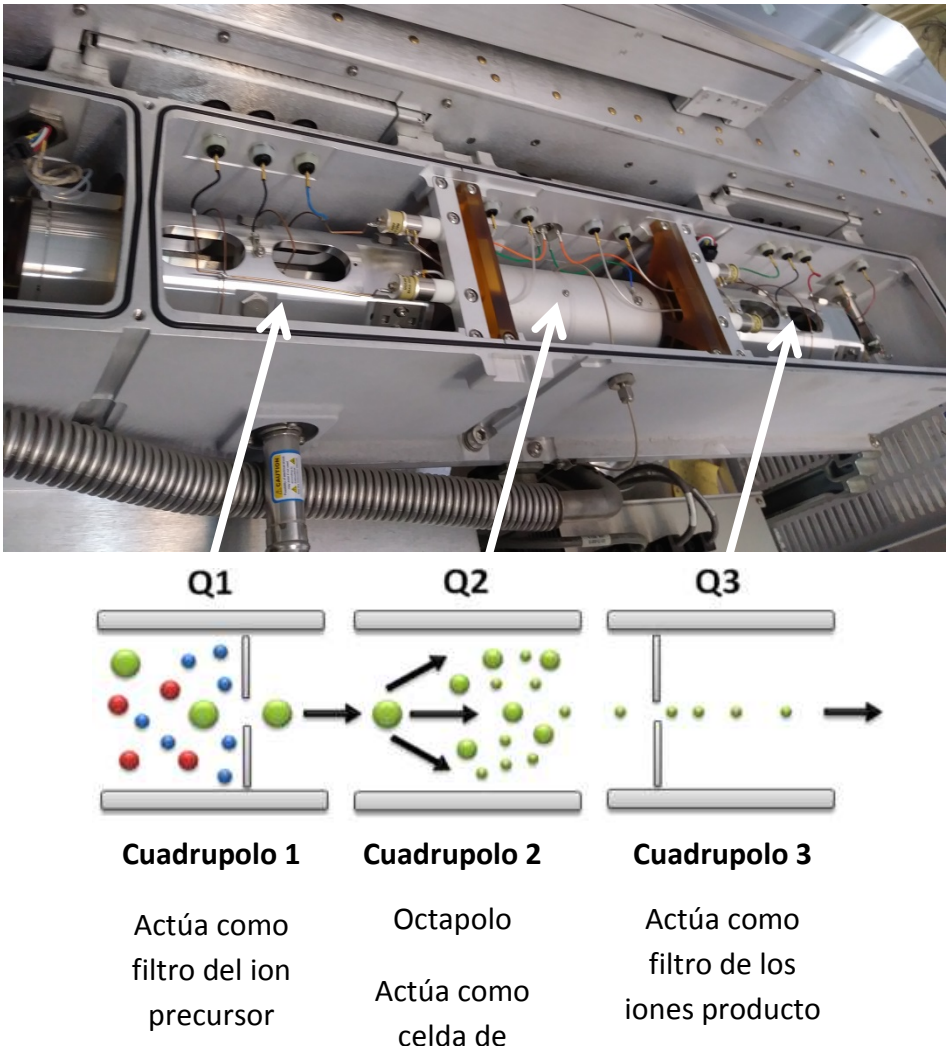

***Figura 25**. Fotografía y esquema de la parte interna de un espectrómetro de masas donde se alojan los 3 cuadrupolos.*

Detectores

Los fragmentos que finalmente son capaces de atravesar el analizador de masas, deben ser detectados. Para ello se necesita un elemento que amplifique la señal producida por el ion y genere una señal eléctrica medible. El elemento más empleado es el *electromultiplicador*, y hace uso

de un proceso conocido como *emisión secundaria de electrones,* mediante el cual el impacto de un ion en un material (oxido de berilio, etc.) desencadena la emisión de electrones. Estos electrones son a su vez acelerados y dirigidos hacia una nueva área de la superficie del dínodo continuo donde colisionan y provocan una nueva cascada de electrones secundarios. Este proceso se repite hasta que al final del detector se genera una cantidad suficiente de electrones que permita una corriente eléctrica medible.

A continuación se muestran ejemplos de detectores:

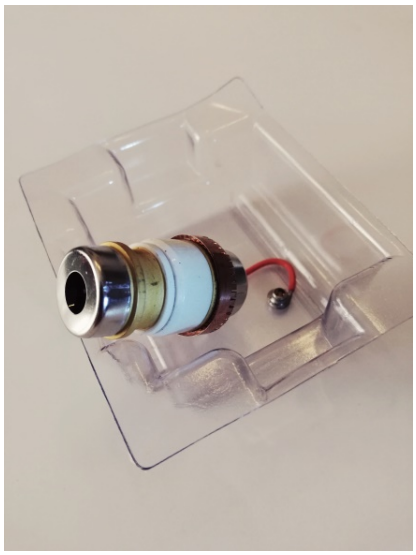

***Figura 26 (izq)**. Electromultiplicador (G3170-80103) para detector de eje triple Agilent. **Figura 27 (drcha.).** Electromultiplicador de dínodo continuo Varian (modelo 4755GM) para espectrómetro Saturn 2000 (colección privada).*

Anexo II. Publicaciones

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Determination of Eight Water-Soluble Vitamins in Supplemented Foods by Liquid Chromatography

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A fast, simple, and reliable method for the isolation and determination of the vitamins thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid in food samples is proposed. The most relevant advantages of the proposed method are the simultaneous determination of the eight more common vitamins in enriched food products and a reduction of the time required for quantitative extraction, because the method consists merely of the addition of a precipitation solution and centrifugation of the sample. Furthermore, this method saves a substantial amount of reagents as compared with official methods, and minimal sample manipulation is achieved due to the few steps required. The chromatographic separation is carried out on a reverse phase C18 column, and the vitamins are detected at different wavelengths by either fluorescence or UV−visible detection. The proposed method was applied to the determination of water-soluble vitamins in supplemented milk, infant nutrition products, and milk powder certified reference material (CRM 421, BCR) with recoveries ranging from 90 to 100%.

KEYWORDS: Water-soluble vitamins; food labeling; supplemented foods; milk; liquid chromatography

INTRODUCTION

Vitamins are crucial for maintaining good health in humans; lack of a sufficient amount of any of them can cause serious diseases (*1*). The human diet does not always contain the amount of vitamins needed for normal development and maintenance of body functions. For this reason, certain food products are supplemented with vitamins, especially those directed to infant nutrition. Moreover, food processing and long periods of food storage may also lead to loss of vitamins. Thus, vitamin fortification allows the nutritional requirements of infant formulas and other baby foods to be met. A rapid and reliable analytical determination of the water-soluble vitamin content in food is needed for food laboratories, manufacturers, and regulatory authorities to confirm the percentage of the recommended dietary allowance (RDA) present in the final food products.

Current official methods (*2*−*12*) for the determination of water-soluble vitamins are based on spectroscopic, chromatographic, chemical, or microbiological techniques that are tedious and time-consuming. Different acid treatments followed by enzymatic digestion before microbiological assay or HPLC procedures have been reported, such as sulfuric acid and amylase (*13*), perchloric acid (*14*), trichloroacetic acid (*15*), or hydrochloric acid and taka-diastase (*16*) treatments. It is important to note that either official methods or HPLC multivitamer methods are not suitable for the simultaneous determination of all the typically supplemented water-soluble vitamins in food.

Separation of water-soluble vitamins has been carried out using reverse-phase liquid chromatography (RP-LC) without ion-pair reagents (*16*−*18*) and also RP-LC with ion-pair chromatography (*15*, *19*, *20*).

Specific HPLC methods have been developed to quantify vitamin C in different foods (*21*−*26*), but only a few HPLC methods simultaneously determining vitamin C and other water-soluble vitamins have been found in the literature (*27*). Similarly, HPLC methods for the determination of pantothenic acid in several food matrices have been reported (*28*−*30*), and attempts to overcome problems of absorbance at low wavelength have utilized postcolumn derivatization (*31*) or mass spectrometry detection with electrospray ionization (*32*). To our knowledge, vitamin C and pantothenic acid have not been included previously in a simultaneous determination of water-soluble vitamins in infant food or vitamin-enriched food products.

In the present work, we describe a simple, fast, and reliable sample treatment procedure previous to the HPLC determination of eight water-soluble vitamins that are usually present in several supplemented food products (infant formulas, infant milk, and vitamin-enriched milks). The vitamins included are thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid.

MATERIALS AND METHODS

Instrumentation. A Megafuge 1.0 centrifuge (Heraeus, Hanau, Germany) was used for sample treatment, and the chromatographic

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Analytical Methods

Improved sample treatment and chromatographic method for the determination of isoflavones in supplemented foods

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ABSTRACT

Isoflavones are a group of substances that belong to the family of phytoestrogens. These natural substances may offer several benefits to human health. One of the most important sources for human isoflavone intake is soy and soybean food derivatives. An improved sample treatment followed by a high performance liquid chromatographic method for the determination of isoflavones in supplemented milk and juices is proposed and compared to the AOAC official method. Detection limits found were between 0.2 and 0.3 mg L^{-1} for daidzein and genistein respectively. Quantification limits found were between 0.7 mg L^{-1} for daidzein to 1.0 mg L^{-1} for genistin, while inter and intra-day variability was under 10% in all cases. Recoveries for spiked samples were over 90% and under 110%. The method was validated by comparison with the AOAC method and by recovery assay methodology.

1. Introduction

Isoflavones are a group of substances that belong to the family of phytoestrogens (Xiao et al., 2009). Many flavonoids are present as flower pigments in most angiosperm families. However, their occurrence is not restricted to the flowers but includes all parts of the plant (Gunatilaka, 2009). From a chemical point of view they comprise a wide group of structurally related compounds with a chromane skeleton provided with a phenyl substituent at the C_2 (flavones) or C_3 (isoflavones) position (Gikas, Alesta, Economou, Karamanos, & Tsarbopoulos, 2008; Patil, Mahajan, & Katti, 2009).

Isoflavones are usually found in plants as glycosides, *i.e.* provided with sugar substituents such as galactose, rhamnose or glucose, or glycoside malonates. The malonates are of biological interest because the plant can use this conjugated form to store the less soluble flavonoid aglycons (Crozier, Jaganath, & Clifford, 2009; de Rijke, Zafra-Gomez, Ariese, Brinkman, & Googier, 2001). If necessary, the organisms generate the aglycon form from these precursors by hydrolysis of the stored form (Sumner, Paiva, Dixon, & Geno, 1996).

These natural substances offer important benefits to human health, such as reduction of cardiovascular risk (Rimbach et al., 2008), osteoporosis (Zhang, Chen, Lai, & Wong, 2008) or hormone – dependent cancers (Messina & Wood, 2008). They have also been shown to stimulate the immune system and to prevent nitration of tyrosine (Oldreive, Zhao, Paganga, Halliwell, & Rice-Evans, 1998). Some other beneficial aspects that have been ascribed to isoflavones include antioxidant activity, metal chelation (Rice-Evans, Miller, & Paganga, 1996) and anticarcinogenic, antiallergic and antiviral effects (Middelton & Kandaswami, 1994, Chapter 15). Ryan-Borchers et al. (2006) have also described that soy isoflavones modulate immune function in health postmenopausal women. In recent years, some authors have described the use of this family of compounds (mainly genistein) in the treatment of genetic diseases like mucopolysaccharidosis or cystic fibrosis (Melin et al., 2004; Piotrowska et al., 2006, 2008; Vandebrouck et al., 2006).

The isoflavones genistein, daidzein, and their glycosides, found in high concentrations in soybeans and soy-protein foods, may have beneficial effects in the prevention or treatment of many hormone-dependent diseases. These bioactive phytoestrogens possess a wide range of hormonal and nonhormonal activities (Setchell, Zimmer-Nechemias, Cai, & Heubi, 1997).

Nevertheless, there is a high controversy in the scientific community about the real health effects of these natural compounds since recent human's studies are inconclusive about the decrease of breast cancer risk on isoflavone high intake (Keinan-Boker, Van Der Schouw, Grobbee, & Peeters, 2004).

One of the most important sources for human isoflavone intake is soy and soybean food derivatives. In the United States, about 25% of infant formula sold are based on soy extracts (Cao et al., 2009). Some of these substances are naturally found in soy grains and are

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ORIGINAL RESEARCH

Simultaneous determination of quinolone antibacterials in bovine milk by liquid chromatography–mass spectrometry

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ABSTRACT: A new liquid chromatography–mass spectrometry (LC–MS) method has been developed and validated for the simultaneous determination of eight quinolone antibacterials for veterinary use in processed bovine milk samples. The quinolones studied included marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid and flumequine. Also, a new sample-treatment procedure was used for extraction and preconcentration of these compounds. It involved defatting by centrifugation, protein precipitation by adding a mixture of glacial acetic acid–acetonitrile and removing acetonitrile with dichloromethane; finally, the acidified aqueous layer was evaporated to dryness in a speed vac system, resuspended in the mobile phase and filtered prior to LC injection. The mobile phase was composed of a formic acid aqueous solution 0.1% (v/v) and acetonitrile, with an initial composition of water–acetonitrile 95: 5 (v/v) and using linear gradient elution. Norfloxacin was used as internal standard. The limits of quantification found (2–7 ng g^{-1}) were in all cases lower than the maximum residue limits tolerated by the European Union for these compounds in milk. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: quinolones; processed milk; liquid chromatography–mass spectrometry (LC–MS)

INTRODUCTION

Quinolones are a group of structurally related antibiotics that are widely used in human and veterinary medicine for the treatment and prevention of pulmonary, urinary and digestive infections (Andriole, 1998; Kuhlmann *et al.*, 1997; Kaartinen *et al.*, 1995; Delépine and Hurtaud-Pessel, 2000). They were introduced for human use in Europe and the USA in the mid-1980s and approved for livestock treatment in the mid-1990s (Anderson *et al.*, 2001). The occurrence of pharmaceuticals—particularly antibiotics—in the environment and in food has generated increasing attention (Tuerk *et al.*, 2006; Kummerer, 2004). Long-term exposure to antimicrobial agents has been associated with an increased risk of the development and spread of antibiotic resistance (Neu, 1992). The 1998 World Health Report of the World Health Organization (WHO) described the increasing occurrence of resistant bacteria and their rapid spread in the world population as one of the biggest health problems of the twenty-first century (World Health Organization, 1998a,b).

In order to ensure the safety of human foodstuffs, the European Union (EU) has set tolerance levels for quinolones in products of animal origin (EU Commission, 1996). Thus, the establishment of sensitive methods for the analysis of residual amounts of these drugs is required for the quality control of food products for consumers and to evaluate the correct application of withdrawal times.

Many studies have been published in the literature on multiresidue analysis of quinolones in biological samples and animal tissues (Carlucci, 1998; Hernández-Arteseros *et al.*, 2002; Samanidou *et al.*, 2005). Most of them involve liquid chromatography with ultraviolet (LC–UV; Gigosos *et al.*, 2000; Pecorelli *et al.*, 2003; Bailac *et al.*, 2004; Christodoulou and Samanidou, 2007), fluorescence (LC–FD; Eng *et al.*, 1998; Hernández-Arteseros *et al.*, 2000; Chu *et al.*, 2002; Espinosa-Mansilla *et al.*, 2005; Hassouan *et al.*, 2007) or mass spectrometric detection (Van Vyncht *et al.*, 2002; Toussaint *et al.*, 2002, 2005a,b; Schneider *et al.*, 2005; Van Hoof *et al.*, 2005; Hermo *et al.*, 2006; Bailac *et al.*, 2006; Rubies *et al.*, 2007).

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Abbreviations used: CIP, ciprofloxacin; DAN, danofloxacin; DIF, difloxacin; ENR, enrofloxacin; FLU, flumequine; MAR, marbofloxacin; MRLs, maximum residue limits; NOR, norfloxacin; OXO, oxolinic acid; SAR, sarafloxacin.

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A novel method for the determination of glycidyl and 3-monochloropropanediol esters in fish oil by gas chromatography tandem mass spectrometry

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ABSTRACT

Today, food security is one of the most important global issues with food quality control and identification of contaminants in foods and beverages, being crucial for human health and safety. In this paper, a novel single-step method for the simultaneous determination of 3-monochloropropanediol (3-MCPD) and glycidyl esters in samples of winterized and non-winterized fish oil by using gas chromatography tandem mass spectrometry (GC-MS/MS) is validated. The method is based on alkaline hydrolysis of esters at room temperature, using only 3-MCPD-d5 as internal standard, and a derivatization step with phenylboronic acid (PBA) at 90 °C. The use of GC-MS/MS results in a simplified sample treatment and improvement of the limits of quantification and precision of the analytical method with no need of additional concentration of the extracts. A backflush tee placed between two HP-5 MS UI columns (15 m×0.25 μm×0.25 mm) was used in order to minimize matrix effects and peak shape degradation usually observed in routine analyses. The method was validated in winterized and non-winterized fish oil, achieving a limit of quantification of 100 ng g^{-1} and 50 ng g^{-1} for 3-MCPD and glycidol, respectively. Method validation was accomplished by comparing our laboratory results with results obtained by an accredited reference laboratory (SGS Germany GmbH) and by calculating the recoveries obtained in an assay with spiked samples. For glycidol quantification, a mathematical equation was developed in order to compensate for the partial conversion of 3-MCPD into glycidol. This expression involves the quantification of 3-MBPD-d5 generated during hydrolysis reaction.

1. Introduction

Food security is a “flexible concept” as is reflected in the many attempts to define it proposed over the years. The United Nations’ Committee on World Food Security defines food security as the condition in which all people, at all times, have physical, social and economic access to sufficient safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life. Over the coming decades, a changing climate, growing global population, rising food prices, and environmental stressors will have significant yet highly uncertain impacts on food security. Economic growth is only sustainable if all countries have food security. Without country-owned food security strategies, there will be obstacles and additional costs to economic growth. The continuing evolution of food security as an operational concept in public policy has reflected the wider recognition of the complexities of the technical and policy issues involved [1]. Foodborne illnesses are a preventable and underreported public health problem. These illnesses are a burden on public health and contribute significantly to the cost of health care. Although anyone can get a foodborne illness, some groups like young children and older adults are at greater risk [2]. In this context, it is crucial to have reliable methods capable of detecting defects or contaminants in foods, either from an external source or formed during food processing and that might pose a hazard to consumers.

Fatty acid esters of 3-monochloropropanediol (3-MCPD) and glycidol are process-induced contaminants found in refined edible oils. The International Agency for Research Cancer (IARC) considers 3-MCPD esters as “possibly carcinogenic to humans (Group 2B)” meanwhile glycidyl esters are considered as “probably carcinogenic to humans (Group 2A)”, making their presence in edible oils a potential health risk [3,4]. These compounds are mainly formed during the refining process in the presence of chloride ions at low pH and high

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Table 4
Proficiency Testing and Inter-Laboratory Comparison to SGS Germany GmbH.

	Accredited Laboratory			Proposed Method				
Sample	3-MCPD (mg kg^{-1})	Δ3-MCPD[a] (mg kg^{-1})	glycidol (mg kg^{-1})	3-MCPD (mg kg^{-1})	Δ3-MCPD[a] (mg kg^{-1})	Glycidol (mg kg^{-1})	% Rec 3-MCPD	% Rec glycidol
B3395	3.27	0.37	0.25	3.04	0.42	0.28	93.0	113.5
B3396	3.25	0.45	0.30	3.12	0.53	0.36	96.0	117.8
B3397	3.40	0.39	0.26	3.23	0.48	0.32	95.0	123.1
B3398	3.26	0.57	0.38	3.32	0.49	0.33	101.8	86.0
B3399	3.36	0.39	0.26	3.60	0.39	0.26	107.1	100.0
B3400	3.25	0.55	0.37	3.21	0.50	0.34	98.8	90.9
DES 2	4.48	0.69	0.46	3.93	0.52	0.35	87.7	75.4
DES 3	3.57	0.44	0.29	3.03	0.36	0.24	84.9	81.8
DES 4	4.31	0.79	0.53	4.05	0.83	0.56	94.0	105.1
DES 5	2.10	0.88	0.59	1.60	0.95	0.64	76.2	108.0
H000421	2.32	0.61	0.41	2.15	0.53	0.36	92.7	86.9
						% Rec	93.4	98.9
						SD (%)	8.4	15.8

Rec: recovery; SD: standard deviation;
[a] Difference between total 3-MCPD sum of free, bounded 3-MCPD and glycidol minus sum of free and bounded 3-MCPD.

Table 5
Inter-day precision for control samples. Glycidol is expressed as 3-MCPD difference for comparing with SGS data.

	3-MCPD (mg kg^{-1})	Glycidol (mg kg^{-1})		3-MCPD (mg kg^{-1})	glycidol[a] (mg kg^{-1})
Day 1	2.90	0.67	Day 30	3.00	0.66
Day 1	3.16	0.51	Day 30	2.80	0.52
Day 1	3.24	0.46	Day 30	3.10	0.57
Day 1	3.40	0.52	Day 30	3.10	0.52
		3-MCPD		Glycidol	
Average (mg kg^{-1})		3.09		0.55	
SD (mg kg^{-1})		0.19		0.07	
RSD (%)		6.1		13.3	
Reference value		3.27		0.57	
Average Recovery (%)		94.4		97.2	

SD: standard deviation; RSD: relative standard deviation.
[a] Expressed as 3-MCPD difference.

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Original research article

Determination of residual lactose in lactose-free cow milk by hydrophilic interaction liquid chromatography (HILIC) coupled to tandem mass spectrometry

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ABSTRACT

Lactose is the major carbohydrate found in milk and dairy products. Lactose intolerance means the body cannot digest foods with this natural sugar in them. In this context, the lactose-free market has experienced a steep increase in recent years. A new method for the determination of residual lactose in lactose-free dairy products using liquid chromatography coupled to tandem mass spectrometry triple quadrupole (HPLC–MS/MS) has been developed. Hydrophilic interaction chromatography (HILIC) has been used for this purpose. An amide chromatographic column with an alkaline mobile phase were selected as optimal. In addition, a fast, cost-effective and reliable sample treatment has been developed for routine analytical laboratory use. The method has been validated by using matrix-matched calibration standards and a recovery assay on a lactose-free milk sample obtained by lactase hydrolysis of regular milk. The limit of quantification (LOQ) was 15 mg L^{-1}, while the recovery was close to 100% with relative standard deviation lower than 9% in all cases. The method was applied to several lactose-free products and the results showed that lactose values in these products are not always below the recommended maximum value of 100 mg L^{-1}.

1. Introduction

Lactose is a major component of milk from mammals and has important nutritional and prebiotic properties. Lactose concentration in human milk is relatively high (7.0%), while in cow milk is about 4.6% (Perati et al., 2016). In the small intestine, it is hydrolysed by the enzyme lactase (β-galactosidase) into glucose and galactose to allow absorption through the intestinal mucosa (van Scheppingen et al., 2017). Approximately 70% of the global adult population and 95% of Asian population have lactase deficiency and are unable to digest lactose (ADILAC, 2016; Schaafsma, 2008). This is known as lactose intolerance (LI). Newborn mammals subsist on milk over the first few months of life, and after weaning there is a genetically-programmed decrease in lactase expression. A large majority of humans show this typical lactase decrease early in life, therefore, adults are unable to properly digest lactose. These individuals are lactose intolerant and are said to have the trait of lactase non-persistence (LNP). Congenital absence of lactase due to a mutation in the gene that is responsible for producing the enzyme is a very rare cause of lactase deficiency, and the symptoms of this type of lactase deficiency begin shortly after birth (Ingram et al., 2009). LI is bothersome but usually not serious and symptoms include abdominal pain, diarrhea, abdominal bloating and distension which reflect the osmotic effects of the unassimilated lactose in the intestinal lumen, plus the fermentation products (such as hydrogen and methane) generated by bacteria in the large intestine.

The demand for lactose-free products is driven by the high prevalence of LI and the worldwide increase in incidences of food intolerances. The dairy industry is continually launching new lactose-free food products. Although the European Food Safety Agency sets the limit of residual lactose content for products labelled as “lactose-free” to 1 g L^{-1}, many dairy companies have set a lower value (0.01%) as a quality feature (Spanish Agency for Consumer Affairs, Food Safety and Nutrition, 2017). In addition, some dairy foods are marketed using the claim “low-lactose”. In that case, the concentration of lactose is < 5 g L^{-1} (Trani et al., 2017). The resulting dairy products contain varying amounts of residual lactose.

In this context, there is a clear need for simple analytical methods to monitor the amount of residual lactose in these products in routine quality control analysis. Different methods have been traditionally applied for the determination of residual lactose such as differential pH

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jfca.2017.11.006.

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Manuscript Details

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Title	Use of QuEChERS and MIP-SPE followed by gas chromatography with tandem mass spectrometry for the quantitative analysis of polycyclic aromatic hydrocarbons in complex dry extracts
Article type	Research Paper

Abstract

A sensitive and selective method based in a combination of QuEChERS and Molecularly Imprinted Polymer technology is validated for the analysis of polycyclic aromatic hydrocarbons in complex dry extracts of Eleutherococcus senticosus, Salvia officinalis, Camellia sinensis, Zingiber officinale, Uncaria tomentosa, Humulus lupulus, Pinus sylvestris L., Spirulina maxima, propolis and royal jelly. The method has been optimized using gas chromatography coupled to tandem mass spectrometry. An additional sample treatment of the dry extracts, based on the combined use of MIP-SPE and QuEChERS, was required because of the strong matrix effect observed related to interferences affecting analyte quantification. Estimation of the method detection limit and quantification limit was carried out for validation. The quantification limits were found to be 0.2 ng g-1 for benzo[a]antracene to 0.4 ng g-1 for benzo[b]fluorantene and chrysene; and the detection limits were found to be range from 0.07 ng g-1 to 0.1 ng g-1. Recoveries are close to 100 % (88 % to 114) % and % RSD was < 14% in all cases. The method was applied in routine analysis to a wide variety of dry extracts from EU and non-EU manufacturers.

Keywords	Polycyclic aromatic hydrocarbons; Dry extracts; Dispersive solid phase extraction (QuEChERS); Molecularly imprinted polymer (MIP); GC-MS/MS
Taxonomy	Analytical Sample Treatment, Analytical Chemistry Analysis, Gas Chromatography Mass Spectrometry, Molecular Imprinted Technique, Solid-Phase Extraction
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