UNIVERSIDAD DE SALAMANCA

FACULTAD DE CIENCIAS QUÍMICAS

Departamento de Química Analítica, Nutrición y Bromatología

ESPECTROMETRÍA DE MASAS ACOPLADA A ELECTROFORESIS CAPILAR Y CROMATOGRAFÍA LÍQUIDA PARA LA DETERMINACIÓN DE CONTAMINANTES Y MICRONUTRIENTES EN ALIMENTOS

MASS SPECTROMETRY COUPLED TO CAPILLARY ELECTROPHORESIS AND LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF CONTAMINANTS AND MICRONUTRIENTS IN FOODSTUFF

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2016

Memoria que para optar al Grado de Doctor por la Universidad de Salamanca presenta la licenciada María Mateos Vivas.

Salamanca, 1 de Junio de 2016

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D.ª Encarnación Rodríguez Gonzalo, Catedrática de Química Analítica de la Universidad de Salamanca, y D. Javier Domínguez Álvarez, Profesor Titular de Química Analítica de la Universidad de Salamanca, ambos directores del trabajo "*Espectrometría de masas acoplada a electroforesis capilar y cromatografía líquida para la determinación de contaminantes y micronutrientes en alimentos*", realizado por la licenciada María Mateos Vivas para optar al grado de Doctor por la Universidad de Salamanca, autorizan la presentación del mismo al considerar que se han alcanzado los objetivos inicialmente previstos.

Salamanca, 1 de Junio de 2016

Fdo: Encarnación Rodríguez Gonzalo   Fdo: Javier Domínguez Álvarez

Deseo expresar mi agradecimiento al Ministerio de Educación por la concesión de una beca de Formación de Profesorado Universitario (FPU, AP-2010-0087), y por la ayuda adicional otorgada dentro del programa FPU (EST13/00852) para la realización de una estancia breve en un centro de investigación extranjero.

Este trabajo ha sido financiado por el proyecto CTQ2011-24075 del Ministerio de Ciencia e Innovación, así como por el programa propio de Financiación de grupos de investigación de la Universidad de Salamanca (USAL2015-16).

El trabajo ha sido apoyado por la Convocatoria Doctor TCUE para el apoyo a la finalización de Tesis Doctorales relacionadas con necesidades empresariales, en el marco del Plan TCUE 2015-2017 de la Universidad de Salamanca, cofinanciado por el Fondo Europeo de Desarrollo Regional (FEDER) y la Junta de Castilla y León.

GRACIAS…

… A los directores de este trabajo, Encarna y Javi, por darme la oportunidad de realizar este bonito proyecto y por guiarme y acompañarme durante estos seis años hasta llegar a conseguirlo. Gracias por confiar en mí, por todos los conocimientos transmitidos, por vuestras ideas y por vuestro tiempo.

… A dos personas que han sido esenciales en el desarrollo de esta tesis: Rita y Diego. Gracias por haber estado para mí a través de cualquier medio de comunicación y casi en cualquier momento. Sería imposible enumerar todo lo que me habéis enseñado, pero si algo he aprendido de vosotros ha sido la pasión por este trabajo. Cada uno, a vuestra manera, habéis sido imprescindibles en lo profesional y grandes apoyos en lo personal. Gracias por apoyarme siempre.

… A todo el departamento de Química Analítica, por haber sido como una gran familia para mí. Con gente así alrededor, da gusto levantarse cada día para ir a trabajar. Gracias a todos mis compañeros "de abajo" por tantos cafés, cenas, fiestas de despedida…por las conversaciones improvisadas en cualquier rincón del laboratorio y, en definitiva, por tantos buenos momentos compartidos. De todos, que habéis sido muchos, me llevo recuerdos maravillosos, pero quiero agradecer de manera especial a los verdaderos amigos que he conocido gracias a esta tesis, a esos que están ahí más allá del laboratorio, más allá de Salamanca e incluso más allá de este país.

… Al Dr. Fanali, a Zeineb y al resto de miembros de su grupo de investigación en el CNR. Gracias por todo lo que me enseñasteis en los tres meses que estuve allí, por acogerme y hacerme sentir como en casa desde el primer día, y por darme la oportunidad de realizar una estancia en una ciudad tan maravillosa como Roma. Sin duda una experiencia que nunca voy a olvidar.

… A Rafa y a Mari Cruz, por su continuo interés y su cariño en todo momento, y a mi nueva familia salmantina, por cuidarme tanto desde el primer día. Estoy encantada de teneros en mi vida. Gracias también a mis abuelos, que espero puedan sentirse orgullosos. Y gracias, por supuesto, a mis amigas (las de siempre, vosotras ya sabéis).

… A la mejor familia que puede existir: Papá, Mamá, Pepe, Esmeralda y mi princesita, Lucía. Para vosotros no hay agradecimientos suficientes ni manera posible de devolveros todo lo que habéis hecho y hacéis por mí día tras día. Vosotros me habéis convertido en la persona que soy y me apoyáis incondicionalmente para que pueda conseguir todo lo que me propongo. Gracias, en definitiva, por sostenerme siempre, por ser mi equilibrio y mi refugio. Os quiero mucho y no podría estar más orgullosa de teneros. Gracias Mamá, especialmente a ti, por ser la madre perfecta y además mi mejor confidente y amiga.

… Al mejor regalo que me ha hecho esta tesis. Gracias Álvaro, por ser mi apoyo y mi calma, por enseñarme a ver la vida de una forma más fácil y por hacerme inmensamente feliz compartiéndola conmigo. Te quiero.

Esta Tesis Doctoral se presenta como un compendio de las publicaciones que se especifican a continuación:

1. *"Capillary electrophoresis coupled to mass spectrometry for the determination of anthelmintic benzimidazoles in eggs using a QuEChERS with preconcentration as sample treatment"*
   Javier Domínguez-Álvarez[1], María Mateos-Vivas[1], Diego García-Gómez[1], Encarnación Rodríguez-Gonzalo[1], Rita Carabias-Martínez[1]. ***Journal of Chromatography A,*** *1278 (2013) 166-174.*

2. ***"****A validated method for the determination of nucleotides in infant formulas by capillary electrophoresis coupled to mass spectrometry"*
   Encarnación Rodríguez-Gonzalo[1], Javier Domínguez-Álvarez[1], María Mateos-Vivas[1], Diego García-Gómez[1], Rita Carabias-Martínez[1]. ***Electrophoresis,*** *35 (2014) 1677-1684.*

3. *"Analysis of free nucleotide monophosphates in human milk and effect of pasteurisation or high-pressure processing on their contents by capillary electrophoresis coupled to mass spectrometry"*
   María Mateos-Vivas[1], Encarnación Rodríguez-Gonzalo[1], Javier Domínguez-Álvarez[1], Diego García-Gómez[1], Rosario Ramírez-Bernabé[2], Rita Carabias-Martínez[1]. ***Food Chemistry,*** *174 (2015) 348-355.*

4. *"Rapid determination of nucleotides in infant formula by means of nano-liquid chromatography"*
   María Mateos-Vivas[1], Salvatore Fanali[3], Encarnación Rodríguez-Gonzalo[1], Rita Carabias-Martínez[1], Zeineb Aturki[3]. ***Electrophoresis,*** *doi:10.1002/elps.201500526.*

5. *"Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotides mono-, di- and triphosphates"*
   María Mateos-Vivas[1], Encarnación Rodríguez-Gonzalo[1], Diego García-Gómez[1], Rita Carabias-Martínez[1]. ***Journal of Chromatography A,*** *1414 (2015) 129-137.*

6. *"Determination of nucleosides and nucleotides in baby foods by hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents"*
   María Mateos-Vivas[1], Encarnación Rodríguez-Gonzalo[1], Javier Domínguez-Álvarez[1], Diego García-Gómez[1], Rita Carabias-Martínez[1]. ***Food Chemistry,*** *211 (2016) 827-835.*

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En relación con la petición de **Dª MARIA MATEOS VIVAS**, solicitando autorización para la presentación de la Tesis Doctoral en el formato de compendio de artículos/publicaciones,

Este Vicerrectorado, teniendo en cuenta el visto bueno de los directores de la tesis así como el informe favorable del departamento de Química Analítica, Nutrición y Bromatología, según lo establecido en el "Procedimiento para la presentación de la tesis doctoral en la Universidad de Salamanca en el formato de compendio de artículos/publicaciones" aprobado por la Comisión de Doctorado y Posgrado el día 15 de febrero de 2013,

HA RESUELTO autorizar lo solicitado.

Salamanca, 27 de enero de 2016

EL VIERRECTOR DE INVESTIGACIÓN Y TRANSFERENCIA

Vicerrectorado - Universidad de Salamanca

Fdo.: Juan Manuel Corchado Rodríguez

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INTRODUCCIÓN GENERAL

1

OBJETO Y ESTRUCTURA DE LA TESIS

1.1. Introducción y Justificación del Tema Objeto de Estudio

La Tesis Doctoral que se presenta está enfocada al desarrollo de nuevos métodos analíticos basados en el acoplamiento de la espectrometría de masas (*Mass Spectrometry,* MS) a técnicas separativas de alta eficacia, como la electroforesis capilar (*Capillary Electrophoresis,* CE) y la cromatografía líquida de alta resolución (*High Performance Liquid Chromatography,* HPLC), que puedan ser de utilidad en la resolución de problemas de especial interés relacionados con la calidad y seguridad alimentaria.

Por ello, este trabajo se dirige a la evaluación de dos tipos de compuestos diferentes: por una parte, a la determinación de residuos de benzimidazoles, fármacos veterinarios de amplio uso como antihelmínticos en animales destinados a la producción de alimentos. Por otro lado, a la determinación de nucleósidos y nucleótidos, nutrientes semi-esenciales presentes de forma natural en alimentos de origen animal. Merece la pena destacar su importancia en el área de la alimentación infantil, ya que estos compuestos están presentes en la leche materna y por este motivo se añaden como suplemento a las fórmulas infantiles y a otros alimentos infantiles complementarios.

Además de su interés dentro del análisis de alimentos, los benzimidazoles y nucleótidos son compuestos con propiedades químicas muy diferentes, por lo que las etapas de tratamiento de muestra que se van a utilizar para su extracción/preconcentración de los alimentos deben contemplar, en cada caso, sus características propias. Se desarrollarán nuevas estrategias de tratamiento de muestra que permitan simplificar estas etapas previas al análisis, de forma que

se acorte el tiempo implicado y a la vez se minimice la manipulación de muestra.

El trabajo se dirige al desarrollo de estrategias novedosas basadas en la metodología QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*) y en la utilización de dispositivos de ultrafiltración asistida por centrifugación (*Centrifugal Ultrafiltration,* CUF) que reduzcan la necesidad de utilización de disolventes orgánicos. También se abordará una técnica altamente consolidada en el análisis de alimentos como es la extracción en fase sólida (*Solid Phase Extraction,* SPE) a través del uso de sorbentes de diferente naturaleza, tanto de tipo polimérico como los basados en interacciones polares o de intercambio catiónico y aniónico. Con estas estrategias se intentará conseguir métodos de tratamiento rápidos, simples, efectivos y compatibles con las técnicas de separación-detección posteriores.

Como técnica de separación, la electroforesis capilar (CE) presenta una alta eficacia y una excelente compatibilidad con medios acuosos, lo que favorece la separación de compuestos de elevada polaridad, iónicos o ionizables. Además, de especial relevancia es el acoplamiento de esta técnica con la detección por espectrometría de masas (CE-MS), que suma a las posibilidades que ofrece la CE el poder de identificación de la detección con MS; presentándose actualmente como una técnica complementaria o alternativa a LC-MS.

Una parte importante de la Tesis se dedicará al desarrollo de innovaciones metodológicas en sistemas de cromatografía líquida. Así, por una parte, se abordará el uso de la cromatografía líquida de interacciones hidrofílicas (*Hydrophilic Interaction Liquid Chromatography,* HILIC) para la separación de compuestos de elevada

polaridad. Además, el acoplamiento de HILIC con la modalidad de espectrometría de masas en tándem (MS/MS) permitirá no sólo la detección e identificación de los analitos, sino el estudio de los procesos de fragmentación y las transiciones que puedan proporcionar una identificación inequívoca de los mismos. Un segundo aspecto abordado es el empleo de la nano-cromatografía líquida (nano-LC) que nos permite conseguir las ventajas inherentes a las técnicas miniaturizadas: mayor eficiencia en la separación, menor tiempo de análisis, bajo consumo de eluyentes y muestras y un buen acoplamiento con MS debido a la reducción del flujo cromatográfico.

En todos los casos, las innovaciones metodológicas que se han abordado a lo largo del desarrollo de esta Tesis Doctoral están enfocadas a conseguir métodos analíticos más rápidos, sensibles y selectivos; que puedan además cumplir los criterios establecidos por la legislación para su validación como métodos cuantitativos de confirmación en el análisis de los compuestos de interés.

1.2. Hipótesis de Trabajo y Principales Objetivos a Alcanzar

Esta Tesis Doctoral presenta dos objetivos bien diferenciados. Por un lado se trata de desarrollar nuevas metodologías que respondan a las demandas que tiene planteadas la Química Analítica tales como la simplificación de los procedimientos analíticos, tanto en etapas de tratamiento de muestra especialmente laboriosas, como en los aspectos relacionados con la separación-detección. El segundo objetivo se dirige a la aplicación de estas metodologías a la resolución de problemas de interés relacionados con el control de calidad de alimentos.

En primer lugar se pondrá a punto un método para la determinación de residuos de fármacos veterinarios, concretamente benzimidazoles antihelmínticos en muestras de huevo, por ser éste un alimento de origen animal de elevado consumo. Se utilizará una técnica de separación de alta eficacia, como es la CE-MS, para obtener separaciones rápidas y con un alto poder de resolución. Se evaluarán diferentes estrategias de tratamiento de muestra basadas en la SPE y en la metodología QuEChERS, que consiste en una extracción con acetonitrilo asistida por sales, de forma que proporcione una extracción eficiente de los benzimidazoles y una adecuada limpieza de una matriz compleja como es el huevo.

Posteriormente los estudios se dirigirán al análisis de micronutrientes como nucleósidos y nucleótidos. Se planteará en primer lugar la utilización de la misma técnica instrumental basada en CE-MS, muy adecuada para la determinación de compuestos hidrofílicos. Los nucleótidos son compuestos de especial importancia en etapas de crecimiento en bebés debido, entre otras cosas, a su función reguladora en la respuesta inmune. Por eso, en este caso, las muestras analizadas estarán relacionadas con la alimentación infantil: fórmulas lácteas de iniciación y leche materna.

También en este apartado se evaluarán diferentes procedimientos de tratamiento de muestra, tanto basados en SPE como en la utilización de dispositivos CUF. Se completará el estudio aplicando el método desarrollado al análisis de muestras de leche materna procedentes de un banco de leche humana. En estas muestras se evaluarán, además, otros parámetros como la influencia de los pre-tratamientos a los que habitualmente se someten estas leches para su almacenamiento y

conservación en dichos bancos: pasteurización y procesado con altas presiones.

Dentro del campo de la cromatografía líquida se abordará un estudio que tendrá como objetivo dilucidar los mecanismos de retención que se generan entre distintas fases estacionarias HILIC, y compuestos de elevada polaridad como nucleótidos. Estos analitos son compuestos fosforilados muy polares que resultan de difícil retención mediante otros mecanismos cromatográficos y cuya determinación puede ser compleja debido a sus posibles interacciones con distintas partes del sistema cromatográfico. Se evaluarán las opciones que consigan evitar estos problemas y que permitan el desarrollo de un método analítico rápido y eficaz para el análisis de un elevado número de nucleósidos y nucleótidos en alimentos infantiles lácteos y no-lácteos.

Como complemento a los métodos desarrollados para la determinación de nucleósidos y nucleótidos mediante las técnicas electroforéticas y cromatográficas disponibles en el Departamento de Química Analítica de la Universidad de Salamanca, se incluye el trabajo llevado a cabo durante la estancia breve realizada en un centro de investigación de reconocido prestigio internacional: *Istituto di Metodologie Chimiche, Consiglio Nazionale delle Ricerche* (CNR, Roma). El grupo de investigación "*Electromigration and Chromatographic Methods*", dirigido por el Dr. Fanali, es pionero en el desarrollo y aplicación de técnicas miniaturizadas de separación, en concreto de la nano-cromatografía líquida (nano-LC). La nano-LC es una técnica miniaturizada de análisis que ha emergido en los últimos años como método de separación complementario o competitivo a la LC

convencional, debido a las numerosas ventajas que ofrece en cuanto a eficiencia, tiempo de análisis, menor cantidad de fase estacionaria así como uso de pequeños volúmenes de fase móvil. De esta forma, la realización de dicha estancia dio lugar a un nuevo método para la determinación de nucleótidos en fórmulas infantiles, tratadas previamente con dispositivos CUF y analizadas posteriormente con nano-LC.

1.3. Metodología

La metodología seguida en el desarrollo del trabajo que se presenta en esta Tesis Doctoral consiste en cuatro pasos fundamentales que se llevarán a cabo de manera secuencial:

1. *Optimización de métodos analíticos para la separación y detección de los analitos objeto de estudio, ya sea con CE-MS, HILIC-MS/MS o nano-LC-UV.*

En CE, la separación se basa en el movimiento de especies cargadas al aplicar una diferencia de potencial entre los dos extremos de una columna capilar rellena de un medio conductor, denominado medio de separación. Por tanto, la optimización del medio de separación (composición, concentración de sales, pH…) será especialmente importante debido a su influencia en la ionización de los analitos. También será decisiva la optimización del medio de inyección y sobre todo, la optimización de los parámetros relativos al acoplamiento CE-MS como la composición del líquido adicional, flujo, presión de nebulización o la ionización en modo positivo o negativo en el ESI.

En HILIC-MS/MS se va a llevar a cabo un estudio con el fin de dilucidar los mecanismos de retención que se producen entre los nucleótidos y las fases estacionarias. Para ello se evaluarán diferentes columnas HILIC y se realizará un estudio detallado de la composición de la fase móvil utilizando diferentes sales y concentraciones de las mismas en una fase móvil rica en componente orgánico. La detección con MS/MS requiere además la optimización de las rutas de fragmentación de los analitos con el fin de asegurar la correcta identificación de los mismos.

La nano-LC es una técnica miniaturizada de análisis que ha emergido en los últimos años como método de separación complementario o competitivo a la LC convencional. La optimización del método basado en nano-LC estará enfocada a la comparación de diferentes fases estacionarias (previo empaquetamiento de las mismas en el propio laboratorio), así como al estudio de los diferentes parámetros experimentales susceptibles de afectar a la separación-detección, como la composición y pH de la fase móvil o la inyección de la muestra.

2. *Desarrollo de estrategias de tratamiento de muestra que faciliten la extracción y/o preconcentración de los analitos de muestras de alimentos de consumo habitual.*

Los alimentos son matrices complejas, por lo que el tratamiento de muestra empleado para la extracción de los analitos y la limpieza de las muestras es un paso crucial en el desarrollo metodológico. En el caso de los benzimidazoles antihelmínticos, fármacos veterinarios de baja polaridad que se administran a animales destinados a la producción de alimentos, la estrategia de tratamiento de muestra se basará en el método QuEChERS (extracción con acetonitrilo asistida por sales). Para

los nucleótidos, nutrientes semi-esenciales de elevada polaridad y que tienen un papel muy importante en la regulación de la respuesta inmune, el proceso se enfocará a la ultrafiltración por centrifugación. También se evaluará, en ambos casos, la extracción en fase sólida utilizando sorbentes de diferentes características: interacciones polares, intercambio aniónico o catiónico.

El objetivo final es encontrar métodos de tratamiento de muestra que sean rápidos, simples, eficientes y compatibles con las técnicas de separación-detección que se van a utilizar.

3. *Validación de los métodos propuestos de acuerdo a la actual legislación europea, a través de la evaluación de los diferentes parámetros que establece dicha legislación.*

Los diferentes métodos analíticos desarrollados a lo largo de esta Tesis se validarán según la Decisión de la Comisión Europea 2002/657, que indica que en los métodos cuantitativos de confirmación es necesario determinar la capacidad de detección (CC_{β}), el límite de decisión (CC_{α}), la veracidad o recuperación, la precisión, la selectividad o especificidad, la aplicabilidad o robustez y la estabilidad.

4. *Aplicación de los métodos desarrollados al análisis de alimentos de elevado consumo con el fin de encontrar respuestas a las problemáticas actuales de la Química Analítica en el área de la calidad y seguridad alimentaria.*

La determinación de benzimidazoles se llevará a cabo en alimentos de elevado consumo como es el caso del huevo, y se comprobará la aplicabilidad del método CE-MS propuesto a través del análisis de diferentes tipos de huevos. El estudio de nucleósidos y

nucleótidos, con CE-MS, HILIC-MS/MS y nano-LC-UV, se centrará en el área de la alimentación infantil llevando a cabo determinaciones en diferentes tipos de leches infantiles comerciales, en leches maternas y en otros tipos de alimentos infantiles complementarios lácteos y no lácteos.

1.4. Estructura de la Tesis

A fin de cumplir los objetivos planteados al inicio de esta investigación y siguiendo en todo momento la metodología propuesta para el desarrollo de la misma, esta Tesis se ha estructurado en dos capítulos generales (I y II) dentro de los cuales se incluyen los diferentes trabajos realizados. A continuación se muestra la temática de cada uno de estos dos capítulos, así como los contenidos que se podrán encontrar en los mismos, poniendo de manifiesto la coherencia y relación existente entre ellos.

En los apartados siguientes se incluye una copia completa de cada una de las publicaciones originales que conforman la Tesis, precedida en cada caso de un resumen en el que se especificarán los objetivos concretos de cada trabajo, la metodología utilizada, los resultados alcanzados y las conclusiones finales.

INTRODUCCIÓN GENERAL

CAPÍTULO I. **APLICACIONES DE LA ELECTROFORESIS CAPILAR ACOPLADA A ESPECTROMETRÍA DE MASAS.**

I.1. *DETERMINACIÓN DE BENZIMIDAZOLES ANTIHELMÍNTICOS EN HUEVO UTILIZANDO QUECHERS CON PRECONCENTRACIÓN COMO TRATAMIENTO DE MUESTRA.*

I.2. *DETERMINACIÓN DE NUCLEÓTIDOS EN FÓRMULAS INFANTILES DE INICIACIÓN TRATADAS PREVIAMENTE CON ULTRAFILTRACIÓN ASISTIDA POR CENTRIFUGACIÓN.*

I.3. *ANÁLISIS DE NUCLEÓTIDOS LIBRES EN LECHE MATERNA Y EFECTO DE LA PASTEURIZACIÓN Y DEL PROCESADO CON ALTAS PRESIONES EN EL CONTENIDO DE LOS MISMOS.*

CAPÍTULO II. **NUEVAS TENDENCIAS EN CROMATOGRAFÍA LÍQUIDA.**

II.1. *DETERMINACIÓN DE NUCLEÓTIDOS CON NANO-CROMATOGRAFÍA LÍQUIDA.*

II.2. *CROMATOGRAFÍA DE INTERACCIONES HIDROFÍLICAS ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM, EN PRESENCIA DE REACTIVOS FORMADORES DE PARES IÓNICOS HIDROFÍLICOS, PARA LA SEPARACIÓN DE NUCLEÓSIDOS Y NUCLEÓTIDOS MONO-, DI- Y TRIFOSFATO.*

II.3. *DETERMINACIÓN DE NUCLEÓSIDOS Y NUCLEÓTIDOS EN ALIMENTOS INFANTILES UTILIZANDO CROMATOGRAFÍA DE INTERACCIONES HIDROFÍLICAS ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM, EN PRESENCIA DE REACTIVOS FORMADORES DE PARES IÓNICOS HIDROFÍLICOS.*

2

BENZIMIDAZOLES ANTIHELMÍNTICOS

2.1. Introducción

Las infecciones parasitarias, tanto en humanos como en animales domésticos, constituyen un problema médico sanitario importante. Estas infecciones por helmínticos ocasionan grandes pérdidas económicas a los propietarios de animales destinados a la producción de alimentos[1]. Es importante destacar la investigación farmacéutica dirigida al desarrollo de nuevos fármacos antihelmínticos para el control de parásitos en animales domésticos[2], que ha dado lugar a un gran número de productos veterinarios para el tratamiento y prevención de estas infecciones. Sin embargo, este desarrollo ha suscitado también la preocupación debido a la amplia disponibilidad de fármacos veterinarios sin prescripción, que puede incrementar el riesgo del uso incorrecto de los mismos y conducir así a la aparición de residuos en alimentos de origen animal[3]. Por todo ello, es imprescindible la determinación de los residuos de estos fármacos que se acumulan en los alimentos, así como respetar los tiempos de retirada necesarios para que estos residuos desaparezcan por completo de los alimentos procedentes de animales tratados con antihelmínticos.

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[1] C. E. Lanusse, R. K. Prichard. *Relationship between pharmacological properties and clinical efficacy of ruminant anthelmintics.* Veterinary Parasitology, 49 (1993) 123-158.

[2] A. F. Loyacano, J. C. Williams, J. Gurie, A. A. DeRosa. *Effect of gastrointestinal nematode and liver fluke infections on weight gain and reproductive performance of beef heifers.* Veterinary Parasitology, 107 (2002) 227-234.

[3] B. Kinsella, S. J. Lehotay, K. Mastovska, A. R. Lightfield, A. Furey, M. Danaher. *New method for the analysis of flukicide and other anthelmintic residues in bovine milk and liver using liquid chromatography–tandem mass spectrometry*. Analytica Chimica Acta, 637 (2009) 196-207.

Los derivados benzimidazólicos son agentes antihelmínticos de amplio uso para el tratamiento de infecciones parasitarias en animales destinados a la producción de alimentos. En general, los residuos antihelmínticos no poseen riesgos para la salud humana siempre que estos sean administrados de una forma adecuada y con la dosis recomendada, respetando los tiempos de retirada[3]. Se han descrito diferentes efectos tóxicos asociados con la exposición crónica a los compuestos benzimidazólicos, como el desarrollo de malformaciones congénitas, diarrea, anemia, edema pulmonar o inflamación de los ganglios linfáticos[4]. La exposición humana a estos agentes tóxicos tiene lugar, principalmente, a través de la ingesta de alimentos contaminados. Para proteger a los consumidores de estos riesgos, la actual legislación[5] proporciona límites máximos de residuos (*Maximum Residue Limits*, MRLs) para ciertos alimentos de origen animal.

2.2. Características generales

El desarrollo de nuevos fármacos antihelmínticos ha sido un área de investigación intensiva desde comienzos del siglo XX, con el propósito de mejorar su eficacia antiparasitaria a la vez que se limitan sus efectos secundarios. Un fármaco antihelmíntico ideal debe tener un

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[4] X. Z. Hu, J. X. Wang, Y. Q. Feng. *Determination of benzimidazole residues in edible animal food by polymer monolith microextraction combined with liquid chromatography-mass spectrometry.* Journal of Agricultural and Food Chemistry, 58 (2010) 112-119.

[5] Commission Regulation, 2010. Commission regulation EU Number 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union L 15, 1-72.

amplio espectro de actividad; ser fácil y práctico de administrar al animal; generar una baja concentración de residuos de forma que los animales tratados y sus productos sean aptos para el consumo humano; ser barato; y que pueda ser administrado de forma segura a los animales sin producir en ellos efectos secundarios perjudiciales. Un compuesto antihelmíntico efectivo debe ser tóxico sólo para el parásito contra el que actúa, lo cual supone un importante desafío en el desarrollo de un fármaco antihelmíntico ideal[1].

De todos los fármacos antiparasitarios, los benzimidazoles son uno de los grupos con mayor espectro de actividad y con elevada efectividad y seguridad. Estos compuestos actúan sobre huevos, larvas y parásitos adultos[6]. Se usan para el control de endoparásitos en aves de corral, reses, ovejas, cabras, cerdos, y otros animales domésticos.

El primer benzimidazol que se introdujo en el mercado como fármaco antihelmíntico de amplio espectro fue el tiabendazol en 1961. En los años siguientes aparecieron nuevos derivados (albendazol, fenbendazol y oxfendazol) con propiedades antihelmínticas mejoradas como una mejor eficacia, un mayor espectro de actividad y una menor toxicidad. Otros compuestos como el febantel o el tiofanato se conocen como pro-benzimidazoles, ya que se transforman en un benzimidazol que aporta la eficacia antihelmíntica después de ser administrados al animal.

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[6] C. Chassaing, M. Berger, A. Heckeroth, T. Ilg, M. Jaeger, C. Kern, K. Schmid, M. Uphoff. *Highly water-soluble prodrugs of anthelmintic benzimidazole carbamates: synthesis, pharmacodynamics, and pharmacokinetics.* Journal of Medicinal Chemistry, 51 (2008) 1111-1114.

2.2.1. Propiedades químicas

Los benzimidazoles presentan una estructura bicíclica compuesta por un anillo de benceno que se fusiona en las posiciones 4 y 5 con un anillo imidazólico, formando el anillo benzoimidazólico[7]. Los distintos benzimidaoles difieren en los sustituyentes que se encuentran en las posiciones R_1 y R_2 del anillo benzoimidazólico. Dependiendo de estos sustituyentes, estos agentes antihelmínticos se pueden clasificar en metilcarbamatos si R_1 es un grupo metilcarbamato; tiazólicos si R_1 es un grupo tiazol; o tiol-halogenados si R_1 es un grupo tiol y R_2 es un halógeno. En la Figura 2.1 se puede observar la estructura química básica de los benzimidazoles.

R2 N R1 N H

Figura 2.1. Estructura química básica de los benzimidazoles.

En la Tabla 2.1 se muestran los benzimidazoles estudiados en este trabajo. Todos son metilcarbamatos, excepto el 5-hidroxi-tiabendazol que tiene un grupo tiazol, y el albendazol-2-aminosulfona y el 2-aminobenzimidazol que son metabolitos del albendazol y el benzimidazol respectivamente, con un grupo amino en la posición R_1.

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[7] L. B. Townsend, D. S. Wise. *The synthesis and chemistry of certain anthelmintic benzimidazoles.* Parasitology Today, 6 (1990) 107-112.

Tabla 2.1. Benzimidazoles estudiados

Benzimidazol	R_1	R_2
Metilcarbamatos		
Albendazol (ABZ)	$-NH-CO-O-CH_3$	$-S-CH_2-CH_2-CH_3$
Albendazol-sulfona ($ABZ-SO_2$)	$-NH-CO-O-CH_3$	$-SO_2-CH_2-CH_2-CH_3$
Carbendazim (CBZ)	$-NH-CO-O-CH_3$	-H
Fenbendazol (FBZ)	$-NH-CO-O-CH_3$	$-S-(C_6H_6)$
Fenbendazol-sulfona ($FBZ-SO_2$)	$-NH-CO-O-CH_3$	$-SO_2-(C_6H_6)$
Oxibendazol (OXI)	$-NH-CO-O-CH_3$	$-O-CH_2-CH_2-CH_3$
Oxfendazol (OFZ)	$-NH-CO-O-CH_3$	$-SO-(C_6H_6)$
Tiazol		
5-hidroxi-tiabendazol (5-OH-TBZ)	$-C_3H_2NS$	-OH
Metabolitos		
Albendazol-2-aminosulfona ($ABZ-NH_2-SO_2$)	$-NH_2$	$-SO_2-CH_2-CH_2-CH_3$
2-aminobenzimidazol ($BZZ-NH_2$)	$-NH_2$	-H

Todos los compuestos estudiados se utilizan generalmente como antihelmínticos, a excepción del carbendazim, que aunque ocasionalmente se utiliza como antihelmíntico, su uso más general es como fungicida.

Estos fármacos se comercializan como polvos blancos cristalinos, con baja solubilidad en agua y elevada solubilidad en disolventes orgánicos como metanol, acetato de etilo o dimetilsulfóxido y tienen naturaleza anfótera[8]. Sus puntos de fusión son ligeramente altos y son estables térmicamente. Los benzimidazoles poseen un anillo imidazólico que contiene átomos de nitrógeno ácidos y básicos. Según

[8] M. Danaher, H. De Ruyck, S. R. H. Crooks, G. Dowling, M. O´Keeffe. *Review of methodology for the determination of benzimidazole residues in biological matrices.* Journal of Chromatography B, 845 (2007) 1-37.

esto, las moléculas se pueden protonar (pH por debajo de aproximadamente 5-6) o desprotonar (pH por encima de aproximadamente 12), como se muestra en la Figura 2.2.

Figura 2.2. Ionización de los benzimidazoles en condiciones ácidas (A) o básicas (B).

Entre los benzimidazoles estudiados, los metabolitos albendazol-2-aminosulfona y 2-aminobenzimidazol presentan otro comportamiento ácido-base al mostrado en la Figura 2, ya que poseen más grupos ionizables, porque tienen una amina primaria en la posición R_1.

El coeficiente de partición (K_{ow}) es también una propiedad importante, dando información sobre la solubilidad de estos residuos en diferentes disolventes. La mayoría de estas moléculas tienen log K_{ow} comprendidos entre 0.8-3.3.

2.2.2. Mecanismo de acción de los benzimidazoles

Los benzimidazoles presentan buena actividad antihelmíntica tanto en humanos como en animales domésticos, actuando frente a parásitos como nematodos (por ejemplo, gusanos redondos), cestodos (por ejemplo, tenias) y trematodos (por ejemplo, gusanos planos); aunque no todos los benzimidazoles son efectivos en una dosis práctica contra todos los tipos de parásitos. Estas diferencias de efectividad se pueden deber a los modos de acción, a diferentes tipos de comportamientos farmacocinéticos, o a ambos[9].

Los antihelmínticos actúan sobre los parásitos a través de dos mecanismos: inhibiendo el metabolismo energético del parásito o inhibiendo la polimerización de la tubulina, que es una proteína globular que al ensamblarse genera los microtúbulos de los parásitos. Los benzimidazoles inhiben el metabolismo energético de los parásitos debido a su capacidad de actuar sobre los sistemas enzimáticos de los mismos, provocando una disminución de la energía disponible necesaria para el funcionamiento normal de sus órganos vitales. Este trastorno enzimático conduce al agotamiento energético completo, provocando la muerte[10].

Los microtúbulos son estructuras celulares presentes en células eucariotas, formados por la polimerización de un dímero de dos

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[9] R. O. McCracken, W. H. Stillwell. *A possible biochemical mode of action for benzimidazole anthelmintics.* International Journal of Parasitology, 21 (1991) 99-104.

[10] P. Tejada, M. Sanchez-Moreno, M. Monteoliva, H. Gomez-Banqueri. *Inhibition of malate dehydrogenase enzymes by benzimidazole anthelmintics.* Veterinary Parasitology, 24 (1987) 269-274.

proteínas globulares, la alfa y la beta tubulina[11]. Estas estructuras tienen numerosas funciones vitales en las células como el movimiento de cromosomas durante la división celular o la absorción y transporte de nutrientes. Los benzimidazoles presentan una elevada afinidad por la tubulina de las células de los parásitos, impidiendo su polimerización y, por lo tanto, ocasionando alteraciones en la estructura y funcionalidad de los microtúbulos. El efecto teratógeno (malformaciones durante el desarrollo) de los benzimidazoles ha sido atribuido a este último mecanismo de acción[8].

2.2.3. Administración y absorción de benzimidazoles

Los benzimidazoles suelen ser administrados de forma oral o a través de los alimentos, metabolizándose en el organismo a otros compuestos con mayor o menor actividad antihelmíntica. Por ejemplo, en las familias del albendazol y el fenbendazol, los metabolitos con mayor actividad antihelmíntica son los derivados sulfóxidos. La biodisponibilidad de estos compuestos depende en gran medida de la ruta de administración y del compuesto en cuestión.

Los fármacos se disuelven en los fluidos intestinales facilitando la absorción de los compuestos en la mucosa gastrointestinal. La velocidad de disolución influye en el ritmo y magnitud de su absorción, en su concentración plasmática máxima y en su posterior distribución y cinética de eliminación. Los compuestos más hidrosolubles se disuelven en los fluidos acuosos alcanzándose rápidamente la máxima

[11] E. Lacey. *Mode of action of benzimidazoles*. Parasitology Today, 6 (1990) 112-115.

concentración en plasma. Los benzimidazoles menos solubles en agua tienen una limitada absorción gastrointestinal, proporcionando tiempos de residencia mayores.

2.2.4. Límites máximos de residuos en alimentos de origen animal para los benzimidazoles antihelmínticos

Se definen los residuos de fármacos veterinarios como "todas las sustancias farmacológicamente activas, ya sean principios activos, excipientes o productos de degradación y sus metabolitos, que permanecen en los productos de alimentación obtenidos de animales a los que se les administró el fármaco en cuestión". Los residuos de fármacos veterinarios en alimentos se consideran un factor de riesgo para la salud pública. Con el fin de proteger a los consumidores, es crucial evaluar la seguridad de estas sustancias incluyendo cualquier efecto toxicológico, farmacológico y microbiológico no deseado que puedan tener.

Por ello, se han establecido límites máximos de residuos para proteger la salud humana. En la Tabla 2.2 se muestran los límites máximos permitidos para los residuos de benzimidazoles en alimentos de origen animal[5].

Tabla 2.2. Límites máximos de residuos (MRLs) permitidos para los benzimidazoles en alimentos de origen animal.

Benzimidazol	Residuo Marcador	Especie Animal	Tejido	MRL
Albendazol (ABZ)	Suma de ABZ-SO, ABZ-SO_2 y ABZ-NH_2-SO_2 expresados como ABZ	Todos los rumiantes	Músculo Grasa Hígado Riñón Leche	100 µg/kg 100 µg/kg 1000 µg/kg 500 µg/kg 100 µg/kg
Fenbendazol (FBZ)	Suma de residuos extraibles que por oxidación se convierten en sulfona de oxfendazol	Todos los ruminates, porcinos y équidos	Músculo Grasa Hígado Riñón	50 µg/kg 50 µg/kg 500 µg/kg 50 µg/kg
		Todos los rumiantes	Leche	10 µg/kg
Flubendazol (FLU)	Suma de flubendazol y 2-aminoflubendazol	Aves de corral y porcinos	Músculo Piel y grasa Hígado Riñón	50 µg/kg 50 µg/kg 400 µg/kg 300 µg/kg
	Flubendazol	Aves de corral	Huevos	400 µg/kg
Mebendazol (MBZ)	Suma de mebendazol, metil (5- (1-hidroxi, 1-fenil) metil-1H-benzimidazol-2-il) carbamato y (2-amino-1H-benzimidazol-5-il) fenilmetanona, expresados como equivalentes de mebendazol	Ovinos, caprinos y équidos	Músculo Grasa Hígado Riñón	60 µg/kg 60 µg/kg 400 µg/kg 60 µg/kg
Oxfendazol (OFZ)	Suma de residuos extraibles que por oxidación se convierten en sulfona de oxfendazol	Todos los ruminates, porcinos y équidos	Músculo Grasa Hígado Riñón	50 µg/kg 50 µg/kg 500 µg/kg 50 µg/kg
		Todos los rumiantes	Leche	10 µg/kg
Oxibendazol (OXI)	Oxibendazol	Porcinos	Músculo Piel y grasa Hígado Riñón	100 µg/kg 500 µg/kg 200 µg/kg 100 µg/kg
Óxido de Albendazol (ABZ oxido)	Suma de ABZ oxido, ABZ-SO_2 y ABZ-NH_2-SO_2 expresados como ABZ	Bovinos y ovinos	Músculo Grasa Hígado Riñón Leche	100 µg/kg 100 µg/kg 1000 µg/kg 500 µg/kg 100 µg/kg
Tiabendazol (TBZ)	Suma de tiabendazol y 5-hidroxi-tiabendazol	Bovinos y caprinos	Músculo Grasa Hígado Riñón Leche	100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg 100 µg/kg

2.3. Presencia de benzimidazoles antihelmínticos en huevos

Los fármacos veterinarios se utilizan en la industria de las aves de corral para prevenir y controlar enfermedades infecciosas y no infecciosas, y para ayudar en situaciones de estrés debidas por ejemplo a condiciones medioambientales extremas o a las que pueden originarse a raíz de procesos de vacunación. Como resultado, siempre existe la posibilidad de que se generen residuos de fármacos en la carne de las aves o en los huevos[12].

Las infecciones por helmínticos se extienden entre las aves de corral debido a la enorme fecundidad y longevidad de los gusanos parasitarios, incrementándose su aparición en las granjas que no utilizan jaulas para el alojamiento de las aves, ya que de esta forma el acceso al material fecal y al suelo es inevitable. Los parásitos necesitan el suelo para refugiarse y también necesitan que las aves consuman heces del suelo, permitiendo así la transmisión, ya sea vía oral o fecal, de los parásitos internos. Las jaulas convencionales de las aves, típicas de las gallinas ponedoras cuyos huevos se utilizan para el consumo humano, presentan menos problemas con los parásitos debido a que el confinamiento evita el contacto con las heces y el suelo[13]. Sin embargo, los pollos, pavos y gallinas para reproducción, que producen huevos fértiles para incubar, y también los pollos para carne y los pavos

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[12] M. Bistoletti, L. Moreno, L. Alvarez, C. Lanusse. *Multiresidue HPLC method to measure benzimidazole anthelmintics in plasma and egg from laying hens. Evaluation of albendazole metabolites residue profiles*. Food Chemistry, 126 (2011) 793-800.

[13] A. A. Permin, M. Bisgaard, F. Frandsen, M. Pearman, P. Nansen, J. Kold*. The prevalence of gastrointestinal helminths in different poultry production systems.* British Poultry Science, 40 (1999) 439-443.

comerciales crecen en sistemas donde el contacto con el estiércol es inevitable. Además, las jaulas convencionales están prohibidas en la Unión Europea desde el año 2012 y actualmente, la mayoría de los consumidores prefieren huevos orgánicos procedentes de gallinas criadas en granjas ecológicas en las que no se utilizan jaulas. Estos factores han provocado un incremento en las infecciones por helmínticos en aves de corral, por lo que se requiere también un mayor número de tratamientos con antihelmínticos[14].

Cuando las aves llegan a ser infectadas por helmínticos, su comportamiento (menos activo) y su salud (pérdida de apetito y de peso corporal, baja producción de huevos e incluso muerte) se ven afectados negativamente ocasionando repercusiones económicas en las empresas[12]. El tratamiento de estas infecciones varía considerablemente dependiendo del tipo de producción, del tipo y edad de las aves y de las preferencias del productor[15]. Es recomendable la evaluación periódica de los antihelmínticos usados debido a que las aves desarrollan resistencia a estos fármacos. De esta forma, el productor puede trazar estrategias efectivas para el control de los helmínticos, previniendo de este modo la pérdida de efectividad en la alimentación, la productividad y el bienestar general de las aves[16].

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[14] F. Kaufmann, G. Daş, B. Sohnrey, M. Gauly. *Helminth infections in laying hens kept in organic free range systems in Germany.* Livestock Science, 141 (2011) 182–187.

[15] T. A. Yazwinski, C. A. Tucker. *Nematodes and acanthocephalans*. En: Y. M. Saif (Ed.), *Diseases of Poultry*. Twelfth ed., Blackwell Publishing, Cambridge, Massachusetts, United States (2008) pp. 1025-1056.

[16] T. A. Yazwinski, C. A. Tucker, E. Wray, L. Jones, F. D. Clark. *Observations of benzimidazole efficacies against Ascaridia dissimilis, Ascaridia galli, and Heterakis gallinarum in naturally infected poultry.* The Journal of Applied Poultry Research, 22 (2013) 75-79.

El flubendazol es el único benzimidazol aprobado en la Unión Europea para el control de infecciones por endoparásitos en aves de corral[17,18,5]. El uso de flubendazol puede generar residuos tanto del fármaco precursor como de sus metabolitos en alimentos. Por ello, se han establecido límites máximos de residuos para la presencia de este compuesto en varios tejidos de aves, incluyendo los huevos[19]. También el fenbendazol se aprobó para el control de nematodos en pavos, más concretamente en hígado y músculo. Sin embargo, por razones económicas, otros benzimidazoles como el albendazol[20] se utilizan para el tratamiento de infecciones por parásitos en aves de corral. Es posible cuantificar los niveles de albendazol o fenbendazol[21,22], tanto de los fármacos precursores como de sus metabolitos sulfona y sulfóxido, en

[17] Codex Alimentarius Commission, 1995. Joint Food and Agriculture Organization of the United Nations/World Health Organization food standards programme on food additives and contaminants. Section 4, 80-82.

[18] The European Agency for the Evaluation of Medicinal Products, 1995.Veterinary Medicines Evaluation Unit, Committee for Veterinary Medicinal Products. Approach towards harmonization of withdrawal periods. EMEA/CVMP/36/95 FINAL.

[19] The European Agency for the Evaluation of Medicinal Products, 2006. Veterinary Medicines and Inspections, Committee for Medicinal Products for Veterinary Use. Flubendazole (extrapolation to poultry). EMEA/CVMP/33128/2006-FINAL.

[20] C. A. Tucker, T. A. Yazwinski, L. Reynolds, Z. Johnson, M. Keating. *Determination of the anthelmintic efficacy of albendazole in the treatment of chickens naturally infected with gastrointestinal helminths*. The Journal of Applied Poultry Research, 16 (2007) 392-396.

[21] R. A. Norton, T. A. Yazwinski, Z. Johnson. *Research note: use of fenbendazole for the treatment of turkeys with experimentally induced nematode infections.* Poultry Science, 70 (1991) 1835-1837.

[22] S. M. Taylor, J. Kenny, A. Houston, S. A. Hewitt. *Efficacy, pharmacokinetics and effects on egg-laying and hatchability of two dose rates of in-feed fenbendazole for the treatment of Capillaria species infections in chickens*. Veterinary Record, 133 (1993) 519-521.

plasma de pollos tratados previamente con estos antihelmínticos[22,23]. De acuerdo con esto, resulta altamente probable la presencia de residuos en la carne y los huevos.

Por otra parte, en la sangre de otras especies animales como rumiantes, cerdos o perros, no es posible detectar el fármaco precursor después de la administración oral[24,25,26,27,28,29]. Esto es debido a que el tracto digestivo de las aves tiene un pH muy bajo que facilita la rápida desintegración de las dosis sólidas administradas oralmente, liberando

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[23] G. Y. Csikó, G. Y. Banhidi, G. Semjén, P. Laczay, G. Ványiné Sándor, J. Lehel, J. Fekete. *Metabolism and pharmacokinetics of albendazole after oral administration to chickens.* Journal of Veterinary Pharmacology and Therapeutics, 19 (1996) 322-325.

[24] S. E. Marriner, J. A. Bogan. *Pharmacokinetics of albendazole in sheep*. American Journal of Veterinary Research, 41 (1980) 1126-1129.

[25] D. R. Hennessy, J. W. Steel, E. Lacey, G. K. Eagleson, R. K. Prichard. *The disposition of albendazole in sheep.* Journal of Veterinary Pharmacology and Therapeutics, 12 (1989) 421-429.

[26] H. A. Benchaoui, E. W. Scott, Q. A. McKellar. *Pharmacokinetics of albendazole, albendazole sulfoxide and netobimin in goats.* Journal of Veterinary Pharmacology and Therapeutics, 16 (1993) 237-240.

[27] L. I. Alvarez, C. A. Saumell, S. F. Sanchez, C. E. Lanusse. *Plasma disposition kinetics of albendazole metabolites in pigs fed different diets.* Research in Veterinary Science, 60 (1996) 152-156.

[28] S. F. Sánchez, L. I. Alvarez, C. E. Lanusse. *Fasting-induced changes to the pharmacokinetic behaviour of albendazole and its metabolites in calves*. Journal of Veterinary Pharmacology and Therapeutics, 20 (1997) 38-47.

[29] S. Sánchez, J. Sallovitz, E. Salvo, Q. McKellar, C. Lanusse. *Comparative availability of two oral dosage forms of albendazole in dogs.* The Veterinary Journal, 160 (2000) 153-156.

así los metabolitos activos[23,30]. Estas características del sistema digestivo mejoran la solubilidad del albendazol y su consecuente absorción intestinal, en comparación con las otras especies.

Algunos autores han encontrado la presencia de los metabolitos sulfóxido y sulfona del albendazol en huevos procedentes de gallinas tratadas con una sola dosis de albendazol. Se encontraron concentraciones que superaban los límites permitidos, incluso dos días después de la administración oral. Normalmente, los tratamientos con benzimidazoles antihelmínticos duran una semana, por lo que el nivel de residuos y de persistencia de los mismos en los huevos será aún mayor[12].

Además, se realizaron estudios adicionales en los que se analizaron por separado la clara y la yema de huevos de gallinas tratadas con albendazol[4] y con flubendazol[31,32,33]. En el primer caso, fue posible detectar albendazol y sus metabolitos en la yema, mientras que en la clara sólo aparecieron niveles muy bajos de los metabolitos. Esto es debido a que la yema está compuesta principalmente de lipoproteínas y

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[30] B. Vermeulen, P. De Backer, J. P. Remon. *Drug administration to poultry.* Advanced Drug Delivery Reviews, 54 (2002) 795-803.

[31] C. A. Kan, H. J. Keukens, M. J. H. Tomassen. *Flubendazole residues in eggs after oral administration to laying hens: determination with reversed phase liquid chromatography.* Analyst, 123 (1998) 2525-2527.

[32] L. Van Leemput, J. Agneessens, K. Vlaminck. *Safety profile of an oral emulsion formulation of flubendazole.* Journal of Veterinary Pharmacology and Therapeutics, 29 (2006) 156 (Supplement 1, Abstract).

[33] G. Balizs. *Determination of benzimidazole residues using liquid chromatography and tandem mass spectrometry.* Journal of Chromatography B: Biomedical Sciences and Applications, 727 (1999) 167-177.

la clara está formada por proteínas solubles en agua. El albendazol es más soluble en lípidos que sus metabolitos y por eso se acumuló en la yema. En el caso del flubendazol, se encontraron concentraciones cinco veces mayores en la yema que en la clara de los huevos recolectados de las gallinas tratadas.

2.4. Antecedentes en la determinación de benzimidazoles antihelmínticos en huevo

A la vista de todo lo anterior, parece obvio que el control de residuos de benzimidazoles y sus principales metabolitos en alimentos de origen animal, supone una cuestión de seguridad para los consumidores. Sin embargo, la determinación de estos compuestos plantea algunas dificultades, ya que a pesar de las similitudes entre las estructuras de las moléculas y el modo de acción, estos compuestos tienen distintas propiedades químicas tales como la polaridad y las características ácido-base.

2.4.1. Cromatografía líquida

Para la separación de los distintos benzimidazoles, lo más habitual es utilizar técnicas cromatográficas, concretamente cromatografía líquida (*High Performance Liquid Chromatography,* HPLC) en sus diferentes modalidades. La determinación de benzimidazoles mediante cromatografía gaseosa (*Gas Chromatography*, GC) no es fácil, debido a la baja volatilidad de estos compuestos; sólo el tiabendazol y el triclabendazol se pueden determinar sin derivatizar[8].

La mayoría de las aplicaciones de GC se han centrado en el análisis en cultivos agrícolas de los benzimidazoles que se usan como fungicidas.

Debido a las propiedades químicas de estos compuestos, la cromatografía líquida en fase inversa (*Reverse Phase Liquid Chromatography*, RPLC) ha sido la técnica más utilizada en el análisis de residuos de benzimidazoles en huevo. En cuanto a la detección, la absorción ultravioleta (UV) a 292 nm ha sido el método tradicional más comúnmente usado[12]. Sin embargo, en los últimos años la detección por espectrometría de masas (*Mass Spectrometry*, MS) ha sido la forma predominante de detección tanto para residuos benzimidazólicos como para otros fármacos veterinarios[34]. El acoplamiento LC-MS en la modalidad de cuadrupolo simple, o en la modalidad en tándem (LC-MS/MS) posibilita el desarrollo de métodos de gran sensibilidad y

[34] H. Sun, Q. W. Yu, H. B. He, Q. Lu, Z. G. Shi, Y. Q. Feng. *Nickel oxide nanoparticle-deposited silica composite solid-phase extraction for benzimidazole residue analysis in milk and eggs by liquid chromatography–mass spectrometry.* Journal of Agricultural and Food Chemistry, 64 (2016) 356-363.

selectividad[35,36,37,38]. Estos métodos permiten la determinación de fármacos polares y no polares; no volátiles y térmicamente inestables sin necesidad de derivatización. La interfaz más utilizada en estos métodos es la ionización por electrospray (*Electrospray Ionization*, ESI). También se han desarrollado métodos basados en la utilización de columnas cada vez de menor diámetro interno y tamaño de partícula, lo que ha originado la modalidad UHPLC (*Ultra High Performance Liquid Chromatography*), que acoplada a espectrometría de masas en tándem incrementa la sensibilidad y disminuye el tiempo de análisis[39].

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[35] M. E. Dasenaki, N. S. Thomaidis. *Multi-residue determination of 115 veterinary drugs and pharmaceutical residues in milk powder, butter, fish tissue and eggs using liquid chromatography–tandem mass spectrometry.* Analytica Chimica Acta, 880 (2015) 103-121.

[36] R. J. B. Peters, Y. J. C. Bolck, P. Rutgers, A. A. M. Stolker, M. W. F. Nielen. *Multi-residue screening of veterinary drugs in egg, fish and meat using high-resolution liquid chromatography accurate mass time-of-flight mass spectrometry.* Journal of Chromatography A, 1216 (2009) 8206-8216.

[37] S. W. C. Chung, C. H. Lam. *Development of a 15-class multiresidue method for analyzing 78 hydrophilic and hydrophobic veterinary drugs in milk, egg and meat by liquid chromatography-tandem mass spectrometry.* Analytical Methods, 7 (2015) 6764-6776.

[38] M. Piatkowska, P. Jedziniak, J. Zmudzki. *Multiresidue method for the simultaneous determination of veterinary medicinal products, feed additives and illegal dyes in eggs using liquid chromatography–tandem mass spectrometry.* Food Chemistry, 197 (2016) 571-580.

[39] A. Garrido Frenich, M. M. Aguilera-Luiz, J. L. Martínez Vidal, R. Romero-González. *Comparison of several extraction techniques for multiclass analysis of veterinary drugs in eggs using ultra-high pressure liquid chromatography–tandem mass spectrometry*. Analytica Chimica Acta, 661 (2010) 150-160.

2.4.2. Electroforesis capilar

En el análisis de fármacos y sus metabolitos, la electroforesis capilar (*Capillary Electrophoresis*, CE) es una buena alternativa a las técnicas cromatográficas. Sus principales ventajas son su alta eficacia en la separación, un tiempo global de análisis corto y un bajo coste operacional. Además, es posible el acoplamiento con espectrometría de masas (CE-MS). En nuestro conocimiento, hasta la fecha, no se han descrito métodos para la cuantificación conjunta de residuos de benzimidazoles y sus metabolitos en muestras de huevo utilizando la electroforesis capilar como técnica de separación.

La electroforesis capilar se ha utilizado para la determinación de los valores de pK_a de los benzimidazoles y sus metabolitos[40,41,42,43]; y también para la determinación de estos analitos y otros compuestos

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[40] Z. Jia, T. Ramstad, M. Zhong. *Medium-throughput pKa screening of pharmaceuticals by pressure-assisted capillary electrophoresis.* Electrophoresis, 22 (2001) 1112-1118.

[41] E. Örnskov, A. Linusson, S. Folestad. *Determination of dissociation constants of labile drug compounds by capillary electrophoresis.* Journal of Pharmaceutical and Biomedical Analysis, 33 (2003) 379-391.

[42] G. Jerez, G. Kaufman, M. Prystai, S. Schenkeveld, K. K. Donkor. *Determination of thermodynamic pKa values of benzimidazole and benzimidazole derivatives by capillary electrophoresis*. Journal of Separation Science, 32 (2009) 1087-1095.

[43] E. Lipka, M. Folly-Klan, J. Charton, M. P. Vaccher, J. P. Bonte, C. Vaccher. *Determination of pKa values of benzimidazole derivatives from mobility obtained by capillary electrophoresis.* Journal of Pharmaceutical and Biomedical Analysis, 53 (2010) 1267-1271.

antihelmínticos en diferentes matrices como orina[44], plasma[45,46,47], fluido cerebroespinal[48], medicamentos[49], piensos[50] y tejidos animales[51]. Se han utilizado distintas modalidades de CE como la electroforesis capilar en medios no acuosos (*Non-Aqueous Capillary Electrophoresis,*

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[44] H. Meier, G. Blaschke. *Capillary electrophoresis–mass spectrometry, liquid chromatography–mass spectrometry and nanoelectrospray-mass spectrometry of praziquantel metabolites*. Journal of Chromatography B, 748 (2000) 221-231.

[45] A. Procházková, M. Chouki, R. Theurillat, W. Thormann. *Therapeutic drug monitoring of albendazole: Determination of albendazole, albendazole sulfoxide, and albendazole sulfone in human plasma using nonaqueous capillary electrophoresis*. Electrophoresis, 21 (2000) 729-736.

[46] W. Thormann, F. Prost, A. Procházková. *Capillary electrophoresis with (R)-(-)-N-(3,5-dinitrobenzoyl)-α-phenylglycine as chiral selector for separation of albendazole sulfoxide enantiomers and their analysis in human plasma*. Journal of Pharmaceutical and Biomedical Analysis, 27 (2002) 555-567.

[47] P. Kowalski, M. Bieniecki, I. Olędzka, H. Lamparczyk. *Validated capillary electrophoretic method for the analysis of ivermectin in plasma after intragastric administration in pigs and horses*. Biomedical Chromatography, 18 (2004) 302-310.

[48] F. O. Paias, V. L. Lanchote, O. M. Takayanagui, P. S. Bonato. *Enantioselective analysis of albendazole sulfoxide in cerebrospinal fluid by capillary electrophoresis.* Electrophoresis, 22 (2001) 3263-3269.

[49] B. Chankvetadze, N. Burjanadze, M. Santi, G. Massolini, G. Blaschke. *Enantioseparation of tetramisole by capillary electrophoresis and high performance liquid chromatography and application of these techniques to enantiomeric purity determination of a veterinary drug formulation of L-levamisole*. Journal of Separation Science, 25 (2002) 733-740.

[50] J. Shen, J. Tong, H. Jiang, Q. Rao, N. Li, L. Guo, S. Deng. *Simultaneous determination of five benzimidazoles in feeds using high-performance capillary electrophoresis*. Journal of AOAC International, 92 (2009) 1009-1015.

[51] X. Z. Hu, M. L. Chen, Q. Gao, Q. W. Yu, Y. Q. Feng. *Determination of benzimidazole residues in animal tissue samples by combination of magnetic solid-phase extraction with capillary zone electrophoresis.* Talanta, 89 (2012) 335-341.

NACE) y la electroforesis capilar zonal (*Capillary Zone Electrophoresis*, CZE) o quiral, con detección principalmente mediante UV o MS.

2.4.3. Tratamiento de muestra

Un aspecto importante en el análisis de cualquier compuesto a nivel de traza en matrices alimentarias es el tratamiento de muestra necesario antes de proceder a su separación y análisis instrumental. En el análisis de compuestos de bajo peso molecular, como fármacos en alimentos, estas etapas tienen como objeto eliminar la interferencia de especies macromoleculares a la vez que se preserva el equipo instrumental. Además, el huevo es una matriz muy compleja debido a su alto contenido lipídico y proteico, lo que provoca que algunos analitos puedan unirse a las lipoproteínas dificultando su extracción[52]. Por otra parte, los disolventes utilizados en la extracción forman emulsiones y espumas con la matriz. Por todo esto, uno de los principales objetivos durante el proceso de extracción es la eliminación de lípidos y proteínas antes del análisis. El acetonitrilo se considera el mejor disolvente extractante ya que precipita proteínas y desnaturaliza enzimas, que podrían degradar a los residuos durante el tratamiento de muestra. Los métodos de extracción requieren normalmente etapas adicionales de limpieza.

Entre los métodos de extracción y limpieza más utilizados para la determinación de benzimidazoles en huevos se encuentran la

[52] V. Jiménez, A. Rubies, F. Centrich, R. Companyó, J. Guiteras. *Development and validation of a multiclass method for the analysis of antibiotic residues in eggs by liquid chromatography–tandem mass spectrometry.* Journal of Chromatography A, 1218 (2011) 1443-1451.

extracción líquido-líquido (*Liquid Liquid Extraction*, LLE) y la extracción en fase sólida (*Solid Phase Extraction*, SPE). En algunos casos, sólo se realiza una de las dos como estrategia de tratamiento de muestra[12,34,35,39], sin embargo lo más común es la combinación de los dos métodos[33,36,37,38,39,53,54]. De esta forma se consigue precipitar las proteínas de la muestra con LLE y llevar a cabo una limpieza posterior de los extractos obtenidos utilizando SPE. Para la LLE se ha utilizado principalmente acetonitrilo[12], mezclas de acetonitrilo-agua[36,37] o disoluciones acuosas de fosfato[4]. También se han usado otras combinaciones más complejas en las que se mezclan disoluciones acuosas de ácido fórmico o de AEDT (ácido etilendiaminotetraacético) con acetonitrilo[38,54] y también con acetonitrilo-metanol[35], o utilizando acetato de etilo en presencia de carbonato potásico y sulfato sódico para llevar a cabo la extracción[33]. Para la SPE se han utilizado diferentes sorbentes, desde los comerciales Oasis HLB[39,53], StrataX[36] o estireno divinilbenceno[33] hasta otros preparados con nanopartículas de óxido de níquel depositadas sobre sílica[34].

También se han desarrollado otros métodos para el tratamiento de muestras de huevo basados en microextracción con polímeros monolíticos (*Polymer Monolith Microextraction,* PMME)[4], extracción en

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[53] X. J. Deng, H. Q. Yang, J. Z. Li, Y. Song, D. H. Guo, Y. Luo, X. N. Du, T. Bo. *Multiclass residues screening of 105 veterinary drugs in meat, milk, and egg using ultra high performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry.* Journal of Liquid Chromatography & Related Technologies, 34 (2011) 2286-2303.

[54] M. Piatkowska, P. Jedziniak, J. Zmudzki. *Comparison of different sample preparation procedures for multiclass determination of selected veterinary drug, coccidiostat and insecticide residues in eggs by liquid chromatography-tandem mass spectrometry.* Analytical Methods, 6 (2014) 3034-3044.

fase sólida con dispersión de la matriz (*Matrix Solid-Phase Dispersion*, MSPD)[39] o utilizando el método QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*)[55], donde los analitos se extraen con acetonitrilo en presencia de sales y se realiza una etapa de limpieza mediante extracción en fase sólida dispersiva (*Dispersive Solid-Phase Extraction*, DSPE) usando PSA (*Primary Secondary Amine*). Merece la pena destacar que el método QuEChERS, ampliamente usado en el análisis de contaminantes mediante HPLC, no ha sido propuesto hasta el momento para su uso en el análisis de contaminantes mediante CE.

Recientemente se ha descrito el uso de la LLE asistida por ultrasonidos en la que se eliminan los lípidos y las proteínas con bajas temperaturas y se utiliza hexano para eliminar la grasa de las muestras[35]; otros métodos utilizan una microextracción asistida por agitación magnética con emulsificación mejorada con surfactantes[56].

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[55] M. Anastassiades, S. J. Lehotay, D. Štajnbaher, F. J. Schenck. *Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce.* Journal of AOAC International, 86 (2003) 412-431.

[56] J. Vichapong, Y. Santaladchaiyakit, R. Burakham, S. Srijaranai. *Determination of benzimidazole anthelminthics in eggs by advanced microextraction with high-performance liquid chromatography*. Analytical Letters, 48 (2015) 617-631.

3

NUCLEÓSIDOS Y NUCLEÓTIDOS

3.1. Introducción

Los nucleósidos y nucleótidos son compuestos intracelulares de bajo peso molecular, esenciales en un gran número de procesos biológicos. Todas las células de mamíferos, bacterias y plantas contienen de forma natural estos compuestos, que son los precursores de los ácidos nucleicos que componen el ADN (ácido desoxirribonucleico) y el ARN (ácido ribonucleico) y que resultan cruciales, por tanto, en el almacenaje, transferencia y expresión de la información genética. Además, los nucleósidos y nucleótidos participan en otras funciones metabólicas ya que forman parte de rutas biosintéticas, actúan en la transferencia de energía química, son componentes de algunas co-enzimas y desempeñan un papel importante como reguladores biológicos[1,2].

El cuerpo humano es capaz de sintetizar de forma endógena estas moléculas, por lo que constantemente se forman y se degradan en el organismo. Así, la formación puede ocurrir a través de dos vías: la síntesis "*de novo*" dentro de células nuevas a partir de aminoácidos precursores; o a través de rutas alternativas de recuperación llamadas "*salvage pathway*", en las que se utilizan los intermedios generados en la degradación de ADN y ARN, como bases y nucleósidos, para la síntesis de nuevos nucleótidos. Algunos tejidos como la mucosa intestinal, las células de la médula ósea o el cerebro, tienen una capacidad limitada para la síntesis de nuevos nucleótidos y utilizan siempre la ruta de

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[1] M. Cosgrove. *Nucleotides*. Nutrition, 14 (1998) 748-751.

[2] J. D. Carver, W. A. Walker. *The role of nucleotides in human nutrition*. Nutritional Biochemistry, 6 (1995) 58-72.

recuperación alternativa, que es más simple y menos costosa energéticamente que las reacciones que ocurren en la síntesis "*de novo*"[1,2,3,4].

Los nucleósidos y nucleótidos también se pueden incorporar al organismo de forma exógena a través de la alimentación, y el cuerpo humano es capaz de absorberlos y hacer uso de ellos[5]. Se pueden considerar nutrientes semi-esenciales o condicionalmente esenciales si el suplemento endógeno resulta insuficiente para que las funciones sean completamente normales, pero su carencia no lleva a un síndrome clásico de deficiencia. Es decir, que las células o el organismo en general cuentan con las rutas bioquímicas necesarias para la síntesis de estos compuestos semi-esenciales, pero un suplemento dietético exógeno puede optimizar las funciones, bien a través del aporte de nutrientes o bien ahorrando a la célula el coste energético de la síntesis[1,2,3,4,5,6].

En definitiva, existen tres vías de formación o incorporación de los nucleósidos y nucleótidos en el organismo, que aparecen sintetizadas en la siguiente figura:

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[3] G. Hu, F. Q. Yang. *Biological activities of nucleosides and their analogues in dietary foods.* Chemical Rapid Communications, 2 (2014) 22-28.

[4] S. Yamamoto, M. F. Wang, A. A. Adjei, C. K. Ameho. *Role of nucleosides and nucleotides in the immune system, gut reparation after injury, and brain function.* Nutrition, 13 (1997) 372-374.

[5] C. T. Van Buren, F. Rudolph. *Dietary nucleotides: a conditional requirement.* Nutrition, 13 (1997) 470-472.

[6] R. L. Tressler, M. B. Ramstack, N. R. White, B. E. Molitor, N. R. Chen, P. Alarcon, M. L. Masor. *Determination of total potentially available nucleosides in human milk from Asian women.* Nutrition, 19 (2003) 16-20.

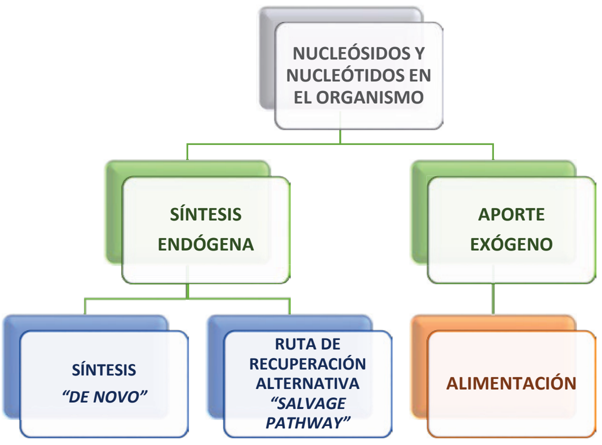

Figura 3.1. Vías de síntesis e incorporación de los nucleósidos y nucleótidos en el organismo.

En ciertas condiciones la demanda de nucleósidos y nucleótidos se incrementa, el suplemento endógeno se vuelve insuficiente y la ingesta de estos compuestos se convierte en esencial para el desarrollo normal de las funciones del organismo. Esto puede ocurrir en ciertos periodos de enfermedad como una lesión intestinal, si disminuye la ingesta de proteínas, si se activa el sistema inmune, en etapas de crecimiento rápido, o en presencia de factores regulatorios o de desarrollo que interfieren con la expresión completa de la capacidad de síntesis endógena[2,5].

Un gran número de estudios previos han demostrado que la presencia de estos compuestos en la dieta tiene efectos beneficiosos sobre diferentes áreas como la respuesta inmune, la absorción de hierro, el metabolismo lipídico, la flora intestinal o la morfología y función tanto intestinal como hepática[1,2,3,4].

Debido a sus importantes funciones, la determinación de nucleósidos y nucleótidos resulta de gran interés en diferentes áreas científicas como la química, bioquímica, medicina, genética, metabolómica o el medioambiente[7,8]. Cabe destacar que su concentración en las células proporciona información para entender el metabolismo energético de las mismas, y su cuantificación en plasma se puede utilizar para evaluar el estrés oxidativo. El estudio de estos compuestos también es útil para obtener información de diferentes patologías, ya que la concentración de algunos nucleósidos y nucleótidos modificados está relacionada con la respuesta inmune en pacientes oncológicos[9,10,11].

Sin embargo, en este capítulo se centrará la atención en los nucleósidos y nucleótidos que actúan como suplemento dietético y en

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[7] S. Studzińska, B. Buszewski. *Effect of mobile phase pH on the retention of nucleotides on different stationary phases for high-performance liquid chromatography*. Analytical and Bioanalytical Chemistry, 405 (2013) 1663-1672.

[8] P. Yeung, L. Ding, W. L. Casley. *HPLC assay with UV detection for determination of RBC purine nucleotide concentrations and application for biomarker study in vivo*. Journal of Pharmaceutical and Biomedical Analysis, 47 (2008) 377-382.

[9] D. Zanini, R. Schmatz, V. Camera Pimentel, J. Martins Gutierres, P. Acosta Maldonado, G. Roberto Thomé, A. Machado Cardoso, N. Stefanello, L. Oliveira, J. Chiesa, D. Bitencourt Rosa Leal, V. M. Morsch, M. R. Chitolina Schetinger. *Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets*. Biomedicine & Pharmacotherapy, 66 (2012) 40-45.

[10] M Rozalski, M. Nocul, C. Watala. *Adenosina diphosphate receptors on blood platelets-potential new targets for antiplatelet therapy*. Acta Biochimica Polonica, 52 (2005) 411-415.

[11] M. B. Donati, A. Falanga. *Pathogenetic mechanisms of thrombosis in malignancy*. Acta Haematologica, 106 (2001) 18-24.

su determinación en diferentes matrices alimentarias, con el objetivo de profundizar en la relación existente entre la dieta y la salud humana[12,13].

3.2. Estructura y propiedades

Un nucleósido consiste en la unión covalente entre una base nitrogenada y una pentosa que puede ser ribosa (ARN) o desoxirribosa (ADN). La base nitrogenada puede ser púrica o pirimidínica y en ambos casos sus átomos proceden principalmente de aminoácidos. Las bases pirimidínicas constan de anillos de seis miembros e incluyen: uracilo, citosina y timina. Las bases púricas tienen además un segundo anillo de cinco miembros y son las siguientes: adenina, guanina, hipoxantina y xantina. El nucleótido es un éster de fosfato formado por la unión del carbono 5' de la pentosa del nucleósido con un grupo mono-, di- o trifosfato[2].

Los nucleósidos, y aún en mayor medida los nucleótidos, debido al grupo o grupos fosfatos que poseen, son compuestos altamente polares con valores de logaritmo del coeficiente de distribución, log D (a pH 9), que varían entre -0.75 y -12.07. Además, cada uno de estos grupos fosfato tiene dos grupos hidroxilo ionizables con valores de pK_a alrededor de 1 y 6, por lo que a valores de pH superiores a 6 (lo que incluye el pH fisiológico) todos los nucleótidos se encuentran con carga

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

[13] J. Bernal, A. M. Ares, J. Pól, S. K. Wiedmer. *Hydrophilic interaction liquid chromatography in food analysis*. Journal of Chromatography A, 1218 (2011) 7438-7452.

neta negativa. Los grupos amino de los anillos de adenina, guanina y citosina se comportan como bases extremadamente débiles susceptibles de protonarse, con valores de pK_a entre 2 y 4.5. Por todo esto, las cargas netas van a variar mucho dependiendo de la especie y del pH. En la Figura 3.2 se muestran las estructuras de los nucleótidos monofosfato con sus valores de pK_a, en la que se pueden apreciar los grupos funcionales susceptibles de ionizarse[14,15,16].

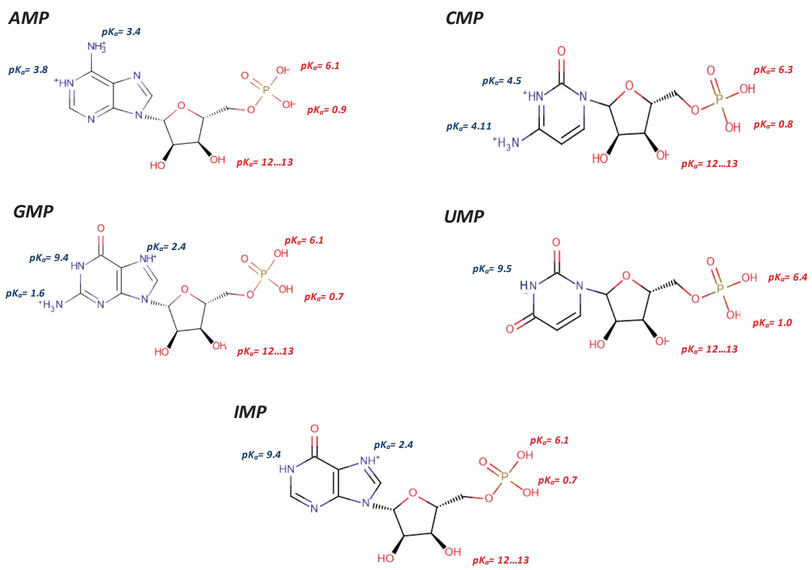

Figura 3.2. Nucleótidos monofosfato y sus valores de pK_a. *AMP:* Adenosina monofosfato, *CMP:* Citidina monofosfato, *GMP:* Guanosina monofosfato, *UMP:* Uridina monofosfato, *IMP:* Inosina monofosfato.

[14] R. H. Garrett, C. M. Grisham. *Biochemistry*. Brooks/Cole, Belmont (2009) pp. 309-335.

[15] R. M. C. Dawson et al. *Data for Biochemical Research*. Clarendon Press, Oxford (1959).

[16] E. Chargaff, J. Baddiley, G. H. Beaven, A. Bendich, D. M. Brown, W. E. Cohn, J. N. Davidson, Z. Dische, E. R. Holiday, E. A. Johnson. *Nucleic acids: chemistry and biology*. Academic Press, New York (1955).

En la Figura 3.3 se muestran las zonas de predominio de las formas ácidas y básicas de los nucleósidos y los nucleótidos monofosfato.

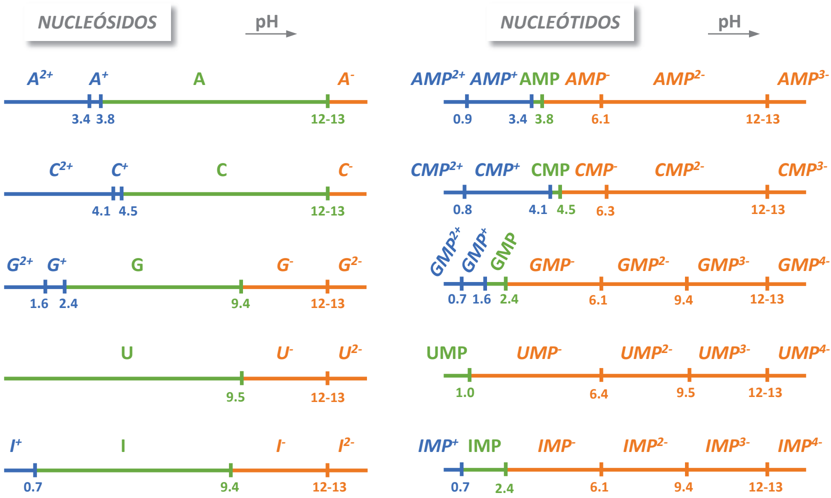

Figura 3.3. Zonas de predominio de las formas ácidas y básicas de los nucleósidos (parte izquierda de la figura) y de los nucleótidos monofosfato (parte derecha de la figura). *A:* Adenosina, *C:* Citidina, *G:* Guanosina, *U:* Uridina, *I:* Inosina, *AMP:* Adenosina monofosfato, *CMP:* Citidina monofosfato, *GMP:* Guanosina monofosfato, *UMP:* Uridina monofosfato, *IMP:* Inosina monofosfato.

El carácter aromático de los anillos de purina y pirimidina hace que estos compuestos presenten absorción en el ultravioleta y que puedan detectarse espectrofotométricamente. Sin embargo, también esta detección va a depender en gran medida del pH debido a la ionización de los analitos.

En esta Tesis la detección se ha realizado utilizando espectrometría de masas (MS), bien con analizador de cuadrupolo simple (Q) o con triple cuadrupolo (QqQ). Con este último analizador se selecciona la transición óptima para cada compuesto, cuyo ion precursor corresponde al ion $[M+H]^+$ en el caso de los nucleósidos adenosina (A), citidina (C), guanosina (G) e inosina (I); y al ion $[M-H]^-$ para la uridina (U) y los nucleótidos mono-, di- y trifosfato. En la Tabla 3.1 se muestran las estructuras de los nucleósidos y nucleótidos estudiados en este trabajo, junto con algunas de sus propiedades más importantes, entre las que se incluyen las transiciones utilizadas para su detección por espectrometría de masas con un triple cuadrupolo.

Tabla 3.1. Propiedades físicas y transiciones MS/MS de los nucleósidos y nucleótidos analizados.

Nucleósidos y Nucleótidos	PM	log D[a] (pH=9)	Estructura	Transición MS/MS
Adenosina (**A**)	267	-0.75	**R1**: Grupo OH	268 → 136
Adenosina monofosfato (**AMP**)	347	-7.68	**R1**: Grupo fosfato	346 → 79
Adenosina difosfato (**ADP**)	427	-8.16	**R1**: Grupo difosfato	426 → 158.9
Adenosina trifosfato (**ATP**)	507	-10.12	**R1**: Grupo trifosfato	506 → 158.9
Citidina (**C**)	243	-1.81	**R2**: Grupo OH	244 → 112
Citidina monofosfato (**CMP**)	323	-9.63	**R2**: Grupo fosfato	322 → 79
Citidina difosfato (**CDP**)	403	-10.1	**R2**: Grupo difosfato	402 → 158.9
Citidina trifosfato (**CTP**)	483	-12.07	**R2**: Grupo trifosfato	482 → 158.9

Tabla 3.1. Continuación.

Guanosina (**G**)	283	-1.47		284 ⟶ 152
			R3: Grupo OH		
Guanosina monofosfato (**GMP**)	363	-8.64	**R3**: Grupo fosfato	362 ⟶ 79
Guanosina difosfato (**GDP**)	443	-9.12	**R3**: Grupo difosfato	442 ⟶ 158.9
Guanosina trifosfato (**GTP**)	523	-11.09	**R3**: Grupo trifosfato	522 ⟶ 158.9
Inosina (**I**)	268	-1.97		269 ⟶ 137
			R4: Grupo OH		
Inosina monofosfato (**IMP**)	348	-9.01	**R4**: Grupo fosfato	347 ⟶ 79
Inosina difosfato (**IDP**)	428	-9.49	**R4**: Grupo difosfato	427 ⟶ 158.9
Inosina trifosfato (**ITP**)	508	-11.46	**R4**: Grupo trifosfato	507 ⟶ 158.9
Uridina (**U**)	244	-1.76		243 ⟶ 110
			R5: Grupo OH		
Uridina monofosfato (**UMP**)	324	-8.1	**R5:** Grupo fosfato	323 ⟶ 79
Uridina difosfato (**UDP**)	404	-8.55	**R5**: Grupo difosfato	403 ⟶ 158.9
Uridina trifosfato (**UTP**)	484	-10.51	**R5**: Grupo trifosfato	483 ⟶ 158.9

[a] Valores calculados usando "*Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2014 ACD/Labs).*"

3.3. Digestión, absorción y metabolismo de los nucleótidos

Sólo una pequeña fracción del total de nucleótidos ingeridos en la dieta se encuentra en forma de nucleótidos libres. La mayoría está presente en forma de nucleoproteínas, que son degradadas enzimáticamente en el tracto intestinal, y convertidas en una mezcla de

ácidos nucleicos y otros componentes. Las nucleasas pancreáticas actúan sobre los ácidos nucleicos para producir una mezcla de mono-, di-, tri- y polinucleótidos; y las polinucleotidasas y fosfodiesterasas intestinales suplementan la acción de las nucleasas pancreáticas para producir mononucleótidos a partir de los ácidos nucleicos. Después, la fosfatasa intestinal alcalina interacciona con el grupo fosfato de los nucleótidos para hidrolizarlos y formar los nucleósidos que serán posteriormente degradados, liberándose así las bases púricas y pirimidínicas[1,2,17].

Del contenido total de nucleótidos ingeridos en la dieta, un porcentaje superior al 90 % se absorbe en forma de nucleósidos y bases. La mayor parte de estos nucleósidos son rápidamente degradados a ácido úrico por el hígado y los enterocitos. Apenas del 2 al 5 % de los nucleótidos ingeridos y sus derivados se utilizan para la síntesis de ácidos nucleicos en los diferentes tejidos, así como para las reservas intracelulares de nucleótidos. Del 25 al 50 % del total retenido y utilizado se encuentra en el tracto gastrointestinal[1,17].

3.4. Fuentes de nucleósidos y nucleótidos

Dados los efectos beneficiosos que pueden derivarse de la ingesta de estos compuestos, resulta interesante saber en qué alimentos se pueden encontrar. Los nucleósidos y nucleótidos están presentes en cualquier alimento compuesto de células vivas, encontrándose mayores concentraciones de los mismos en aquellos alimentos que tengan una

[17] J. R. Hess, N. A. Greenberg. *The role of nucleotides in the immune and gastroinstestinal systems: potential clinical applications.* Nutrition in clinical practice, 27 (2012) 281-294.

mayor densidad celular o que procedan de tejidos metabólicamente activos. Esto significa que, generalmente, los alimentos procedentes de animales contienen mayor cantidad que los derivados de plantas, a excepción de las habas y otras legumbres que poseen cantidades relativamente altas en sus semillas. Diversos estudios realizados demostraron que los órganos internos de animales como el hígado, el riñón o el corazón, también los pescados pequeños y el marisco son fuentes ricas en nucleósidos y nucleótidos. En menor medida también contribuyen las legumbres, los champiñones y los vegetales[2,18,19].

Algunos nucleótidos como el IMP y el GMP se usan como potenciadores del sabor, ya que producen "mayor cuerpo y suavidad" en los productos líquidos. En caldos, por ejemplo, estas características se asocian con productos que contienen derivados de la carne. El "quinto sabor", conocido como *umami*, incluye sustancias como el IMP y el ácido glutámico, y se asocia también con alimentos ricos en proteínas. El efecto de los nucleótidos como potenciadores del sabor implica su presencia en una amplia variedad de alimentos y bebidas, lo que aumenta el nivel de ingesta de estos compuestos[2,20,21].

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[18] A. J. Clifford, D. L. Story. *Levels of purines in foods and their metabolic effects in rats*. The Journal of Nutrition, 106 (1976) 435-442.

[19] R. Verkerk. *Nucleotides: Speculation on lifestyle-induced essentiality*. NHD Clinical, 64 (2011) 29-32.

[20] K. Kojima. *Safety evaluation of disodium 5'-inosinate, disodium 5'-guanylate and disodium 5'-ribonucleate*. Toxicology, 2 (1974) 185-206.

[21] S. Fuke, S. Konosu. *Taste active components in some foods: a review of Japanese research.* Physiology behaviour, 49 (1991) 863-868.

Los ingredientes alimenticios refinados como azúcar, harina o aceites vegetales de elevado consumo en las dietas actuales no contienen nucleótidos. Aunque no existen recomendaciones sobre la cantidad de nucleósidos y nucleótidos que puede ser beneficiosa en la dieta, sí existen sugerencias sobre que es mejor cierta cantidad que ninguna[19].

3.5. Importancia en la alimentación infantil

El aporte exógeno de nucleósidos y nucleótidos resulta especialmente importante para bebés y niños, ya que se encuentran en etapas de crecimiento rápido[22]. Como se ha explicado anteriormente, en estas condiciones la demanda de estos compuestos se incrementa y la inclusión de los mismos a través de la alimentación permite que actúen como complemento a la síntesis endógena que tiene lugar en el organismo, ayudando así a optimizar las funciones celulares; además de influir positivamente en la regulación de la respuesta inmune. De esta forma, el análisis de nucleósidos y nucleótidos en alimentos infantiles supone un área de aplicación de elevado interés.

La leche materna es la mejor fuente de nucleótidos[23,24], y éstos forman parte de la fracción de nitrógeno no proteica de la leche. El

[22] B. D. Gill, H. E. Indyk. *Determination of nucleotides and nucleosides in milks and pediatric formulas: a review.* Journal of AOAC International, 90 (2007) 1354-1364.

[23] M. Sugawara, N. Sato, T. Nakano, T. Idota, I. Nakajima. *Profile of nucleotides and nucleosides of human milk.* Journal of Nutrition Science and Vitaminology, 41 (1995) 409-418.

[24] L. Thorell, L. B. Sjöberg, O. Hernell. *Nucleotides in human milk: sources and metabolism by the newborn infant.* Pediatric Research, 40 (1996) 845-852.

nitrógeno no proteico constituye alrededor del 25 % del total de nitrógeno que contiene la leche materna, e incluye otros compuestos como amino azúcares y carnitina, que juegan papeles específicos en el desarrollo neonatal. Al contrario, en la leche de vaca, el nitrógeno no proteico supone solamente un 2 % del total de nitrógeno[2,22,25,26].

Por estas razones, el Comité Científico de Alimentación de la Comunidad Europea (SCF, 1993) recomienda suplementar las fórmulas infantiles con nucleótidos en concentraciones similares a las que se encuentran en la leche materna. En la actualidad, la mayoría de estas fórmulas infantiles están elaboradas con leche bovina, y el nitrógeno no proteico representa menos del 20 % de su composición total. Los nucleótidos libres que se encuentran en mayor concentración en la leche materna son los mononucleótidos: citidina monofosfato (CMP), uridina monofosfato (UMP), adenosina monofosfato (AMP), guanosina monofosfato (GMP) e inosina monofosfato (IMP)[23]. Algunos autores han encontrado que la citidina difosfato (CDP) también es un nucleótido predominante en algunas leches maternas[25], y en menor concentración también se han encontrado guanosina difosfato manosa y uridina disfosfato hexosa[27].

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[25] K. Y. Liao, T. C. Wu, C. F. Huang, C. C. Lin, I. F. Huang, L. Wu. *Profile of nucleotides and nucleosides in Taiwanese human milk.* Pediatrics and Neonatology, 52 (2011) 93-97.

[26] C. Oliveira, I. M.P.L.V.O. Ferreira, E. Mendes, M. Ferreira. *Development and application of an HPLC/Diode array methodology for determination of nucleotides in infant formulae and follow-up milks.* Journal of Liquid Chromatography & Related Technologies, 22 (1999) 571-578.

[27] A. Gil, F. Sánchez-Medina. *Acid-soluble nucleotides of human milk at different stages of lactation.* Journal of Dairy Research, 49 (1982) 301-307.

Las fórmulas infantiles se suplementan únicamente con los cinco mononucleótidos ya que son las formas principales de absorción intestinal. En la leche materna se encuentran otras formas además de las formas libres, que también son asequibles nutricionalmente para los bebés: ácidos ribonucleicos poliméricos, nucleósidos y aductos que contienen nucleótidos[28]. De acuerdo con esto, el contenido total de nucleótidos se expresa con las siglas inglesas TPAN (*Total potentially available nucleotide*)[29,30,31]. La distribución de estas cuatro formas principales en las que se pueden encontrar los nucleótidos depende de la etapa de lactancia y de la localización geográfica de la muestra analizada. Más del 80 % del contenido total de nucleótidos (TPAN) está presente como nucleótidos poliméricos (43.3 %) y nucleótidos libres (39.9 %). El resto se encuentran como nucleósidos y aductos de nucleótidos[29,31].

A la hora de comparar los contenidos de nucleótidos en la leche materna y en las fórmulas infantiles, también se debe tener en cuenta que los nucleótidos pueden interaccionar con otros componentes de la leche materna y esto puede afectar a la biodisponibilidad y a la acción biológica de los mismos[2]. Por otra parte, las enzimas endógenas de la

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[28] P. Aggett, J. L. Leach, R. Rueda, W. C. MacLean. *Innovation in infant formula development: A reassessment of ribonucleotides in 2002*. Nutrition, 19 (2003) 375-384.

[29] B. D. Gill, H. E. Indyk, M. Manley-Harris*. Determination of total potentially available nucleosides in bovine milk.* International Dairy Journal, 21, (2011) 34-41.

[30] B. D. Gill, H. E. Indyk, M. Manley-Harris*. Determination of total potentially available nucleosides in bovine, caprine, and ovine milk.* International Dairy Journal, 24, (2012) 40-43.

[31] J. L. Leach, J. H. Baxter, B. E. Molitor, M. B. Ramstack, M. L. Masor. *Total potentially available nucleosides of human milk by stage of lactation.* The American Journal of Clinical Nutrition, 61 (1995) 1224-1230.

leche pueden degradar los nucleótidos monofosfato durante la producción de las fórmulas infantiles, disminuyendo así la concentración de nucleótido suplementado y aumentando consecuentemente la del nucleósido[22]. Esto se atribuye a un proceso de defosforilación del nucleótido por parte de la fosfatasa alcalina residual activa[32].

La Organización Mundial de la Salud (OMS) recomienda prolongar la lactancia exclusiva hasta el sexto mes de vida de un bebé, momento a partir del cual se hace necesario la introducción de alimentos complementarios con el objetivo de cubrir las necesidades energéticas del bebé para un desarrollo y crecimiento adecuados. Estos alimentos complementarios no lácteos deben tener una composición adecuada en lo que se refiere a energía y nutrientes, ya que en algunas ocasiones suponen la única fuente de nutrición para bebés entre 6 y 12 meses[33].

Existen diferentes tipos de alimentos infantiles complementarios, los que se clasifican como no grasos y cuya composición principal es a base de frutas y verduras; y los considerados grasos, compuestos de carne, huevos, queso o cereales[34]. Aunque actualmente no existe información sobre los niveles de nucleótidos deseables en este tipo de

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[32] B. D. Gill, H. E. Indyk. *Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas*. International Dairy Journal, 17 (2007) 596-605.

[33] A. Mir-Marqués, A. González-Masó, M. L. Cervera, M. de la Guardia. *Mineral profile of Spanish commercial baby food*. Food Chemistry, 172 (2015) 238-244.

[34] P. Viñas, N. Campillo, I. López-García, S. Martínez-López, M. I. Vasallo, M. Hernández-Córdoba. *Anion Exchange Liquid Chromatography for the Determination of Nucleotides in Baby and/or Functional Foods.* Journal of Agriculture and Food Chemistry, 57 (2009) 7245-7249.

alimentos, su estudio despierta interés debido a que cada vez se incluyen más ingredientes diferentes en las dietas de los bebés; y como se ha dicho anteriormente, todos los ingredientes de origen animal o vegetal que contienen material celular son una fuente potencial de nucleótidos[35].

3.6. Antecedentes en la determinación de nucleósidos y nucleótidos en alimentos

Tal como se acaba de describir, los nucleósidos y nucleótidos se convierten en muchas ocasiones en nutrientes esenciales para el organismo. Teniendo en cuenta los numerosos efectos positivos que aportan, parece lógico que su control sea un área de importante aplicación en el análisis de alimentos en general y, más concretamente, en el ámbito de la alimentación infantil. A continuación, se recogen algunos de los antecedentes bibliográficos que existen para la determinación de estos compuestos en diferentes matrices alimentarias, poniendo especial atención en los trabajos que utilizan cromatografía líquida (*High Performance Liquid Chromatography*, HPLC) o electroforesis capilar (*Capillary Electrophoresis*, CE), por ser éstas las dos técnicas separativas que se han utilizado en el desarrollo de la Tesis.

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[35] P. Viñas, N. Campillo, G. Férez-Melgarejo, M. I. Vasallo, I. López-García, M. Hernández-Córdoba. *Ion-pair high-performance liquid chromatography with diode array detection coupled to dual electrospray atmospheric pressure chemical ionization time-of-flight mass spectrometry for the determination of nucleotides in baby foods.* Journal of Chromatography A, 1217 (2010) 5197-5203.

3.6.1. Cromatografía Líquida

La cromatografía líquida ha sido la técnica más comúnmente utilizada para el análisis de estos compuestos, sin embargo la separación cromatográfica de nucleósidos y nucleótidos no resulta una tarea fácil. Son compuestos altamente polares, por lo que el uso de las columnas convencionales C_{18} que se emplean normalmente en la cromatografía en fase inversa *(Reversed Phase Liquid Chromatography*, RPLC) se encuentra, en este caso, limitado. En este sentido, se ha intentado mejorar la retención a través del uso de fases estacionarias con modificadores polares, las denominadas "*polar embedded*" o "*polar encapped*"[36,37]. Actualmente, la cromatografía líquida de par iónico (*ion-pair liquid chromatography,* IP-LC) y la de intercambio iónico (*ion-exchange liquid chromatography,* IE-LC) se han convertido en unas de las más usadas ya que, trabajando a un pH adecuado, la naturaleza iónica del éster de fosfato de los nucleótidos permite la interacción con reactivos catiónicos capaces de formar pares iónicos[24,35] y con fases estacionarias de intercambio aniónico[34,38]. También se ha propuesto en los últimos años el uso de cromatografía líquida de interacciones hidrofílicas (*Hydrophilic Interaction Liquid Chromatography,* HILIC)

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[36] R. Lorenzetti, S. Lilla, J. L. Donato, G. de Nucci. *Simultaneous quantification of GMP, AMP, cyclic GMP and cyclic AMP by liquid chromatography coupled to tandem mass spectrometry.* Journal of Chromatography B, 859 (2007) 37-41.

[37] Z. J. Lin, W. Li, G. Dai. *Application of LC-MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides.* Journal of Pharmaceutical and Biomedical Analysis, 44 (2007) 330-341.

[38] K. Inoue, R. Obara, T. Akiba, T. Hino, H. Oka. *Determination of nucleotides in infant formula by ion-exchange liquid chromatography.* Journal of Agricultural and Food Chemistry, 56 (2008) 6863-6867.

para la separación de estos analitos polares[39,40,41,42]. Los detectores más usados para el acoplamiento con HPLC han sido el array de diodos (*Diode Array Detection,* DAD)[32,34,38,43], y la espectrometría de masas (*Mass Spectrometry,* MS)[35,40,44].

Es necesario profundizar en el hecho de que en el análisis de compuestos fosforilados (como es el caso de nucleótidos mono-, di- y trifosfato) con LC-MS, los analitos pueden interaccionar con partes específicas de la configuración instrumental[45]. Estas interacciones se producen de dos maneras diferentes, bien por la adsorción de los

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[39] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka. *Separation efficiencies in hydrophilic interaction chromatography.* Journal of Chromatography A, 1184 (2008) 474-503.

[40] K. Inoue, R. Obara, T. Akiba, T. Hino, H. Oka. *Development and application of an HILIC-MS/MS method for the quantitation of nucleotides in infant formula*. Journal of Agricultural and Food Chemistry, 58 (2010) 9918-9924.

[41] K. Inoue, D. Dowell. *HILIC-MS/MS Method for the Quantitation of Nucleotides in Infant Formula and Adult Nutritional Formula: First Action 2011.21.* Journal of AOAC International, 95 (2012) 603-605.

[42] D. García-Gómez, E. Rodríguez-Gonzalo, R. Carabias-Martínez. *Stationary phases for separation of nucleosides and nucleotides by hydrophilic interaction liquid chromatography.* Trends in Analytical Chemistry, 47 (2013) 111-128.

[43] I. M. P. L. V. O. Ferreira, E. Mendes, A. M. P. Gomes, M. A. Faria, M. A. Ferreira. *The determination and distribution of nucleotides in dairy products using HPLC and diode array detection.* Food Chemistry, 74 (2001) 239-244.

[44] Y. Ren, J. Zhang, X. Song, X. Chen, D. Li. *Simultaneous Determination of 5'-Monophosphate Nucleotides in Infant Formulas by HPLC-MS*. Journal of Chromatography Science, 49 (2011) 332-337.

[45] R. Tuytten, F. Lemière, E. Witters, W. Van Dongen, H. Slegers, R.P. Newton, H. Van Onckelen, E.L. Esmans. *Stainless steel electrospray probe: A dead end for phosphorylated organic compounds.* Journal of Chromatography A, 1104 (2006) 209-221.

analitos sobre los grupos silanol de las fases estacionarias cuando se trabaja en medio ácido, o a través de la formación de complejos metálicos de los residuos fosforilados con iones metálicos generados en distintas partes del sistema cromatográfico. Ambos procesos conducen a la disminución de las señales cromatográficas y a la aparición de colas en los picos o "*peak tailing*"[46]. Se han propuesto diferentes estrategias para prevenir la formación de enlaces por puentes de hidrógeno entre los grupos silanol de la superficie interna de los capilares de sílica y los compuestos fosforilados, como la derivatización de los grupos silanol libres[47] o la desactivación de la sílica con dimetilclorosilano[46].

La interacción entre los compuestos fosforilados y los metales, que proceden principalmente del acero inoxidable de los sistemas HPLC-ESI-MS o de los propios disolventes, afecta a la detección debido a la formación de complejos de los compuestos fosforilados con el hierro (III)[48]. Para prevenir esta interacción se han propuesto estrategias como la sustitución del acero inoxidable por polieterétercetona (PEEK), el uso de fases móviles con pH alto[45], el pretratamiento del sistema

[46] J. Kim, D.G. Campo, R.D. Smith. *Improved detection of multi-phosphorylated peptides in the presence of phosphoric acid in liquid chromatography/mass spectrometry*. Journal of Mass Spectrometry, 39 (2004) 208-215.

[47] F. Gritti, Y.V. Kazakevich, G. Guiochon. *Effect of the surface coverage of endcapped C18-silica on the excess adsorption isotherms of commonly used organic solvents from water in reversed phase liquid chromatography.* Journal of Chromatography A, 1169 (2007) 111-124.

[48] S. Liu, C. Zhang, J.L. Campbell, H. Zhang, K.K.C. Yeung, V.K.M. Han, G.A. Lajoie. *Formation of phosphopeptide-metal ion complexes in liquid chromatography/electrospray mass spectrometry and their influence on phosphopeptide detection*. Rapid Communications in Mass Spectrometry, 19 (2005) 2747-2756.

cromatográfico con ácido fosfórico[49], y el uso de fases móviles que contienen aniones carbonato[50] o agentes quelatantes como el AEDT[48].

A pesar de la problemática asociada a la determinación de nucleósidos y nucleótidos utilizando HPLC, se han llevado a cabo aplicaciones en matrices alimentarias muy variadas. Existen antecedentes sobre el análisis de estos compuestos en diferentes tipos de hongos[51,52], y también en cerveza, coliflor y ciertos tipos de sopas en las que se utilizan nucleótidos como potenciadores del sabor[53]. También se ha detectado la presencia de nucleósidos y nucleótidos en las hojas y

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[49] A. Wakamatsu, K. Morimoto, M. Shimizu, S. Kudoh. *A severe peak tailing of phosphate compounds caused by interaction with stainless steel used for liquid chromatography and electrospray mass spectrometry*. Journal of Separation Science, 28 (2005) 1823-1830.

[50] Y. Asakawa, N.Tokida, C. Ozawa, M. Ishiba, O. Tagaya, N. Asakawa. *Suppression effects of carbonate on the interaction between stainless steel and phosphate groups of phosphate compounds in high-performance liquid chromatography and electrospray ionization mass spectrometry*. Journal of Chromatography A, 1198-1199 (2008) 80-86.

[51] A. Ranogajec, S. Beluhan, Z. Smit. *Analysis of nucleosides and monophosphate nucleotides from mushrooms with reversed phase HPLC*. Journal of Separation Science, 33 (2010) 1024-1033.

[52] F. Q. Yang, D. Q. Li, K. Feng, D. J. Hu, S. P. Li. *Determination of nucleotides, nucleosides and their transformation products in Cordyceps by ion-pairing reversed-phase liquid chromatography–mass spectrometry.* Journal of Chromatography A, 1217 (2010) 5501-5510.

[53] N. Yamaoka, Y. Kudo, K. Inazawa, S. Inagawa, M. Yasuda, K. I. Mawatari, K. Nakagomi, K. Kaneko. *Simultaneous determination of nucleosides and nucleotides in dietary foods and beverages using ion-pairing liquid chromatography–electrospray ionization-mass spectrometry.* Journal of Chromatography B, 878 (2010) 2054-2060.

frutos de algunas plantas[54,55]. En otros casos el estudio de estos analitos está relacionado con la frescura del alimento en sí, por lo que su papel como indicadores de calidad resulta de gran interés. Es el caso, por ejemplo, de la determinación realizada en atún fresco y enlatado[56], de la comparación llevada a cabo entre muestras de jalea real naturales o adquiridas en el supermercado[57,58,59]; o del estudio de la presencia y

[54] H. Liu, Y. Jiang, Y. Luo, W. Jiang. *A simple and rapid determination of ATP, ADP and AMP concentrations in pericarp tissue of litchi fruit by high performance liquid chromatography.* Food Technology and Biotechnology, 44 (2006) 531-534.

[55] S. Guo, J. Duan, D. Qian, H. Wang, Y. Tang, Y. Qian, D. Wu, S. Su, E. Shang. *Hydrophilic interaction ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry for determination of nucleotides, nucleosides and nucleobases in Ziziphus plants.* Journal of Chromatography A, 1301 (2013) 147-155.

[56] M. T. Veciana-Nogues, M. Izquierdo-Pulido, M. C. Vidal-Carou. *Determination of ATP related compounds in fresh and canned tuna fish by HPLC*. Food Chemistry, 59 (1997) 467-472.

[57] X. F. Xue, J. H. Zhou, L. M. Wu, L. H. Fu, J. Zhao. *HPLC determination of adenosine in royal jelly.* Food Chemistry, 115 (2009) 715-719.

[58] L. Zhou, X. F. Xue, J. H. Zhou, Y. Li, J. Zhao, L. M. Wu. *Fast determination of adenosine 5′-triphosphate (ATP) and its catabolites in royal jelly using ultraperformance liquid chromatography*. Journal of Agricultural and Food Chemistry, 60 (2012) 8994-8999.

[59] L. Wu, L. Chen, J. N. Selvaraj, Y. Wei, Y. Wang, Y. Li, J. Zhao, X. Xue. *Identification of the distribution of adenosine phosphates, nucleosides and nucleobases in royal jelly.* Food Chemistry, 173 (2015) 1111-1118.

relación de ciertos nucleótidos y nucleósidos para evaluar la calidad de muestras de carne[60,61].

Respecto a la alimentación infantil, se han llevado a cabo determinaciones en fórmulas infantiles[22,26,32,38,40,41,43,44,62,63,64,65], leche materna[6,22,32,65] y otros alimentos infantiles complementarios[34,35]; utilizando las diferentes modalidades cromatográficas que se han señalado anteriormente. Sin embargo, la mayoría de estas aplicaciones se han dirigido al estudio de nucleósidos o nucleótidos por separado, debido posiblemente a las dificultades de la determinación conjunta de estos compuestos. Por otra parte, algunos de los trabajos que sí se han enfocado a la separación simultánea, se han visto limitados por los elevados tiempos de análisis[32,43,51,53,59] o por la pérdida de sensibilidad

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[60] N. Battle, M. C. Aristoy, F. Toldrá. *Early postmortem detection of exudative pork meat based on nucleotide content.* Journal of Food Science, 65 (2000) 413-416.

[61] L. Mora, A. S. Hernández-Cázares, M. C. Aristoy, F. Toldrá, M. Reig. *Hydrophilic interaction chromatography (HILIC) in the analysis of relevant quality and safety biochemical compounds in meat, poultry and processed meats.* Food Analytical Methods, 4 (2011) 121-129.

[62] C. Perrin, L. Meyer, C. Mujahid, C. J. Blake. *The analysis of 5′-mononucleotides in infant formulae by HPLC.* Food Chemistry, 74 (2001) 245-253.

[63] B. D. Gill, H. E. Indyk, M. C. Kumar, N. K. Sievwright, M. Manley-Harris. *A liquid chromatographic method for routine analysis of 5-mononucleotides in pediatric formulas*. Journal of AOAC International, 93 (2010) 966-973.

[64] B. D. Gill, H. E. Indyk, M. Manley-Harris. *Analysis of nucleosides and nucleotides in infant formula by liquid chromatography–tandem mass spectrometry*. Analytical and Bioanalytical Chemistry, 405 (2013) 5311-5319.

[65] A. G. Sfakianaki, C. D. Stalikas. *Selective microextraction of mononucleotides from milk using alumina and stannia hollow fibers prior to their determination by hydrophilic interaction liquid chromatography-diode array detection*. Food Chemistry, 184 (2015) 188-195.

derivada de la problemática inherente al análisis de compuestos fosforilados[64].

3.6.2. Electroforesis capilar

La electroforesis capilar (CE) se presenta como una opción adecuada, eficiente y versátil para la separación de nucleósidos y nucleótidos[66], ya que son compuestos altamente polares y además, se encuentran cargados en un amplio intervalo de pH. Para la determinación de estos compuestos en alimentos se han utilizado diferentes modalidades de esta técnica como la electroforesis capilar zonal (*Capillary Zone Electrophoresis*, CZE), cromatografía electrocinética micelar (*Micellar Electrokinetic Chromatography*, MEKC) o la electrocromatografía capilar (*Capillary Electrocromatography*, CEC). Aunque la CZE es la modalidad más simple y la que se utiliza con mayor frecuencia, es posible conseguir una gran flexibilidad en la selectividad de la separación sólo con cambiar la composición del electrolito de fondo (*Background electrolyte*, BGE) y/o el tipo de capilar utilizado, según se trabaje en una modalidad electroforética[67] u otra. Es

[66] W. F. Smyth, V. Rodriguez. *Recent studies of the electrospray ionisation behaviour of selected drugs and their application in capillary electrophoresis–mass spectrometry and liquid chromatography–mass spectrometry*. Journal of Chromatography A, 1159 (2007) 159-174.
[67] X. J. Chen, F. Q. Yang, Y. T. Wang, S. P. Li. *CE and CEC of nucleosides and nucleotides in food materials*. Electrophoresis, 31 (2010) 2092-2105.

común la utilización de tampones de separación en medio básico y también la presencia de modificadores orgánicos[68].

Los detectores más frecuentemente utilizados han sido los de absorción con DAD trabajando a longitudes de onda entre 250-260 nm, más concretamente 254 nm[69,70]; y la detección por MS, en la que la investigación se ha centrado en el estudio de la interfaz utilizada para el acoplamiento CE-MS. Aunque se han desarrollado diferentes tipos como las que incluyen líquido adicional, las que no necesitan líquido adicional, las de unión líquida o las de electrodo directo, los sistemas que emplean líquido adicional siguen siendo actualmente los acoplamientos más utilizados[71,72]. En menor medida, también se han propuesto

[68] Y. X. Gong, S. P. Li, P. Li, J. J. Liu, Y. T. Wang. *Simultaneous determination of six main nucleosides and bases in natural and cultured Cordyceps by capillary electrophoresis*. Journal of Chromatography A, 1055 (2004) 215-221.

[69] S. Cortacero-Ramírez, A. Segura-Carretero, C. Cruces-Blanco, M. L. Romero-Romero, A. Fernández-Gutiérrez. *Simultaneous determination of multiple constituents in real beer samples of different origins by capillary zone electrophoresis*. Analytical and Bioanalytical Chemistry, 380 (2004) 831-837.

[70] F. Q. Yang, S. Li, P. Li, Y. T. Wang. *Optimization of CEC for simultaneous determination of eleven nucleosides and nucleobases in Cordyceps using central composite design*. Electrophoresis, 28 (2007) 1681-1688.

[71] M. Haunschmidt, W. Buchberger, C. W. Klampfl*. Investigations on the migration behaviour of purines and pyrimidines in capillary electromigration techniques with UV detection and mass spectrometric detection*. Journal of Chromatography A, 1213 (2008) 88-92.

[72] F. Q. Yang, L. Ge, J. W. H. Yong, S. N. Tan, S. P. Li. *Determination of nucleosides and nucleobases in different species of Cordyceps by capillary electrophoresis–mass spectrometry.* Journal of Pharmaceutical and Biomedical Analysis, 50 (2009) 307-314.

aplicaciones que emplean fluorescencia inducida por láser (*Laser Induced Fluorescence*, LIF)[73] y detección por conductividad[74].

En cuanto a las muestras de alimentos analizadas, merece la pena señalar que aunque existe una amplia variedad de alimentos que pueden actuar como fuentes potenciales de nucleósidos y nucleótidos, no existen sin embargo un gran número de aplicaciones concretas enfocadas a la determinación de estos compuestos utilizando esta técnica. La CE se ha usado para estudiar la separación de estándares de nucleótidos[75,76,77] y también en el análisis de matrices enriquecidas con IMP y GMP[78] debido a su efecto como potenciadores del sabor. Existe un

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[73] W. Wang, L. Zhou, S. Wang, Z. Luo, Z. Hu. *Rapid and simple determination of adenine and guanine in DNA extract by micellar electrokinetic chromatography with indirect laser-induced fluorescence detection*. Talanta, 74 (2008) 1050-1055.

[74] J. J. Xu, H. Y. Zhai, Z. G. Chen, W. F. Lin, P. X. Cai, J. Y. Mo. *Determination of effective constitutions in natural cordyceps sinensis and cultured cordyceps mycelia by capillary electrophoresis with high frequency conductivity detection.* Chemical Research and Application, 5 (2005) 644-647.

[75] W. G. Kuhr, E. S. Yeung. *Optimization of sensitivity and separation in capillary zone electrophoresis with indirect fluorescence detection.* Analytical Chemistry, 60 (1988) 2642-2646.

[76] A. P. McKeown, P. N. Shaw, D. A. Barrett. *Electrophoretic behaviour of oligonucleotides and mono-, di- and triphosphate nucleotides by capillary zone electrophoresis*. Electrophoresis, 22 (2001) 1119-1126.

[77] M. Cornelius, C. G. C. T. Wörth, H. C. Kliem, M. Wiessler, H. H. Schemeiser. *Detection and separation of nucleoside-5'-monophosphates of DNA by conjugation with the fluorescent dye BODIPY and capillary electrophoresis with laser-induced fluorescence detection.* Electrophoresis, 26 (2005) 2591-2598.

[78] C. F. Yeh, S. J. Jiang. *Determination of monophosphate nucleotides by capillary electrophoresis inductively coupled plasma mass spectrometry.* Analyst, 127 (2002) 1324-1327.

gran número de aplicaciones para la determinación de estos analitos en diferentes tipos de hongos y algunas determinaciones en otras matrices como cerveza, té, extractos de pescado, maíz o soja y también en algunos alimentos funcionales[67]. Recientemente también se ha descrito la aplicación de la CE en el estudio del metabolismo celular de la levadura *Saccharomyces Cerevisiae*[79], muy utilizada en los procesos de fermentación industrial de cerveza, vino, pan o antibióticos. En el área de la alimentación infantil, hasta la fecha sólo se ha descrito una determinación de nucleótidos en leche materna utilizando CE-UV[80].

3.6.3. Tratamiento de muestra

Los alimentos son mezclas complejas de compuestos naturales orgánicos como lípidos, carbohidratos, proteínas, vitaminas, compuestos fenólicos, ácidos orgánicos y aromas; e inorgánicos como agua y minerales. Además, también suelen estar presentes otras sustancias generalmente originadas en el procesado, en los tratamientos agroquímicos, en el empaquetado y en el almacenamiento[81]. De esta forma, los alimentos son matrices complejas en las que el tratamiento de muestra previo a la separación y detección

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[79] P. Zhu, S. Wang, J. Wang, L. Zhou, P. Shi. *A capillary zone electrophoresis method for adenine nucleotides analysis in Saccharomyces cerevisiae*. Journal of Chromatography B, 1008 (2016) 156-163.

[80] J. Cubero, J. Sánchez, C. Sánchez, D. Narciso, C. Barriga, A. B. Rodríguez. *A new analytical technique in capillary electrophoresis: studying the levels of nucleotides in human breastmilk*. Journal of Applied Biomedicine, 5 (2007) 85-90.

[81] L. Mondello, G. Dugo, P. Dugo. *Recent applications in LC–MS: food and flavours*. LC-GC Europe, 2 (2002) 2-8.

de los analitos, es una etapa crucial. Los métodos de extracción utilizados requieren normalmente etapas adicionales de limpieza de la muestra, y en algunos casos puede ser necesaria la preconcentración de los extractos obtenidos.

Debido a la elevada polaridad de los nucleósidos y nucleótidos, el método más comúnmente utilizado para la extracción de estos analitos de diferentes muestras de alimentos ha sido la extracción líquido-líquido o sólido-líquido, utilizando principalmente agua y también disolventes orgánicos polares como etanol o metanol. Si bien la extracción se puede llevar a cabo simplemente con agua u otro disolvente polar[52,55], lo que en algunos casos se ha denominado inmersión cuando la muestra tratada es sólida[82]; también es frecuente realizar la extracción asistida por temperatura[51,52,58], ultrasonidos[68,72], extracción Soxhlet[83,84,85] o extracción con líquidos presurizados (PLE)[70]. Además, en ocasiones puede ser recomendable la utilización de

[82] S. Y. Lin, W. H. Chen, C. Y. Liu. *Nucleoside monophosphates recognition using macrocyclic polyamine bonded phase in capillary electrochromatography*. Electrophoresis, 23 (2002) 1230-1238.

[83] S. P. Li, P. Li, T. T. X. Dong, K. W. K. Tsim. *Determination of nucleosides in natural Cordyceps sinensis and cultured Cordyceps mycelia by capillary electrophoresis.* Electrophoresis, 22 (2001) 144-150.

[84] S. P. Li, Z. H. Song, T. T. X. Dong, Z. N. Ji, C. K. Lo, S. Q. Zhu, K. W. K. Tsim. *Distinction of water-soluble constituents between natural and cultured Cordyceps by capillary electrophoresis*. Phytomedicine, 11 (2004) 684-690.

[85] Y. Gengliang, L. Haiying, L. Haiyan, W. Dexian, L. Baohui, C. Yi. *Determination of adenosine, adenine and uracil in Cordyceps Inensis (Berk.) Sacc and Artificial Cordyceps by capillary zone electrophoresis.* Chinese Journal of Analytical Chemistry, 30 (2002) 1081-1084.

disoluciones salinas[58] o de diferentes mezclas de disolventes como agua-etanol[57] o agua-etanol-acetona[59].

Para la limpieza de las muestras, una de las estrategias más utilizadas hasta la fecha ha sido la adición de medios ácidos para precipitar las proteínas y eliminar de esta forma las posibles interferencias causadas por las macromoléculas presentes en las matrices alimentarias[54,56,60]. Entre los ácidos más frecuentemente utilizados se encuentran el ácido perclórico[43,62], tricloroacético[34,35], acético[32] y fórmico[44]. Este procedimiento ha sido muy utilizado para el tratamiento de alimentos infantiles, sin embargo, merece la pena destacar que debido a la inestabilidad que sufren los nucleótidos a pH bajo, se requiere una etapa adicional de neutralización del ácido para que los analitos conserven sus propiedades antes de llevar a cabo el análisis. En el caso de las leches maternas, el protocolo seguido suele incluir diferentes digestiones enzimáticas con el objetivo de convertir las diferentes formas de nucleótidos presentes en la leche en sus correspondientes nucleósidos[29,31]. Por otra parte, es importante señalar el uso de la ultrafiltración por centrifugación (CUF) por tratarse de un procedimiento simple y eficaz para la limpieza de muestras complejas como son las matrices alimentarias, tanto de alimentos en general como de alimentos infantiles, a través de la retención de las especies macromoleculares[38,40,53].

En función de los niveles de nucleósidos y nucleótidos en las muestras, y dependiendo también de la técnica de separación-detección que se vaya a utilizar, puede ser recomendable o necesaria la realización de una etapa de preconcentración de los analitos estudiados. Por ejemplo, en el análisis de compuestos a nivel traza con CE suele ser

necesario incluir una etapa adicional de preconcentración con el objetivo de mejorar la sensibilidad de la técnica. Se han probado diferentes estrategias como el barrido, *stacking*, isotacoforesis o extracción con membranas[67]. Sin embargo, ya sea con CE o con HPLC, la extracción en fase sólida (SPE) es uno de los métodos más utilizados para la limpieza y preconcentración de muestras. Tal cómo se explicó anteriormente en el apartado de estructura y propiedades, los nucleótidos se encuentran con carga neta negativa en un amplio intervalo de pH, por lo que lo más habitual ha sido la realización de SPE con sorbentes de intercambio aniónico[62,63,67]. Especial atención merece un procedimiento novedoso descrito recientemente para la extracción y preconcentración de nucleótidos de muestras de fórmulas infantiles y leche materna, en el que se realiza una microextracción selectiva utilizando fibras huecas de aluminio y estaño[65].

Para finalizar este capítulo y destacar la importancia del análisis de nucleósidos y nucleótidos en alimentos infantiles, merece la pena señalar la reciente colaboración de B. D. Gill y H. E. Indyk con 12 laboratorios de diferentes países, que ha dado lugar a un método oficial de la AOAC[86]. Este método es aplicable al análisis de nucleótidos 5'-monofosfato en fórmulas infantiles y formulas nutricionales pediátricas y para adultos. La extracción de los analitos se lleva a cabo con sorbentes SPE de intercambio aniónico y para la separación cromatográfica se usa una fase estacionaria C_{18} con un gradiente de elución. Se realiza detección UV y cuantificación con la técnica de patrón interno (timidina 5'-monofosfato).

[86] B. D. Gill, H. E. Indyk. *Analysis of nucleotide 5′-monophosphates in infant formulas by HPLC-UV: Collaborative study, Final Action 2011.20.* Journal of AOAC, 98 (2015) 971-979.

Capítulo I

Aplicaciones de la Electroforesis Capilar Acoplada a Espectrometría de Masas

DETERMINACIÓN DE BENZIMIDAZOLES ANTIHELMÍNTICOS EN HUEVO UTILIZANDO QUECHERS CON PRECONCENTRACIÓN COMO TRATAMIENTO DE MUESTRA

I.1

ARTÍCULO DE INVESTIGACIÓN I.1

RESUMEN

I.1. Determinación de benzimidazoles antihelmínticos en huevo utilizando QuEChERS con preconcentración como tratamiento de muestra

Los derivados benzimidazólicos son agentes antihelmínticos de amplio uso para el tratamiento de infecciones parasitarias en animales destinados a la producción de alimentos. En general, los residuos antihelmínticos no poseen riesgos para la salud humana siempre que estos sean administrados de una forma adecuada y con la dosis recomendada, respetando los tiempos de retirada. Sin embargo, el importante desarrollo en la investigación de nuevos antihelmínticos ha suscitado cierta preocupación debido a la amplia disponibilidad de fármacos veterinarios sin prescripción, que puede incrementar el uso incorrecto de los mismos. La exposición humana a estos agentes tóxicos tiene lugar, principalmente, a través de la ingesta de alimentos contaminados; por ello, para proteger a los consumidores de estos riesgos, la actual legislación proporciona límites máximos de residuos (MRLs) para ciertos alimentos de origen animal.

A la vista de lo anterior, parece obvio que el control de residuos de benzimidazoles antihelmínticos y sus metabolitos en alimentos de origen animal es especialmente importante. Sin embargo, el desarrollo de un método para el control de estos compuestos a los niveles establecidos por la legislación no resulta una tarea fácil, ya que a pesar de las similitudes en sus estructuras y en el modo de acción, las propiedades químicas de los benzimidazoles son muy diferentes. La mayoría de los métodos descritos hasta el momento para el análisis de residuos benzimidazólicos en diferentes tipos de alimentos de origen animal utilizan cromatografía líquida (HPLC) como técnica de

separación, acoplada principalmente a espectrometría de masas (MS). Por otra parte, aunque la electroforesis capilar (CE) ha demostrado ser una buena alternativa en el análisis de fármacos y metabolitos en alimentos, existen sólo algunos métodos para la determinación de fármacos antihelmínticos, principalmente aplicados al análisis de fluidos biológicos.

Por todo ello, el objetivo principal de este trabajo fue el desarrollo de un método analítico simple y eficiente basado en CE-MS, que permitiese la cuantificación de un amplio número de benzimidazoles y sus metabolitos en muestras de huevo; por ser este un alimento de elevado consumo. Además, el huevo es una matriz compleja que requiere de un estudio detallado para la extracción/preconcentración de los analitos y para su posterior limpieza, por lo que la optimización del tratamiento de muestra supuso un objetivo adicional para el desarrollo del método completo. En nuestro conocimiento, la CE no había sido utilizada hasta la fecha para el análisis de benzimidazoles en muestras de huevo. Tampoco el método QuEChERS (*Quick, Easy, Cheap, Effective, Rugged and Safe*), ampliamente usado en el análisis de contaminantes con HPLC, había sido propuesto en análisis con CE.

Para llevar a cabo los objetivos planteados, el primer paso fue la optimización de la separación de los analitos en CE y su cuantificación con CE-MS. Los 10 benzimidazoles estudiados en este trabajo tienen valores de pK_a entre 5 y 6, por lo que la utilización de medios ácidos favorece su separación electroforética como cationes. En relación a la detección con ESI-MS se optimizaron diferentes parámetros, poniendo especial atención en la composición del líquido adicional, ya que debido a la insignificante contribución del medio de separación al flujo total que

llega al electrospray, este es el medio principal en el que tiene lugar la ionización de los analitos. Los mejores resultados en cuanto a sensibilidad se obtuvieron utilizando una mezcla de isopropanol/agua al 50 %. Además, se aplicó una presión de nebulización programada, que proporcionó una alta resolución y estabilidad de la señal de masas a lo largo del análisis.

Posteriormente se realizó un estudio detallado de la composición relativa del medio de separación e inyección, estudiándose diferentes combinaciones de ambos medios, tanto en acuoso como en orgánico, así como de la presencia de aditivos en el medio de separación. Se comprobó que la inyección y separación de los analitos en medio acuoso originaba una mejor resolución en la separación electroforética, pero una peor sensibilidad que cuando se utilizaban medios de inyección y separación orgánicos. Así, se decidió combinar la inyección en medio orgánico para conseguir la mejor sensibilidad, con la separación en medio acuoso para no perder la eficiencia en la separación. Además, la inyección en medio orgánico permitió que el método fuese compatible con los tratamientos de muestra utilizados para la extracción de los benzimidazoles. Por otra parte, con la presencia de un modificador orgánico en el medio de separación se consiguió ampliar la ventana de separación en CE y conseguir de esta forma una mayor eficiencia en la separación. Finalmente, la mejor combinación fue utilizar una concentración 0.4 M de ácido fórmico en acetonitrilo como medio de inyección; y 6 M de disolución acuosa de ácido fórmico con un 30 % de isopropanol como medio de separación. En estas condiciones, trabajando con inyección hidrodinámica y utilizando un patrón interno, se obtuvieron resultados satisfactorios para la calibración de los 10

analitos, con límites de detección (LODs) instrumentales comprendidos entre 2 y 103 μg L^{-1}.

Se evaluaron diferentes tratamientos para la extracción y preconcentración de los benzimidazoles de las muestras de huevo. Cabe destacar que en todos ellos se procedía a la separación previa de la clara y la yema del huevo y se efectuaba el tratamiento sobre la yema, por ser en esta parte, compuesta de proteínas liposolubles, donde tienden a acumularse los analitos estudiados. Los dos primeros procedimientos se basaron en el uso de extracciones con diferentes disolventes, en el primer caso se realizó una doble extracción, primero con acetato de etilo y luego con hexano, y en el segundo método se llevó a cabo una extracción simple con una disolución acuosa de fosfato. En los dos casos se realizó una etapa de limpieza posterior con sorbentes de extracción en fase sólida (SPE) de tipo ABN, que consiste en un co-polímero de estireno divinilbenceno con diferentes grupos funcionales que permiten la retención de compuestos ácidos, básicos y neutros. El tercer método se basó en la conocida metodología QuEChERS en la que se realiza una extracción con acetonitrilo asistida por sales seguida de una limpieza mediante extracción en fase sólida dispersiva (DSPE) usando PSA (*Primary Secundary Amine*). Con este último tratamiento se consiguieron los mejores resultados en cuanto a simplicidad, reproducibilidad y eficiencia de la extracción, por lo que se seleccionó para los estudios posteriores de cuantificación y validación. Con el fin de obtener una mayor sensibilidad se ensayaron distintos procedimientos, encontrándose los mejores resultados al efectuar una preconcentración por evaporación del extracto obtenido en la etapa de limpieza con PSA.

Con el procedimiento de extracción optimizado se determinaron la eficacia de la extracción, el efecto de matriz y la eficiencia total del proceso. Se observó un claro efecto de matriz para la mayoría de los benzimidazoles estudiados y además, el rendimiento de la extracción no alcanzó en ningún caso el 100 %. A la vista de estos resultados se pudo concluir que la cuantificación en muestras de huevo con la metodología propuesta requería realizar la calibración en la modalidad de matriz real (*matrix-matched calibration*). De esta forma, se llevó a cabo la validación del método a través de la evaluación de los parámetros establecidos por la directiva europea 2002/657: linealidad de la calibración, límites analíticos (de detección, LOD; de cuantificación, LOQ; de decisión, CC_{α}; y capacidad de detección, CC_{β}), repetibilidad, reproducibilidad y recuperación. Se obtuvieron LODs entre 3 y 51 ng g^{-1}, inferiores en todos los casos a los límites máximos de residuos establecidos por la legislación para algunos benzimidazoles y sus metabolitos. Se encontraron también buenos resultados para la repetibilidad y reproducibilidad, comprobándose que los valores de precisión mejoraban cuando se utilizaba patrón interno. Al no disponer de muestras certificadas de referencia (CRM), se determinó la exactitud evaluando las recuperaciones del método, obteniéndose valores comprendidos entre el 86 y el 107 % con precisiones que variaron entre el 10 y el 20 %.

Finalmente, el método propuesto se aplicó al análisis de benzimidazoles en muestras de yema procedentes de huevos de diferente naturaleza: faisán, gallina enana, codorniz y huevos de gallina ecológicos. Ninguna de las muestras contenía a los benzimidazoles en niveles superiores a los límites de detección del método, por lo que todas ellas fueron dopadas antes de realizar los análisis posteriores. Se

obtuvieron, en todos los casos, valores satisfactorios de recuperación y precisión que pusieron de manifiesto la robustez del método y su aplicabilidad en diferentes muestras de huevo.

Para concluir, es necesario indicar que un método simple y sensible basado en CE-MS ha sido propuesto para la determinación de 10 benzimidazoles en muestras de huevo, y una variante de la metodología QuEChERS en la que se incluye una etapa de preconcentración ha sido aplicada por primera vez para el análisis de muestras reales con CE. Los resultados obtenidos en este trabajo indicaron que CE-MS puede ser un método alternativo a los métodos cromatográficos para la determinación de derivados benzimidazólicos en muestras de huevos de distinta naturaleza con las ventajas y condicionantes inherentes a esta técnica de separación.

ARTÍCULO DE INVESTIGACIÓN I.1

Journal of Chromatography A, 1278 (2013) 166-174

Journal of Chromatography A, 1278 (2013) 166–174

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

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

Capillary electrophoresis coupled to mass spectrometry for the determination of anthelmintic benzimidazoles in eggs using a QuEChERS with preconcentration as sample treatment

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ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 4 December 2012
Accepted 23 December 2012
Available online 8 January 2013

Keywords:
Anthelmintics
Benzimidazoles
Egg
QuEChERS with preconcentration
Programmed nebulizing-gas pressure
Capillary electrophoresis–electrospray ionization-mass spectrometry

ABSTRACT

Benzimidazoles (BZDs) are anthelmintic agents widely used in veterinary medicine. Their use in food-producing animals increases the possibility of residues appearing in animal tissues and products. Most analytical procedures reported for the determination of BZDs have been developed based on liquid chromatography (LC) because of their polar nature – zwitterionic – and thermal lability. To our knowledge, the determination of these compounds by capillary electrophoresis coupled to mass spectrometry (CE–MS) has not yet been described. In this work CE–MS is proposed for the identification and simultaneous quantification of several benzimidazoles in egg samples. The target compounds were 2-aminobenzimidazole, carbendazim, albendazole-2-aminosulphone, 5-hydroxy-thiabendazole, oxibendazole, albendazole, fenbendazole, oxfendazole, albendazole-sulphone, fenbendazole-sulphone. Optimization of the composition and nature – organic/aqueous – of both the electrophoretic separation buffer and the injection medium was carried out with a view to obtaining the best sensitivity and separation efficiency for the CE–MS coupling. A comparative study was carried out on different sample treatments for analyte extraction from egg samples. Two of them comprised a solvent extraction step followed by clean-up using a new commercial polymeric sorbent (Evolute ABN©), and the third was a particularization of the general extractive method so called Quick, Easy, Cheap, Effective, Rugged and Safe (*QuEChERS*). Different modifications of the *QuEChERS* method were assayed, which included a later preconcentration step based on either SPE with MCX© sorbents or evaporation. The whole optimized method (*QuEChERS* with preconcentration prior to CE–MS) was validated according to the 2002/657/EC decision obtaining a CE–MS method sufficiently reliable and robust to determine residues of these compounds in egg samples of different origins with limits of detection between 3 and 51 μg L^{-1} (S/N = 3) and recoveries in the 74–112% range.

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1. Introduction

Anthelmintic drugs are used to treat parasitic infections and include benzimidazoles (BZDs), flukicides, levamisole, macrocyclic lactones and morantel. In general, it may be proposed that anthelmintic residues pose no human health risk if veterinary drugs are properly administered and the recommended doses are correctly adhered to. However, there may be a concern that if withdrawal periods are not adhered to, or if products are administered to animals in unapproved applications, the levels may exceed maximum residue limits (MRLs) in foods. Some of these drugs possess toxicological properties namely teratogenicity, embryotoxicity; neurotoxicity, hyperplasia, goitrogenicity and mutagenicity [1].

Broad-spectrum benzimidazole-derived drugs have been used since the beginning of the sixties in animal health and crop protection. The metabolism of benzimidazoles is extensive. The metabolites found depend on the structure of the parent drug, the tissue, and the animal species [2]. The EU has MRLs for benzimidazoles and their metabolites in animal products [3]. For liver tissue, the range is 100–1000 μg kg^{-1}, depending on the benzimidazole; for kidney and muscle, 50–500 μg kg^{-1}; for fat 50–200 μg kg^{-1}, while milk has lower MRLs (10–100 μg kg^{-1}), the pertinent analytical methods requiring greater sensitivity.

In light of the above, it seems evident that the control of residues of benzimidazole anthelmintics and their main metabolites in foods of animal origin is especially important. However, it is extremely difficult to develop a single multi-residue method for the control of the whole set of residues at the MRL level prescribed by law because

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0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2012.12.064

despite the similarities in their chemical structure and mode of action, their chemical properties are very different [4], such that their joint extraction is a complex problem.

Most methods used for the analysis of one or more benzimidazoles as residues in a variety of foods types use LC as the separation technique, mainly coupled to mass analysers, although there are several coupled to UV detectors [5–9] or fluorescence detectors [10]. Regarding the LC–MS and LC–MS/MS methods, 21 benzimidazoles have been isolated from the muscle and liver tissue of different animals [11] employing pressurized liquid extraction (PLE) and analysed by HPLC–MS/MS; 10 benzimidazole residues and some of their metabolites have been determined in eggs, milk, chicken and pork by polymer monolith microextraction (PMME) followed by HPLC–MS [12], and 15 benzimidazoles and two metabolites have been determined in pig muscle by solvent extraction and SPE followed by HPLC–MS/MS [13]. Kinsella et al. [1] have developed a LC–MS/MS method capable of isolating 16 benzimidazoles and metabolites from bovine milk and liver, and Kaufmann et al. have developed ultra high performance liquid chromatography–high resolution mass spectrometry (UPLC–HRMS) methods for the determination of benzimidazoles in different meat matrices [14] and more recently in tissue and honey [15].

Capillary electrophoresis (CE) has proved to be a powerful alternative to chromatographic techniques in the analysis of drugs and metabolites in foods. Its main advantages are high separation efficiency, short global-analysis times and low operational costs. There are a few methods in the literature for the determination of different anthelmintic drugs by CE–MS, mainly in body fluids [16–20] and feed [21], by different CE modalities, such us chiral CE, capillary zone electrophoresis (CZE) and non-aqueous capillary electrophoresis (NACE). Recently, Hu et al. [22] have proposed the determination of benzimidazole compounds in pig liver and muscle by CZE–DAD.

In the analysis of low-molecular weight compounds in foods such as drugs in eggs, the sample treatments used aim to avoid the interference deriving from the presence of macromolecular species and to preserve the instrumental set-up. Owing to their high lipid and protein content eggs constitute a very complex matrix, since some analytes may bind to lipoproteins, thereby hindering their extraction, and the extraction solvents form emulsions and foams with the matrix. Therefore, one of the main objectives during the extraction procedure is the removal of lipids and proteins prior to analysis.

Extraction methodologies usually require additional clean-up steps and different approaches have been employed: Garrido-Frenich et al. [23] have compared different isolation techniques for the extraction of 5 benzimidazoles from eggs analysed by UHPLC–MS/MS. In light of all the above, the main aim of this work was to develop a simple and efficient sample treatment leading to the development of a reliable method for the separation, identification, and quantification of a broad range of benzimidazole derivatives and their metabolites in eggs by use of CE–MS. This target was achieved in three steps: (i) optimization of the electrophoretic separation of the analytes and their quantification via CE–ESI-MS; (ii) comparison of different solvent extraction methods, two of them including liquid–liquid extraction (LLE) steps and a third based on *QuEChERS* methodology, and (iii) validation of the proposed method based on the *QuEChERS* approach but modified with the aim of reaching higher sensitivities.

Here we developed a simple method that allows quantification at the $\mu g\,kg^{-1}$ level of benzimidazole residues in eggs. To our knowledge CE–MS has not been applied to the analysis of a broad set of benzimidazoles and their metabolites in egg samples. Additionally, the *QuEChERS* approach, widely used in the analysis of pollutants by means of HPLC, has not been proposed for CE–MS analyses.

2. Experimental

2.1. Chemicals and reagents

Analytical standards of *Albendazole* (ABZ), methyl[6-(propylthio)-1H-benzoimidazol-2-yl]carbamate, CAS RN [54965-21-8]; *Albendazole-sulfone* ($ABZ-SO_2$), methyl(5-(propylsulfonyl)-1H-benzimidazol-2-yl)carbamate, CAS RN [75184-71-3], *albendazole-2-aminosulfone* ($ABZ-NH_2-SO_2$), (5-propylsulfonyl-1H-benzimidazol-2-yl)amine, CAS RN [80983-34-2] and *carbendazim* (CBZ), methyl(1H-benzimidazol-2-yl)carbamate, CAS RN [10605-21-7] were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Fenbendazole (FBZ), methyl(N-6-phenylthio-1H-benzoimidazol-2-yl)carbamate, CAS RN [53571-02-1]; *oxfendazole* (OFZ), methyl(N-6-phenylsulfinyl-1H-benzoimidazol-2-yl)carbamate, CAS RN [53716-50-0]; *fenbendazole-sulfone* ($FBZ-SO_2$), methyl(N-6-phenylsulfonyl-1H-benzoimidazol-2-yl)carbamate, CAS RN [54029-20-8]; *oxibendazole* (OXI), methyl(N-6-propoxy-1H-benzimidazol-2-yl)carbamate, CAS RN [20559-55-1]; *2-aminobenzimidazole* ($BZZ-NH_2$), CAS RN [934-32-7]; *thiabendazole-5-hydroxy* (5-OH-TBZ), 4-hydroxy-2-(1,3-thiazol-4-yl) benzimidazole, CAS RN [948-71-0] were obtained from Sigma–Aldrich (Steinheim, Germany). The internal standard *Carbendazim D3* (CBZ D3) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

The organic solvents – acetonitrile (ACN) and methanol (MeOH) (Merck, Darmstadt, Germany), isopropanol, ethyl acetate and ethanol (Scharlau, Barcelona, Spain) were of HPLC grade and were used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab UHQ water purification system (Noáin, Spain).

All chemicals used for the preparation of the buffer and all other chemicals were of analytical reagent grade.

Different commercial polymeric based SPE materials were tested in the sample treatments: Evolute® ABN, styrene–divinyl benzene copolymer (Biotage, Uppsala, Sweden); Oasis® MCX, sulfonated divinyl benzene-N-vinylpyrrolidone copolymer (Waters, Massachusetts, USA); Oasis® HLB, divinyl benzene-N-vinylpyrrolidone copolymer (Waters, Massachusetts, USA); and Isolute® ENV+, hydroxylated styrene–divinyl benzene copolymer (Biotage, Uppsala, Sweden). Primary–secondary amine exchange material (PSA) was purchased from Supelco, Bellefonte, USA.

2.2. Apparatus and software

All experiments were carried out with a Hewlett-Packard HP3D CE instrument (Agilent Tehcnologies, Waldbron, Germany) equipped with a UV–visible DAD device working at 214 nm with a bandwidth of 16 nm. MS was performed using an Agilent LC/MSD SL mass spectrometer (Agilent Technologies) equipped with a single quadrupole analyser. Both the UV–visible DAD detector and the MS device were controlled by Agilent HP ChemStation software, version B.02.01 SR1.

Fused-silica capillaries (50 μm I.D.) with a total length of 87.5 cm and 20 cm to the UV detector were from Polymicro Technologies (Phoenix, AZ, USA), and were used throughout the work for CE–MS analysis. The fused-silica capillaries employed in the CE–DAD analysis were of 75 μm I.D. with a total length of 57 cm and 50 cm to the UV detector. All of them were purchased from CM Scientific (Shipley, West Yorkshire, United Kingdom).

CE separation buffers and injection samples were placed in 1700 μL disposable amber glass vials with polyurethane pre-perforated caps (Agilent, Waldbroon, Germany).

A centrifuge, model Kokusan H-103N series from Eurocommercial S.A (Madrid, Spain), was used for the separation of the liquid phases. A vortex model ZX Classic from Velp Scientifica (Milan, Italy) was used to dissolve the dry residues obtained after evaporations.

The conditioning of the SPE sorbents, sample passage and cartridge drying were accomplished in a vacuum pump (Alfa, Barcelona, Spain) coupled to a 20-port manifold for sample preparation (Varian, Harbor City, USA).

For pH measurements, a pH-meter model 2001 (Crison, Barcelona, Spain) was employed. The rest of the instrumentation and materials were those routinely used in analytical laboratories: analytical scales, an ultrasonic bath and volumetric calibrated glassware.

2.3. CE separation conditions

Standard solutions were injected using a pressure of 50 mbar over 30 s. In all cases, the injection medium was a mixture of ACN/formic acid (99:1, vol.). Electrophoretic separation was achieved with a voltage of 22 kV (positive CE mode), with an initial ramp of 7 s. The running buffer (BGE) was a mixture of UHQ water/isopropanol (70:30, vol.) containing 6.0 mol L^{-1} of formic acid. The temperature of the capillary was kept constant at 25 °C. Before the first use, the capillary was conditioned by flushing with the BGE for 5 min at 4 bar. This was also implemented as a daily start-up procedure. After each run, the capillary was prewashed at 4 bar for 2 min with fresh running buffer.

2.4. ESI-MS conditions

MS was performed using an Agilent LC/MSD SL mass spectrometer equipped with a single quadrupole analyser. An Agilent coaxial sheath-liquid sprayer was used for CE–ESI-MS coupling. The sheath liquid, consisting of a 50:50, vol. isopropanol:UHQ water mixture, was delivered at a flow rate of 1.00 mL min^{-1} by an Agilent 1100 series pump equipped with a 1:100 flow-splitter. The ESI voltage was set at +4000 V. Other electrospray parameters under optimum conditions were: drying-gas flow rate, 7.0 L min^{-1}; drying-gas temperature, 350 °C. The nebulizing-gas pressure was switched off during injection, after which the minimum pressure possible (1 psi) was applied during separation, finally applying the optimum pressure for ionization (4 psi) 1.0 min before the analytes arrived at the detector, using a programmed nebulizing-gas pressure approach [24]. The mass spectrometer was operated in the positive-ion mode (ESI+). The optimized fragmentor voltage was 120 V for all analytes.

2.5. Sample treatments

Hen (*Gallus gallus*) and quail (*Coturnix coturnix*) eggs acquired at a market were used, together with bantam (*Gallus gallus*) and pheasant (*Phasianus colchicus*) eggs from a free-range farm. They were known not to have been subject to any treatment and that they came from birds that had not been treated with anthelmintic drugs. Before performing each method it was necessary to separate the yolk from the white, since in the analysis of eggs collected from animals treated with anthelmintic drugs it is known that the concentration of the analytes studied is greater in the egg yolk [12]. Fortification of the samples, when necessary, was performed directly on the yolk once this had been separated from the white, and a period of about 12 h was allowed to elapse before continuing with any of the extraction processes in order to favour the interaction between the analytes and the matrix compounds.

Three different sample treatments were tested and compared. Two of them were based on solvent extraction and SPE clean-up – named A and B – and a third, which was a *QuEChERS* method – named C. Several modifications were introduced in the implementation of this latter (C1, C2 and C3) to improve sensitivity.

2.5.1. Solvent extraction-based procedures: methods-A and -B

For these methods, the sample extraction steps were those from either Balizs [13] (method-A) or Hu et al. [12] (method-B). The first is based on a double-extraction of the egg sample, employing ethyl acetate followed by *n*-hexane, and the second one is performed employing an aqueous monosodium phosphate solution. In method-A the organic extract was evaporated to dryness in a gentle stream of nitrogen and re-dissolved in 10 mL of a 0.010 mol L^{-1} aqueous ammonium acetate solution.

The clean-up procedure employed in the present work for both method A and B was as follows: the extract was subjected to SPE (Evolute® ABN), the sorbent being conditioned with 3 mL of ACN followed by 3 mL of UHQ water; the passage of the organic phase was gravity-driven; the cartridges were dried under a vacuum for 15 min at 20 mmHg (1 mmHg = 133.322 Pa), and the elution of the retained components was accomplished with 2.0 mL of an ACN/formic acid (99:1, vol.) solution. One millilitre of the eluate was spiked with 100 μL of the internal standard solution – 15 mg L^{-1} of CBZ D3 – and injected into the CE–MS system.

2.5.2. QuEChERS-based procedure: method C

This was a particularization of the general-purpose extraction method called "*Quick Easy Cheap Effective Rugged Safe*". Briefly, an aliquot of 3.00 g of egg yolk was placed in a 50 mL centrifuge tube and UHQ was added up to 10.0 g; then, 10.0 mL of ACN was added and the capped tube was shaken for 1 min. Finally, 4.0 g of magnesium sulphate, 1.0 g of sodium chloride and 1.0 mL of ammonium formate solution (2.5×10^{-3} mol L^{-1}, pH = 7.5) were added, the capped tube was shaken again for 1 min and then centrifuged for 5 min at 1500 × *g*.

DSPE was used for the clean-up: 1 mL of the supernatant was placed in a tube containing 0.025 g of primary–secondary amine (PSA) together with 0.150 g of magnesium sulphate, and the capped tube was shaken for 30 s and centrifuged for 2 min at 1500 × *g*. The cleaned supernatant was decanted, spiked with 100 μL of the internal standard solution – 15 mg L^{-1} of CBZ D3 – and injected into the CE–MS system.

2.5.3. Modified-QuEChERS procedures: methods C1, C2 and C3

The extraction step was the same as that employed in the *QuEChERS* – method designated C – incorporating the variations from the clean-up step.

Method C1: The clean-up involved SPE instead of DSPE and employed all the supernatant obtained in the extraction step. Oasis® MCX sorbents previously conditioned with 3 mL of ACN were employed and the passage of the organic phase was gravity-driven. The cartridges were dried under a vacuum for 15 min at 20 mmHg (1 mmHg = 133.322 Pa), and the elution of the retained components was accomplished with 2.0 mL of a solution (90:10, vol.) containing ACN and aqueous ammonium hydroxide solution (>25%).

The eluate was evaporated to dryness in a gentle stream of nitrogen and the dry residue was vortex-shaken with 2.0 mL of an ACN/formic acid (99:1, vol.) solution and filtered through a 0.22 μm filter. One mL of the filtrate was spiked with 100 μL of the internal standard solution – 15 mg L^{-1} of CBZ D3 – and injected into the CE–MS system.

Method C2: In this case, both the extraction and the clean-up steps were the same as those employed in the *QuEChERS* method designated C. DSPE was carried out employing all the supernatant obtained in the extraction step.

Following this, to achieve preconcentration the whole supernatant was evaporated to dryness in a gentle stream of nitrogen and the dry residue was vortex-shaken in 2.0 mL of an ACN/formic acid (99:1, vol.) solution. One mL of this solution was spiked with

100 μL of the internal standard solution – 15 mg L^{-1} of CBZ D3 – and injected into the CE–MS system.

Method C3: The only difference with method C2 was that the dry residue was dissolved in 1.0 mL instead of 2.0 mL. Eight hundred μL of this solution was spiked with 80 μL of the internal standard solution – 15 mg L^{-1} of CBZ D3 – and injected into the CE–MS system.

2.6. Quantitative parameters

2.6.1. Standard preparation and quantification procedure

Stock solutions at 500 mg L^{-1} (ABZ, CBZ, BZZ-NH_2, OXI, FBZ and OFZ) and 100 mg L^{-1} (ABZ-SO_2, ABZ-NH_2-SO_2, 5-OH-TBZ, FBZ-SO_2 and CBZ D3) were prepared by dissolving the compounds in MeOH. All solutions were kept at 4 °C in amber glass bottles. Working solutions containing the analytes at variable concentrations were prepared daily by mixing the appropriate amounts of the stock solution/s and diluting in a suitable medium.

Analyte quantification was carried out in the SIM acquisition mode, using protonated molecules: $[M+H]^+$. The peak area was obtained by manual base-to-base integration of the peak. The quantitative variable was the ratio between the peak area generated by that ion and the peak area generated by the ion of the internal standard (CBZ D3), both expressed in arbitrary peak area units.

2.6.2. Extraction parameters

The matrix effect (ME) and extraction parameters, namely extraction recovery (ER) and process efficiency (PE) were investigated based on the approach proposed by Matuszewski et al. and used by Badoud et al. [25]. The definitions of the three parameters are as follows: Matrix Effect (ME) = *b*/*a*; Extraction Recovery (ER) = *c*/*b*; and Process Efficiency (PE) = *c*/a, where *a* stands for the analytical signal provided by standard solutions at 600 μg L^{-1} in ACN/formic acid (99:1, vol.); *b* stands for the analytical signal provided by samples spiked after the method C3 had been applied to egg yolk aliquots, and *c* refers to samples spiked before the method C3 had been applied to egg yolk aliquots.

2.7. Method validation

The *QuEChERS* method with preconcentration developed – method C3 – was validated, according to European Legislation [26] evaluating the following parameters: calibration curves and analytical limits; repeatability and with-in laboratory reproducibility; accuracy and/or recovery; and ruggedness. In addition, the specificity – by comparing the migration times and the signals of standard electropherograms against yolk samples spiked with 1 identification point per analyte – and the stability – by controlling the storage conditions of the analytes and samples – were also evaluated, maintaining the conservation parameters within the values recommended by the supplier.

3. Results and discussion

As mentioned earlier, the aim of this work was to develop a simple and efficient sample treatment to achieve a reliable CE–MS separation of benzimidazole residues in eggs. For this purpose, three steps were carried out sequentially: (i) optimization of the CE separation of the analytes and their quantification by CE–ESI-MS; (ii) comparison of several sample extraction procedures; and (iii) validation of the whole method proposed.

3.1. Optimization of electrophoretic separation and mass spectrometric detection

3.1.1. Preliminary studies and ESI-MS optimization

The benzimidazole compounds studied here have pKa values between 5 and 6 and hence in acidic media they remain protonated as cations. Thus, the use of acidic media can be seen as useful when attempting their electrophoretic separation. Preliminary separation studies were done in CE–UV under different volatile BGEs, obtaining good separations upon employing 0.100 mol L^{-1} formic acid and 10% (vol.) ACN as a modifier.

For the optimization of ESI-MS detection, the ESI parameters of capillary voltage, drying-gas flow and drying-gas temperature were set to the values recommended by the supplier to achieve a stable electrospray for a sheath liquid flow of 10 μL min^{-1}–1 mL min^{-1} and 1:100 split. However, special attention was paid to the composition of the sheath liquid, since in light of the negligible contribution of the BGE to the total flow arriving at the ESI in CE–ESI this is the main medium in which the ionization of the analytes takes place.

Four different solvents and mixtures were assayed as sheath liquids: pure isopropyl alcohol and methanol, and mixtures of isopropyl alcohol/UHQ water 50% (vol.) and methanol/UHQ water 50% (vol.). The best sensitivity was obtained with the isopropyl alcohol/UHQ water 50% (vol.) mixture for most of the 10 analytes under study; only for BZZ–NH_2 was a 33% signal of the maximum achieved, in this case in pure isopropyl alcohol. Hence, the mixture of isopropyl alcohol/UHQ water 50% (vol.) was selected as the sheath liquid as a compromise for the 10 analytes. The presence of acetic acid in the sheath liquid, in the 0.005–0.020 mol L^{-1} range, did not improve sensitivity. These results can be explained considering that the analytes had already arrived at the ESI in their cationic forms and hence did not require the presence of an acidic media to facilitate their ionization and, in addition, the presence of salts in the sheath liquid could be a source of ion suppression in the electrospray.

Special attention was also paid to the nebulizing pressure of the ESI since it has been reported [24] that a programmed nebulizing pressure (PNP) sometimes becomes crucial in order to maintain the separation achieved in CE and for separation to remain stable. The optimized conditions found were a PNP from 1 to 4 psi, as indicated in Section 2.4.

Study of the curves obtained upon varying the cone voltage of the MS between 60 and 120 V provided the best results for all the analytes at 120 V.

The optimized values for the ESI-MS parameters proved to be as indicated in Section 2.4.

3.1.2. Influence of the composition of the BGE and the solution for injection on the sensitivity and separation efficiency

The composition of the solution for injection becomes crucial in CE in order to obtain stable analyses of real complex matrices and to achieve the best sensitivity and separation efficiency. Hence, different compositions of the solution for injection were assayed in combination with different volatile BGEs. The most significant results are shown in the total ion electropherograms (TIE) of Fig. 1.

When a sample in aqueous medium was injected in an aqueous BGE, the TIE obtained (Fig. 1A) showed poor sensitivity but better separation efficiency in comparison with those obtained when both the solution for injection and the BGE were organic (Fig. 1B). Accordingly, we attempted to achieve the better sensitivity obtained with the organic media without losing the separation efficiency achieved with the aqueous media. Fig. 1C shows the TIE obtained by CE–MS when the solution for injection was in organic medium and the BGE remained in aqueous medium. Under these

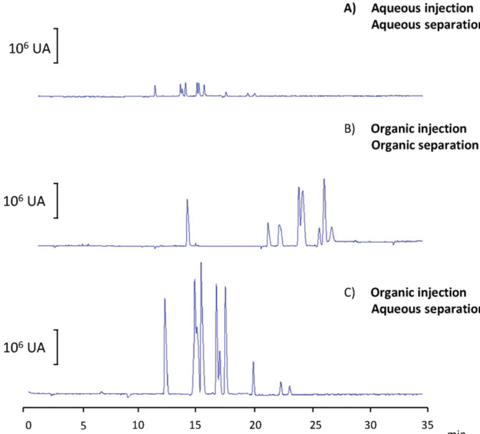

Fig. 1. Total ion electropherograms (TIE) for the benzimidazoles studied at a concentration level of 10 μg kg^{-1}. Separation medium: **A** and **C**, 100 mM formic acid with 10% of acetonitrile; **B**, 300 mM formic acid and 50 mM ammonium formate in acetonitrile/methanol (30/70, vol.). Injection medium: **A**, 0.4 M formic acid in water; **B** and **C**, 0.4 M formic acid in acetonitrile. Electrokinetic injection: **A** and **B**, 10 kV, 5 s; **C**, 10 kV, 15 s.

conditions, the CE–MS method provided much more sensitivity and efficiency remained fairly similar to that achieved earlier. In addition, this combination in which the solution for injection is organic-based makes the potential sample treatments more compatible with the further CE separation without the need to change solvents, which is an improvement over the CE–MS method.

Even better separations were achieved when different modifiers were included in the BGE. Two different BGE modifiers were tested: urea and isopropyl alcohol. It is well known that the presence of urea at relatively high concentrations usually tends to expand the separation window in CE. However, urea, with a boiling point of 133 °C and a decomposition temperature between 150 and 160 °C, can be considered a semi-volatile compound [27]. Hence, a study was carried out with different concentrations of urea up to 2.0 mol L^{-1}, observing that concentrations lower than 0.5 mol L^{-1} had no effect on the separation already achieved, while higher concentrations greatly improved the CE separation. However, after nine sequential injections the TIE's obtained were poorly reproducible and the migration times of the analytes were progressively increased due to the deposits of non-volatile compounds blocking the ESI needle tip.

By contrast, the isopropyl alcohol as a BGE modifier afforded good results; the TIE obtained revealed better separation efficiency with respect to that obtained in its absence and very high reproducibility between runs. Finally, the best combination was as follows: an injection solution of 0.4 mol L^{-1} formic acid in ACN; BGE, 6.0 mol L^{-1} aqueous formic acid solution with 30% vol. isopropyl alcohol.

Working with this medium, electrokinetic injection provided better sensitivity than hydrodynamic injection. However, even when an internal standard was used the relationship between the property and concentration was not suitable ($R^2 \approx 0.52$). Upon working with hydrodynamic injection we obtained good calibration results for the 10 analytes studied ($R^2 \approx 0.99$). On using hydrodynamic injection, the limits of detection (S/N = 3) ranged between 2 and 103 μg L^{-1} for BZZ-NH_2 and 5-OH-TBZ respectively.

3.2. Egg samples: extraction, preconcentration and clean-up steps

3.2.1. Preliminary studies on SPE sorbents for clean-up and preconcentration

The low sensitivity shown by CE means that it is necessary to perform a preconcentration and clean-up of the extracts obtained prior to their analysis with CE–MS. We thus performed several previous studies with standards using SPE with sorbents of different types. SPE materials were selected to match the chemical properties of the analytes studied, mainly their hydrophilicity and their cationic form at low pH.

Initially we selected ABN sorbents. These materials, made of a divinylbenzene styrene copolymer, have different functional groups that allow the retention of acid, basic and neutral compounds. The average pore diameter (40 Å) of that material is significantly smaller than those of other commercial polymeric reversed-phase SPE materials (60 Å). This narrower pore size reduces the retention of interfering high-molecular weight compounds [15]. Under optimum elution conditions (see Section 2), we obtained recovery values close to 100% as long as the solvent percolating along the sorbent was aqueous. However, the recovery values decreased dramatically upon increasing the percentage of acetonitrile in the sample, such that the use of these sorbents for the preconcentration of the analytes studied is only suitable when the analytes are dissolved in an aqueous phase or when there is a minimum percentage of organic phase (<10%).

Since the ABN cartridges did not provide suitable retention when the sample was organic, a study was made of the behaviour of the analytes when they were present in acetonitrile as an organic solvent. We decided to use a polymeric sorbent with sulphonic functional groups in which retention may occur through cationic exchange interactions (MCX), together with a polymeric cartridge with polar residues (HLB) and a polymeric cartridge with hydroxyl groups that has a high specific surface area (ENV+). Under the previous conditions found for the elution of the analytes – see method-C1, Section 2.5.3 – with the MCX sorbent we obtained recovery values closet to 100%, such that it was concluded that they were the most suitable ones for retaining the benzimidazoles dissolved in acetonitrile.

3.2.2. Comparative study of sample treatments

In this work a comparative study of different procedures for the extraction and clean-up of benzimidazoles and their metabolites in egg yolk samples was carried out. Three different methods were tested – methods A, B and C – as well as three variants – C1, C2 and C3; all of them are described in Section 2.5. As indicated in Section 2.5, the simple separation of the egg yolk from the egg white seems to be a good method for avoiding unwanted dilution of the analytes [12].

In brief, the method-A extraction step is based on that reported by Balizs [13], comprising a double extraction with ethyl acetate followed by *n*-hexane, and for – B, the step is based on that of Hu et al. [12], comprising a single extraction step with an aqueous monosodium phosphate solution. The subsequent clean-up proposed here for both methods, A and B, was an SPE with Evolute® ABN sorbents. Method-C is a particularization of the general *QuEChERS* method.

Several replicates of blank egg yolk samples spiked with the analytes at the mg kg^{-1} level were subjected to either method-A, -B, or -C and analysed by CE–ESI-MS. The peak areas obtained were compared with those of standard solutions containing the analytes at the theoretically expected concentrations for each method. Fig. 2 shows the recoveries for each analyte in each of the three methods normalized to those of method-C.

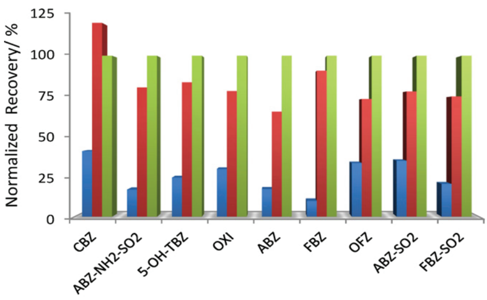

Fig. 2. Sample treatment recoveries obtained with methods -A, -B and -C normalized against method-C.

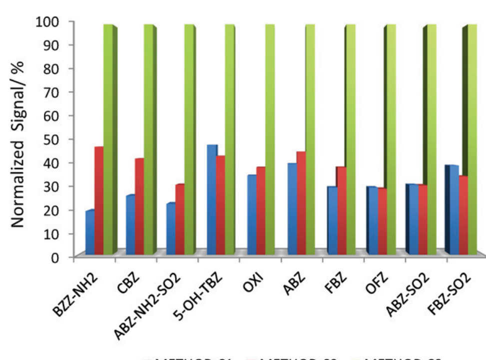

Fig. 3. Peak areas obtained with the sample treatment methods -C1, -C2 and -C3 normalized against method -C3.

It is worth mentioning that although method-A provided cleaner extracts, the recoveries were poorer in comparison with those achieved with method-B and -C (see Fig. 2). In addition, method-A was not very reproducible among replicates and somewhat time-consuming. Method-B was experimentally simpler and the recoveries were slightly lower than those obtained with method-C. However, it should be noted that after the extraction step with the aqueous monosodium phosphate solution the extracts obtained were not clean and were difficult to filter, leading to blockade of the SPE cartridges in the clean-up step.

Method-C (*QuEChERS*) proved to be simple and fast and was reproducible among replicates. Additionally, it provided the highest efficiency in analyte extraction – Fig. 2. Accordingly, this method was selected to carry out further studies. The results obtained with the *QuEChERS* method can be attributed to the use of acetonitrile as the extraction solvent, since it precipitates proteins and denatures enzymes, which could degrade drug residues during sample treatment [28].

The *QuEChERS* method does not involve preconcentration and, in addition, only a small fraction of the whole extract is subjected to a clean-up process in the original *QuEChERS* method since it is mainly oriented towards analysis by gas chromatography (GC). In order to improve the sensitivity found in method-C, three variants, -C1, -C2 and -C3, were tested.

The variations of method-C, namely -C1, -C2 and -C3, started from the clean-up step: SPE – employing MCX sorbents – instead of DSPE in method C1; and DSPE of the whole supernatant, followed by concentration by evaporation in methods C2 (final volume 2.0 mL) and C3 (final volume 1.0 mL).

In Fig. 3 the peak areas of the analytes obtained in the three variants are compared with those of method C3 at a spiking level of 200 $\mu g\,kg^{-1}$.

Method-C1, based on a preconcentration by SPE with MCX afforded similar results regarding sensitivity as method-C2, except for BZZ-NH_2, CBZ and ABZ-NH_2SO_2. Time consumption was also similar for both methods. For these two reasons, method-C2 was selected as simpler, cheaper and better for the group of analytes studied.

Finally, the best result was obtained with method-C3, which maintained the expected double preconcentration factor achieved with the -C2 variant.

In light of the results found – simplicity, good sensitivity and reproducibility – the *QuEChERS* variant with preconcentration by evaporation (method-C3) was selected for further quantification and validation studies. With this method enrichment factors from 1.7 to 3 were achieved.

3.3. Extraction parameters and CE–MS matrix effects

The approach developed by Matuszewski et al. and employed by Badou et al. [25] was followed. Three types of samples were analysed as described in Section 2.6.2, applying method-C3 and the optimized CE–ESI-MS conditions. The matrix effect (ME), extraction recovery (ER) and overall process efficiency (PE) were calculated and the results are shown in Fig. 4.

We observed matrix effects for all the analytes, except for BZZ-NH_2 and CBZ, which were those with higher charge-radius ratios. Moreover, as expected, the ER was lower than 100% for all the analytes, varying between 45% for BZZ-NH_2 and 81% for ABZ. It should be noted that for those analytes showing high matrix effects – ABZ, FBZ-SO_2 – this effect was compensated by their higher ER yields, and the overall process efficiency of the method remained in a band ranging from 45 to 65% for all the compounds studied. In light of these results it can be concluded that quantification in egg samples with the proposed methodology requires a matrix-matched calibration procedure.

3.4. Validation of the QuEChERS method with preconcentration prior to use of the CE–ESI-MS method

Validation of the proposed method was accomplished following the 2002/657/EC decision, which establishes the validation criteria of analytical methods for the determination of organic residues or contaminants in foods of animal origin.

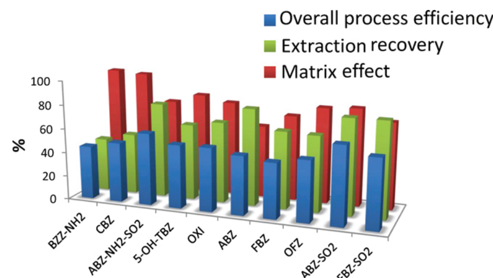

Fig. 4. Results obtained, for all analytes, in the study of extraction parameters.

Table 1
Validation parameters according to the 657/2002/EC decision for the method developed (*QuEChERS* with preconcentration prior to CE–MS).

2002/657/ EC validation		$BZZ-NH_2$	CBZ	$ABZ-NH_2-SO_2$	5-OH-TBZ	OXI	ABZ	FBZ	OFZ	$ABZ-SO_2$	$FBZ-SO_2$
Calibration parameters	Intercept/10^{-2}	9 ± 6	9 ± 8	−2 ± 4	0 ± 1	0 ± 6	0 ± 3	−3 ± 3	−1 ± 4	0 ± 3	1 ± 3
	Slope/10^{-3} g ng^{-1}	1.91 ± 0.07	2.8 ± 0.1	1.5 ± 0.1	0.28 ± 0.01	3.1 ± 0.1	0.77 ± 0.07	3.37 ± 0.05	1.81 ± 0.08	1.37 ± 0.07	1.44 ± 0.06
	LOD[a]/ng g^{-1}	3	3	14	51	7	20	15	16	23	15
	CC_α [b]/ng g^{-1}	18	7	27	29	16	62	14	28	27	24
	CC_β [c]/ng g^{-1}	30	13	45	50	27	105	24	48	45	41
Repeatability[d] and (reproducibility) as RSD/%	50 ng g^{-1}	5	8	15	–[e]	7	17	18	12	14	16
	100 ng g^{-1}	6 (9)	6 (12)	11 (8)	12 (14)	6 (7)	18 (16)	17 (16)	8 (9)	15 (13)	13 (13)
	200 ng g^{-1}	11	7	10	14	9	9	8	6	12	17
Recovery[f]/ng g^{-1}	50 ng g^{-1}	50 ± 9	50 ± 5	51 ± 9	–[e]	48 ± 8	51 ± 9	51 ± 10	51 ± 8	49 ± 8	43 ± 9
	100 ng g^{-1}	100 ± 16	97 ± 8	93 ± 13	94 ± 13	96 ± 14	104 ± 10	101 ± 14	97 ± 19	87 ± 14	97 ± 16
	200 ng g^{-1}	203 ± 19	204 ± 9	192 ± 32	209 ± 22	192 ± 20	172 ± 29	205 ± 17	197 ± 29	210 ± 20	214 ± 24

[a] LOD: Limits of detection calculated for a signal to noise ratio of 3.
[b] CC_α: Decision limits calculated according to 2002/657/EC decision.
[c] CC_β: Detection capability calculated according to 2002/657/EC decision.
[d] Repeatability calculated as intraday precisions (6 injections per level). Reproducibility: Calculated as interday precisions (2 different days, 6 injections per level each day).
[e] Concentration below LOD.
[f] Recoveries calculated as the average of 3 injections per concentration level.

Table 2
Recoveries[a] found in the analyses, of egg samples of different origins spiked at 100 μg kg^{-1} using the method developed.

Analyte	Common pheasant	Bantam	Common quail	Free-range hen
$BZZ-NH_2$	82 ± 15	91 ± 15	77 ± 15	79 ± 15
CBZ	84 ± 7	86 ± 7	85 ± 7	84 ± 7
$ABZ-NH_2-SO_2$	96 ± 13	99 ± 13	88 ± 13	90 ± 13
5-OH-TBZ	108 ± 22	112 ± 22	97 ± 22	104 ± 22
OXI	96 ± 13	102 ± 13	100 ± 13	91 ± 13
ABZ	99 ± 14	99 ± 14	92 ± 14	78 ± 14
FBZ	80 ± 14	96 ± 14	92 ± 14	95 ± 14
OFZ	81 ± 18	93 ± 18	98 ± 18	92 ± 18
$ABZ-SO_2$	74 ± 18	82 ± 25	86 ± 25	80 ± 25
$FBZ-SO_2$	84 ± 19	82 ± 25	88 ± 25	92 ± 25

[a] Recoveries calculated as the average of 4 injections.

3.4.1. Calibration curves, decision limits and detection capabilities

Two analytical limits are recommended in the 2002/657/EC decision: the decision limit (CC_α), which is defined as "the lowest concentration level of the analyte that can be detected in a sample with a chance of 1% of a false positive decision", and the detection capability (CC_β), which is "the smallest content of the analyte that can be detected in a sample with a chance of 5% of a false negative decision".

These parameters were determined for the ten benzimidazoles studied from the calibration curves obtained in the 50–400 μg kg^{-1} concentration range, whose characteristics are shown in Table 1. Values between 7 μg kg^{-1} and 62 μg kg^{-1} for CC_α, and between 13 μg kg^{-1} and 105 μg kg^{-1} for CC_β were obtained, calculated according to the criterion of Verdon et al. [29]. Since no legislation limits for these compounds in eggs have yet been proposed, and taking into account the daily ingestion of eggs, it has been considered that the methods should have LODs of around 50–100 μg kg^{-1}, which are the lowest limits proposed by the EU legislation as MRLs for foods of animal origin, with the exception of milk. The MRL for the albendazole family, calculated as the sum of concentrations of ABZ, $ABZ-NH_2-SO_2$, and $ABZ-SO_2$ and expressed as ABZ, was 100 μg kg^{-1}. Under our conditions, the limit of detection (LOD) for the albendazole family was 56 μg kg^{-1}. This value is lower than that established by the legislation as the MRL for this family.

In the case of the fenbendazole family (FBZ, OFZ and $FBZ-SO_2$), according to the legislation, the limits should be calculated as the sum of all three compounds. Under these conditions, the limit of detection (LOD) for this family was 46 μg kg^{-1}, which is again lower than that established by the legislation.

3.4.2. Repeatability and reproducibility

In order to determine the intra-laboratory repeatability and reproducibility of the proposed method, blank egg samples were spiked with all the analytes at three concentration levels: 50, 100 and 200 μg kg^{-1}. Repeatability was calculated as the precision on the same day (intraday), analysing six consecutive replicates of spiked egg samples, while reproducibility was checked as the precision on different days (interday), analysing the samples from two consecutive days – six replicates each day. The relative standard deviations (RSD) for the three concentrations studied are shown in Table 1. The values found were lower than 20%.

3.4.3. Recoveries

Validation of a method requires estimation of its accuracy by analysing certified reference materials (CRM). However, when these are not available, as in the present case, the legislation allows this parameter to be substituted by recovery, which is defined as the real concentration of a substance recovered along the whole analytical procedure.

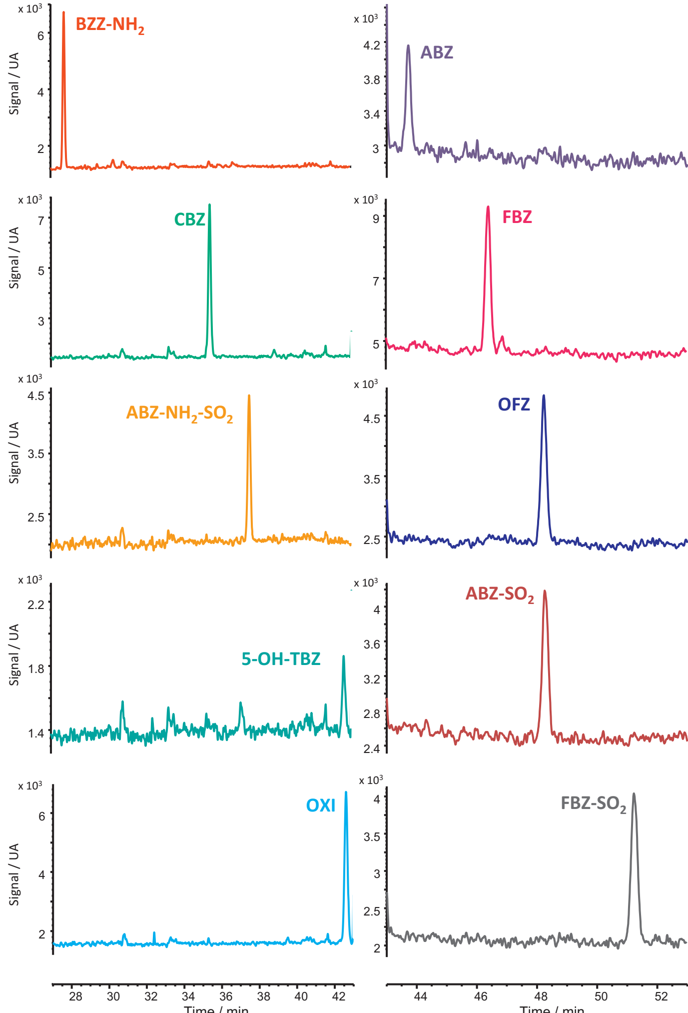

Fig. 5. Extracted ion electropherogram (XIE) for an egg sample from a free-range hen spiked at the 100 μg kg^{-1} level.

The recoveries of egg samples spiked at 50, 100 and 200 μg kg^{-1} were determined. The results obtained are shown in Table 1. It may be seen that for the three concentrations studied the recoveries were in the accepted range of the Codex Guidelines (CAC/GL 71-2009): 70–120%, with relative standard deviations of less than 20%.

3.5. Application to real samples

In order to validate the applicability and ruggedness of the method developed, four different egg samples that had not been used previously were analysed. The origin and nature of the egg samples used are described in Section 2.5. None of the samples contained benzimidazole compounds at concentrations above the LOD and hence for further analyses they were spiked at 100 μg kg^{-1}. The signal obtained for each of the analytes was introduced into the corresponding calibration line. Table 2 shows the recovery values (found/spiked) obtained and their precision. In all cases, satisfactory recoveries were obtained, which points to the ruggedness of the method and its applicability in the analysis of different egg samples.

Fig. 5 shows an extracted ion electropherogram for an egg sample from a free-range hen spiked at the 100 μg kg^{-1} level.

4. Conclusions

A simple and sensitive CE–MS method for the determination of 10 benzimidazoles and their metabolites in egg samples has been established and the *QuEChERS* approach has been applied for the first time to capillary electrophoresis in real samples. A variant of this method that includes a preconcentration step can be seen as the most sensitive and reliable to carry out such analyses.

Joint optimization in CE of both the composition of the background electrolyte and the solution for injection allowed the injection of organic solutions, making the method more compatible with the treatment necessary for egg samples.

The entire method, *QuEChERS* with preconcentration prior to CE–ESI-MS, was validated according to the 2002/657/EC decision, affording a reliable and rugged method for determining residues of these compounds in egg samples of different origins.

The results obtained here indicate that electrophoresis coupled to mass spectrometry (CE–MS) offers an alternative to other chromatographic methods of benzimidazole derivatives in egg samples, with the merits and demerits inherent to this separation technique.

Acknowledgements

The *Ministerio de Ciencia e Innovación*, Spain (Project CTQ 2011-24075) is gratefully acknowledged for financial support of this work. M.M.V. and D.G.G. gratefully acknowledge financial assistance from the Ministerio de Educación, Spain (FPU grant).

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DETERMINACIÓN DE NUCLEÓTIDOS EN FÓRMULAS INFANTILES DE INICIACIÓN TRATADAS PREVIAMENTE CON ULTRAFILTRACIÓN ASISTIDA POR CENTRIFUGACIÓN

I.2

ARTÍCULO DE INVESTIGACIÓN I.2

RESUMEN

I.2. Determinación de nucleótidos en fórmulas infantiles de iniciación tratadas previamente con ultrafiltración asistida por centrifugación

Los nucleótidos son los precursores de los ácidos nucleicos que componen el ADN y el ARN y resultan cruciales, por tanto, en el almacenaje, transferencia y expresión de la información genética. Además, participan en otras funciones metabólicas y desempeñan un papel importante como reguladores biológicos. El cuerpo humano es capaz de sintetizar de forma endógena estas moléculas, sin embargo también se pueden incorporar al organismo de forma exógena a través de la alimentación. Se pueden consideran nutrientes semi-esenciales ya que en ciertas condiciones la demanda de nucleótidos se incrementa, el suplemento endógeno se vuelve insuficiente y la ingesta de estos compuestos se convierte en esencial para el desarrollo normal de las funciones del organismo. Esto puede ocurrir en etapas de crecimiento rápido en bebés y niños, dónde los nucleótidos juegan un papel muy importante, entre otras cosas, en la mejora de la respuesta inmune.

Los nucleótidos están presentes de forma natural en la leche materna y, por este motivo, se añaden a las fórmulas infantiles sustitutivas de la lactancia materna según lo recomendado por diferentes organizaciones entre las que se encuentra el Comité Científico para la Alimentación de la Comunidad Europea. Los nucleótidos permitidos en fórmulas infantiles están limitados a los cinco nucleótidos monofosfato: adenosina 5'-monofosfato (AMP), citidina 5'-monofosfato (CMP), guanosina 5'-monofosfato (GMP), inosina 5'-monofosfato (IMP) y uridina 5'-monofosfato (UMP). Por ello, resulta de

especial interés la determinación de estos cinco derivados AMP, CMP, GMP, IMP y UMP en fórmulas infantiles comerciales.

Para la determinación de estos compuestos se ha propuesto la cromatografía líquida de par iónico, de intercambio aniónico y más recientemente de interacciones hidrofílicas, debido a que el uso de columnas convencionales de fase inversa se encuentra limitado por la elevada polaridad de los nucleótidos. Sin embargo, la electroforesis capilar (CE) se presenta como una técnica muy adecuada para la separación de compuestos polares e ionizados en un amplio intervalo de pH. Aunque la electroforesis capilar acoplada a espectrometría de masas (CE-MS) ha sido aplicada en otro tipo de matrices, hasta la fecha no ha sido propuesta para la determinación de nucleótidos en fórmulas infantiles.

En este trabajo se describe, por primera vez, un método simple y eficiente basado en CE-MS con ionización por electrospray (ESI) para la identificación y cuantificación de nucleótidos monofosfato en fórmulas infantiles. Para ello, en primer lugar se llevó a cabo la optimización de la separación en CE y de la detección con ESI-MS. Se estudiaron diferentes medios de separación volátiles con valores de pHs entre 9 y 11, zona en la que los analitos se encuentran cargados negativamente y donde el flujo electroosmótico es alto y estable, reduciendo de esta forma el tiempo de análisis y haciendo más robusto el acoplamiento con la fuente de ionización. Se obtuvieron los mejores resultados en cuanto a separación electroforética y tiempo total de análisis utilizando como tampón de separación una disolución acuosa de formiato amónico 30 mM a pH 9.6.

También se realizaron diversos estudios encaminados a optimizar la ionización de los analitos en la fuente del ESI, en modo positivo o negativo. En modo positivo sólo se observaron señales apreciables cuando se añadía medio ácido en el líquido adicional de la interfaz del electropray, si bien la relación señal/ruido siempre fue menos favorable que la obtenida en el modo negativo. Utilizando un líquido adicional compuesto por isopropanol-agua al 50 %, se encontró que el ion más abundante para todos los nucleótidos era siempre el $[M-H]^-$, que fue el elegido finalmente para llevar a cabo la cuantificación en el modo SIM. También se optimizaron el resto de parámetros relativos a la detección en ESI-MS, poniendo especial atención en la presión de nebulización. Se comprobó que cuando se utiliza un capilar de 100 μm de diámetro interno es necesaria la utilización de una presión de nebulización programada (PNP), para mantener la separación electroforética y conseguir una alta resolución y una señal estable de masas a lo largo del análisis. Esto es debido a que el flujo de líquido adicional alrededor del extremo del capilar en la interfaz del ESI puede crear succión, sobre todo en la etapa de inyección, lo que puede generar perfiles de flujo parabólico y menores eficiencias en la separación. Sin embargo, este efecto no es significativo cuando se utilizan capilares de 50 μm de diámetro interno, que fueron los seleccionados finalmente en el método desarrollado en este trabajo.

El método CE-ESI-MS así propuesto presenta valores de límites de detección (LODs) entre 15 y 26 μg L^{-1} y valores de reproducibilidad entre el 5 y el 7 % para un nivel de concentración de 100 μg mL^{-1}. De esta forma, se procedió a buscar un tratamiento de muestra simple evitando etapas de evaporación de disolvente, es decir, en el que la composición del medio en el que se encuentra la muestra fuese

totalmente compatible con la inyección en el sistema electroforético. Para este propósito se realizaron diversos estudios preliminares, basados en la precipitación de proteínas en medio ácido y en la preconcentración y limpieza de la muestra utilizando extracción en fase sólida (SPE) con sorbentes de distinta naturaleza. Sin embargo, los resultados no fueron satisfactorios con ninguno de los procedimientos estudiados.

Finalmente se seleccionó un tratamiento de muestra que incluye una etapa de ultrafiltración asistida por centrifugación (CUF) que permite obtener la muestra disuelta en agua, de forma que el procedimiento resulta totalmente compatible con el análisis en CE-MS. Se optimizó la cantidad de muestra y la cantidad de agua en la que se disuelve dicha muestra, encontrándose los mejores resultados cuando se disuelven 0.5 g de fórmula infantil en 15 mL de agua. Estudios adicionales demostraron que la reproducibilidad y la sensibilidad del método mejoraban notablemente cuando se realizaba una centrifugación previa a la etapa de ultrafiltración para eliminar la grasa, y cuando se incorpora un acondicionamiento inicial del dispositivo CUF con agua. En estas condiciones, se obtuvieron recuperaciones para la extracción comprendidos entre el 98 y el 122 %.

El método completo fue validado de acuerdo a lo establecido en la directiva europea 2002/657, utilizando para ello una muestra de leche infantil que sólo contenía uno de los nucleótidos estudiados. Se encontraron LODs entre 0.8 y 1.8 μg g^{-1}, valores de repetibilidad y reproducibilidad inferiores en todo caso al 20 %; y recuperaciones, determinadas a través de muestras dopadas a tres niveles de

concentración diferentes, comprendidas entre el 90 y el 106 % con valores de precisión inferiores al 15 %.

Para comprobar la aplicabilidad del método se analizaron cuatro fórmulas infantiles comerciales que contenían los cinco nucleótidos monofosfato, y se compararon los valores obtenidos con los contenidos etiquetados por el fabricante.

En conclusión, en este trabajo se describe por primera vez un método simple y altamente eficiente basado en CE-ESI-MS para la determinación de nucleótidos monofosfato en fórmulas infantiles. Se utiliza un tratamiento de muestra totalmente compatible con la separación electroforética que evitaba el uso de disolventes orgánicos para la extracción de los compuestos. El método completo fue validado y propuesto como un método fiable y robusto para la determinación de estos compuestos en diferentes muestras comerciales de leches infantiles, poniendo de manifiesto la aplicabilidad de la CE-MS como alternativa a los métodos cromatográficos de análisis.

ARTÍCULO DE INVESTIGACIÓN I.2

Electrophoresis, 35 (2014) 1677-1684

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Received November 8, 2013
Revised January 9, 2014
Accepted January 9, 2014

Research Article

A validated method for the determination of nucleotides in infant formulas by capillary electrophoresis coupled to mass spectrometry

In this work CE-ESI-MS is proposed for the identification and simultaneous quantification of several ribonucleotide 5′-monophosphates in infant formula (IF) samples. The target compounds were adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, uridine 5′-monophosphate, and inosine 5′-monophosphate. To our knowledge, the application of CE for the determination of these bioactive compounds in IFs has not yet been described. Optimization of the composition of the electrophoretic separation buffer and -mainly- the injection medium was carried out with a view to obtaining the best sensitivity and separation efficiency for the CE-MS coupling. Different sample treatments were assayed and one based on centrifugal ultrafiltration proved to be the simplest and most compatible with CE separation of the analytes and their ionization by the electrospray source. The whole optimized method (centrifugal ultrafiltration treatment prior to CE-MS) was validated according to the 2002/657/EC decision, obtaining a reliable and robust CE-MS method to determine these compounds in IF samples, with LODs between 0.8 and 1.8 μg/g (S/N = 3) and recoveries in the 90–106% range.

Keywords:
CE-ESI-MS / Centrifugal ultrafiltration / Infant formulas / Nucleotide 5′-monophosphates / Programmed nebulizer pressure

DOI 10.1002/elps.201300550

1 Introduction

Owing to their role in the storage and transmission of genetic information nucleic acids are some of the most important biomolecules. There are two types of nucleic acids, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA). Each is a polymer chain with similar units -nucleotides- connected by covalent bonds -phosphodiester links- which make up the backbone of the molecule.

Among others, ribonucleotides are bioactive components of human breast milk and may be significant in the improvement of human health. The influence of nucleotides in pediatric growth and nutrition and their composition in milk are productive areas of research [1]. Although nucleotides are not considered essential nutrients they may become essential during periods of rapid growth and may be responsible for an enhanced immune response in infants.

The majority of infant formulas (IFs) are bovine milk based. Human milk is the best source of nucleotides for young infants [2, 3]. Since nucleotides are present in higher amounts in human milk [2,4], pediatric formulas are increasingly supplemented with them. Supplementation of adapted-milk formulas with adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), uridine 5′-monophosphate (UMP), guanosine 5′-monophosphate (GMP), and inosine 5′-monophosphate (IMP), using concentrations within the ranges found in human milk, has been recommended by several organizations, among others, the Scientific Committee for Food of the European Community (SCF, 1993). This supplementation is done exclusively with 5′-mononucleotides because this is the main form for intestinal absorption.

It should be remarked that endogenous milk enzymes may degrade nucleotide monophosphate during the production of IFs, lowering the supplemented nucleotide concentrations and increasing that of nucleosides [1]. This can be attributed to a process of dephosphorylation of nucleotides by residual active alkaline phosphatase [5].

For the quantitation of nucleotides supplemented in IFs, the addition of acid followed by centrifugation of the

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Abbreviations: AMP, adenosine 5′-monophosphate; **CMP**, cytidine 5′-monophosphate; **CUF**, centrifugal ultrafiltration; **GMP**, guanosine 5′-monophosphate; **IF**, infant formula; **IMP**, inosine 5′-monophosphate; **MWCO**, molecular weight cut-off; **PNP**, programmed nebulizer pressure; **UHQ**, ultra high quality; **UMP**, uridine 5′-monophosphate

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precipitated proteins is a common approach. The acids employed are mainly perchloric [6, 7] and trichloroacetic [8, 9] although acetic [5] and formic [10] acids have also been used. Owing to the uncertain stability of nucleotides at low pH, acid neutralization is advocated after the centrifugation step for storage prior to analysis. Regarding other sample treatments, Gill et al. [11] used EDTA and carried out a SPE step based on anion exchange. Also, centrifugal ultrafiltration [12, 13] (CUF) has been applied directly in water-reconstituted IFs.

All the above techniques analyse the extracts by HPLC. RPLC is somewhat limited with conventional C_{18} columns because of the inherently poor interaction of the highly polar analytes with the nonpolar stationary phase, resulting in poor retention and resolution. In this sense, some efforts have been made to retain these polar compounds in polar modified reverse phase stationary phases such as polar embedded or polar encapped columns. Currently, ion-pair and ion-exchange LC have become the prevalent techniques for the analysis of nucleotides in milk and pediatric products. At the appropriate pH the ionic nature of the phosphate ester facilitates strong interactions with cationic ion-pair reagents [3, 8] and with anion-exchange stationary phases [9, 13], thereby enhancing nucleotide retention and resolution. Moreover, HILIC has recently been proposed for the quantitation of nucleotide monophosphates in infant and adult-nutritional formulas [12, 14]. The detectors coupled to HPLC are mainly diode arrays (DAD), for UV absorption [5, 7, 9, 13, 15], and MS [8, 10, 12].

CE offers another approach for the separation of highly polar compounds. This technique has the advantage of separating charged analytes that are often not well separated by conventional RP LC. Since ribonucleotides are charged in a broad range of pH, CE is a very adequate, efficient and versatile technique for the analysis of this type of compound. In recent years, several review articles have been published regarding CE and CE-MS for food analysis [16–18]. Specifically, CE has been used to separate mixtures of nucleotide standards [19–21] and to determine them in IG (IMP and GMP)-enriched monosodium glutamates [22]. Also, Cubero et al. [23] proposed a CE-UV method for the determination of nucleotides in human breast milk but the electropherograms obtained revealed a high risk of interferences.

The goal of this work was to develop and validate a CE-MS method that uses a fairly simple sample treatment technique that allows the quantification of ribonucleotide 5′-monophosphates in IFs. As far as we know, CE has not yet been applied to the quantification of nucleotides in pediatric formulas.

2 Materials and methods

2.1 Chemicals and reagents

Analytical standards of AMP disodium salt, CAS RN [457 8-31-8]; CMP, CAS RN [63-37-6]; GMP disodium salt hydrate, CAS RN [5550-12-9]; UMP disodium salt, CAS RN [3387-36-8], and IMP disodium salt, CAS RN [352195-40-5] were purchased from Sigma-Aldrich (Steinheim, Germany).

Isopropanol (Scharlau, Barcelona, Spain) was of HPLC grade and was used as received. Ammonium formate and 25% ammonia were purchased from Riedel de Häen (Hannover, Germany) and Scharlau, respectively. Ultra high quality (UHQ) water was obtained with a Wasserlab UHQ water purification system (Noáin, Spain). All other chemicals used were of analytical reagent grade.

CUF devices (CUF; Spin-X UF 20, 5k molecular weight cut-off, polyethersulfone, nonsterile, 20 mL) were purchased from Corning (NY, USA) and Chromabond SB polypropylene strong-anion exchange SPE cartridges, 1000 mg, were purchased from Scharlau.

2.2 Apparatus and software

All experiments were carried out with a Hewlett-Packard HP3D CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a UV-visible DAD device working at 214 nm with a bandwidth of 16 nm. MS was performed using an Agilent LC/MSD SL mass spectrometer (Agilent Technologies) equipped with a single quadrupole analyzer. Both the UV-visible DAD detector and the MS device were controlled by Agilent HP ChemStation software, version B.04.01.

Fused-silica capillaries (50 and 100 μm id) with total lengths of 87.5 and 20 cm to the UV detector were purchased from CM Scientific (Shipley, West Yorkshire, United Kingdom). CE separation buffers and injection samples were placed in 1700-μL disposable amber glass vials with polyurethane pre-perforated caps (Agilent Technologies).

A centrifuge, model Kokusan H-103N series from Eurocommercial S.A. (Madrid, Spain) was used for CUF. For pH measurements, a pH-meter model 2001 (Crison, Barcelona, Spain) was employed. The rest of the instrumentation and materials was that routinely used in analytical laboratories: analytical scales and calibrated volumetric glassware.

2.3 CE separation conditions

Standard solutions were injected using a pressure of 50 mbar over 30 s. In all cases, the injection medium was UHQ water. Electrophoretic separation was achieved with a voltage of 30 kV (positive CE mode), with an initial ramp of 7 s. The BGE was a 30 mM ammonium formate-ammonia medium (pH 9.6). The temperature of the capillary was kept constant at 25°C. Before the first use, the capillary was conditioned by flushing with the BGE for 5 min at 4 bar. This was also implemented as a daily startup procedure. After each run, the capillary was prewashed at 4 bar for 2 min with fresh running buffer.

2.4 ESI-MS conditions

MS was performed using an Agilent MSD SL mass spectrometer equipped with a single quadrupole analyzer. An

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Agilent coaxial sheath-liquid sprayer was used for CE-ESI-MS coupling. The sheath liquid, consisting of a 50:50, v/v isopropanol:UHQ water mixture, was delivered at a flow rate of 1.00 mL/min by an Agilent 1100 series pump equipped with a 1:100 flow-splitter. The ESI voltage was set at −4000 V. Other electrospray parameters under optimum conditions were: drying-gas flow rate, 7.0 L/min; drying-gas temperature, 350°C and nebulizing-gas pressure, 10 psi. The mass spectrometer was operated in the negative-ion mode (ESI−). The optimized fragmentor voltage was 100 V for all analytes. For quantification, SIM mode was selected. The *m/z* corresponding to the $[M-H]^-$ ions were 346 for AMP, 322 for CMP, 362 for GMP, 323 for UMP, and 347 for IMP with an isolation width of 1 u and a dwell time of 47 ms.

2.5 Sample treatment

Five different brands of powdered IF acquired on the market were used: an unsupplemented cow's milk based IF (IF1), two ribonucleotide 5′-monophosphate-supplemented starting IFs (IF2-IF3) and two ribonucleotide 5′-monophosphate-supplemented follow-on IFs (IF4-IF5). Their composition, per 100 g, varied between 2003 and 2140 kJ of energy value; 9.9 and 12.5 g protein; 54.1 and 63.4 g carbohydrates, and 20 and 27 g fat. All these were analyzed, and it was found that they all contained the five nucleotides studied with the exception of IF1, which only had CMP at a concentration value close to the quantification limit. Accordingly, this was used as a matrix for the development and later validation of the proposed method. IF2-IF5 were used to check the applicability and robustness of the method.

To implement the proposed method, 0.5 g of infant milk was placed in a centrifuge tube (50 mL) and ultrapure water was added up to 15 g. The mixture was shaken manually and stored at 5°C for 15 min. After that, it was centrifuged for 15 min (2800 × *g*). A 10-mL aliquot of the supernatant was collected, attempting to avoid collecting the sheet of fat present at the top and this was passed through the CUF device (30 min, 2800 × *g*), previously conditioned with 5 mL of ultrapure water (15 min, 2800 × *g*), to accomplish ultrafiltration. The analytes studied were present in the filtrate, which was injected directly into the CE-MS system.

Fortification of the IF1 samples was performed directly on the IF, and a period of about 12 h was allowed to elapse before continuing with any of the extraction processes in order to favor the interaction between the analytes and the matrix compounds.

2.6 Quantitative parameters

Stock solutions of ribonucleotide 5′-monophosphates at 500 mg/L were prepared by dissolving the compounds in acidified methanol (10 mM $HClO_4$). All solutions were kept at −18°C in amber glass bottles. Working solutions containing the analytes at variable concentrations were prepared daily by mixing the appropriate amounts of the stock solution/s and diluting in UHQ water.

Analyte quantification was carried out in the SIM acquisition mode, using deprotonated molecules: $[M\text{-}H]^-$. The quantitative variable was the peak area generated by the respective ion expressed in arbitrary peak area units. Quantification of the IFs (IF2-IF5) was accomplished with the standard additions method: the milk samples were fortified after the CUF treatment by the addition of an adequate volume (20–60 μL) of standard solutions of the mononucleotides to 200-μL aliquots of the single CUF extract. The six levels at which the samples were fortified ranged from 0.1 to 2 μg/g. The unspiked and spiked samples were analyzed by CE-MS, obtaining three replicas per sample.

2.7 Method validation

The method developed was validated according to European Legislation, evaluating the following parameters: calibration curves and analytical limits; repeatability and within laboratory reproducibility; accuracy and/or recovery. In addition, specificity, by comparing the migration times and the signals of standard electropherograms against spiked IF samples (1 identification point per analyte), and stability, by controlling the storage conditions of the analytes and samples maintaining the conservation parameters within the values recommended by the supplier, were also evaluated.

3 Results and discussion

To this end, three steps were carried out sequentially: (i) optimization of the CE separation of the analytes and their quantification by CE-ESI-MS; (ii) checking for the compatibility of several sample treatments with CE separation and ESI ionization; and (iii) validation of the whole method proposed.

3.1 Optimization of electrophoretic separation and mass spectrometric detection

3.1.1 Preliminary MS studies and optimization of CE separation

The possibilities of CE-MS analysis may be very diverse owing to the different ionic forms of the nucleotide compounds versus pH (Fig. 1) [24].

Regarding MS analysis, it is possible to carry out analyte ionization at the ESI source in positive or negative mode. Different studies were conducted, introducing individual aqueous solutions of each analyte under pressure (50 mbar) at the ESI source and recording the corresponding MS spectra. In positive ESI-MS mode, no appreciable signal was observed in the MS spectra except when acid medium (formic acid 10 mM) was added to the sheath liquid. However, this signal had a less favorable S/N than in negative mode. It was

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Figure 1. Ribonucleotide 5′-monophosphates and its pK_a values.

observed that for all the analytes assayed the most abundant ion was always the monoanion [M-H]⁻, the signal corresponding to the dianionic form proving to be lower than 10% and the trianionic form being completely undetectable. Thus, the monitoring of the nucleotides for quantification was carried out in single ion monitoring (SIM) mode, recording the [M-H]⁻ signal of each analyte.

Regarding CE separation, although it would be possible to separate the nucleotides as cations or anions, from a practical point of view it is best to perform separation in CE with a high and stable EOF in order to reduce analysis times and make the coupling to the ESI source more robust. Thus, separation was confined to the high-pH zone, in which all the analytes were present in different anionic forms.

CE separation studies were conducted with different volatile BGEs, obtaining acceptable separations for a standard aqueous mixture upon employing 30 mM ammonium formate in the 9–11 pH range (adjusted with concentrated ammonia solution). Figure 2 shows the variation in the migration times when the pH was varied between 9 and 10.

A differential behavior was observed in migration for the nucleotides UMP, GMP, and IMP. The retention times of the nucleotides UMP, GMP, and IMP increased with the rise in pH. This behavior could be due to the fact that for these nucleotides in the working pH range the net negative charge of the analytes increases, passing from having one negative charge to having two negative charges in the case of UMP. By contrast, for the nucleotides GMP and IMP, the predominance of the form with two negative charges changes to a form with three negative charges. This acid-base behavior leads their electrophoretic mobilities to increase in the opposite sense to the EOF and their migration times increase. In the pH range studied here, the predominant species for AMP and CMP have two negative charges and therefore they do not undergo any appreciable variation in their electrophoretic mobilities. From a practical point of view, a pH of 9.6 is sufficient to achieve complete separation among the five compounds with a shorter total analysis time than at higher pH.

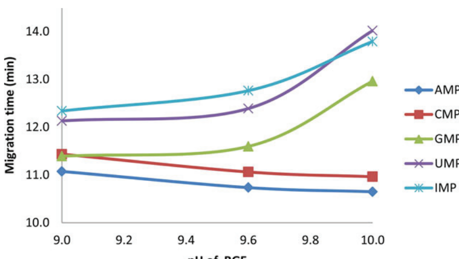

Figure 2. Influence of the pH of the separation medium (30 mM ammonium formate) on the migration times for the electrophoretic separation of the five analytes studied.

3.1.2 ESI-MS optimization

For the optimization of ESI-MS detection, the ESI parameters of capillary voltage, drying-gas flow, and drying-gas temperature were set to the values recommended by the supplier to achieve a stable electrospray for a sheath liquid flow of 10 μL/min (1 mL/min at 1:100 split). Two different sheath liquids were tested (50:50, v/v isopropanol:UHQ water and 50:50, v/v methanol:UHQ water) the best sensitivity being seen with the first one. The curves obtained upon varying the cone voltage of the MS between 60 and 120 V did not provide different sensitivities and a value of 100 V was selected.

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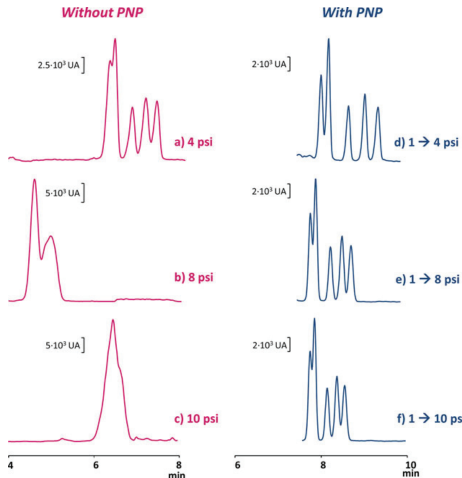

Figure 3. Total ion electropherogram (TIE) of a standard aqueous solution containing the analytes at a concentration level of 5 mg/L. Capillary: 100 μm (id). Separation medium: 7.5 mM ammonium formate (pH 9.6). Seven seconds hydrodynamic injection at 50 mbar; (a–c) electrophoretic separation for different electrospray pressures without PNP; (d–f) electrophoretic separation for different electrospray pressures with PNP.

Special attention was also paid to the nebulizing pressure of the ESI since it has been reported [25] that a programmed nebulizer pressure (PNP) sometimes becomes necessary to maintain the separation achieved in CE, using a UV detector, and for separation to remain stable. Figure 3 shows the results obtained for a 100-μm ID CE capillary with/without the PNP approach. It can be observed that maintaining a PNP was necessary in CE separation for higher final nebulizing pressures. This effect was mainly due to the overlapping hydrodynamic flow caused by holding the ESI nebulizing pressure along the CE separation. For a 50-μm ID CE capillary this was much less pronounced and the separation of the analytes was not affected, the only effect being a slight reduction in their migration times. This 50-μm ID CE capillary was finally selected and used in the validation of the method since the use of 100-μm ID CE capillary presented just a slightly higher sensitivity. Finally, the optimized values for the CE-ESI-MS parameters proved to be as indicated in the experimental Section "ESI-MS conditions."

3.1.3 Characteristics of the method

The whole optimized CE-ESI-MS method was initially applied to standard aqueous solutions of the analytes, obtaining linear calibration curves for all compounds across the range tested (100–5000 μg/L). Calibration curves were prepared at seven levels and each calibration level was replicated four times. The LODs (S/N of 3) ranged from 15 μg/L for CMP to 26 μg/L for UMP. The RSD values (intraday, $n = 6$) for the analytical signal ranged from 5.0% for CMP to 7.0% for UMP at a concentration level of 100 μg/L.

3.2 Treatment of IF samples prior to CE-ESI-MS

3.2.1 Preliminary studies. Influence of the composition of the injection medium

The composition of the injection medium is a parameter that must be optimized in CE in order to obtain stable analyses of real complex matrices and to achieve the best sensitivity and separation efficiency. With the aim of proposing a simple procedure that would not include solvent evaporation steps and in which the composition of the sample would be compatible with CE-MS, different preliminary studies were performed. As mentioned in the Introduction, most sample treatments of IFs were based on the addition of nonvolatile perchloric or other volatile (trichloroacetic, formic, acetic) acids to the milk before phase separation. In initial assays, these acids were tested as protein precipitants prior to centrifugation and, for the samples studied here, the only one that produced an effective and clear separation of phases after centrifugation was perchloric acid at 0.15 M. However, the extract obtained did not afford a good electrophoretic separation when injected into the CE-MS system.

In addition, the compatibility of the sample treatment based on strong-anion exchange SPE [11] with CE was checked. This treatment was accomplished employing a standard solution of nucleotides and distorted signals for the analytes were found in the electropherogram. In this treatment, phosphate buffer (pH 3) was employed to elute the retained nucleotides. Hence, a subsequent study was conducted, injecting a series of standard aqueous mixtures containing phosphate at different concentrations (Fig. 4). Again, similar poorly resolved electropherograms were obtained as compared with those obtained after SPE. These results indicate that a minimal amount of this salt (phosphate) in the injection medium strongly affects CE separation. The results obtained indicate that with the most commonly used treatments for samples of milk and their later determination by HPLC a change of medium must be implemented before being used in CE-MS.

3.2.2 Optimization of the ultrafiltration sample treatment

In light of the results reported above, two treatments employing CUF were assayed. These treatments were simple and did not add any precipitant to the milk [12, 13]. The second one incorporated an additional centrifugation of the milk prior to the ultrafiltration step. Both were tested and the best results were obtained with this second treatment.

The treatment selected [13] was subsequently optimized. First, the amount of powdered milk sample was studied (5.0, 1.0, and 0.5 g). It was observed that on decreasing the

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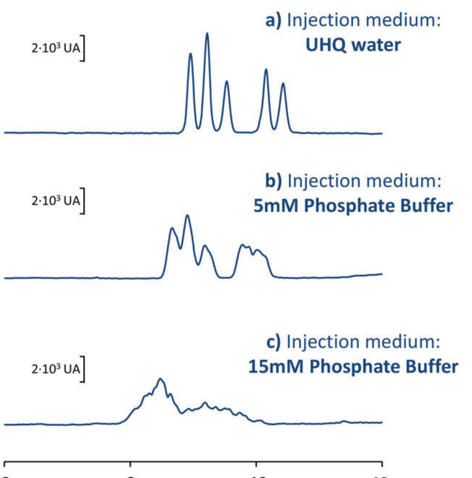

Figure 4. Total ion electropherograms (TIE) for standard solutions containing the analytes at a concentration level of 5 mg/L. Capillary: 50 μm (id). Separation medium: 30 mM ammonium formate (pH 9.6). Thirty seconds hydrodynamic injection at 50 mbar; (a), aqueous standard solution and (b) and (c) aqueous standard solutions containing 5 mM and 15 mM phosphate buffer at pH 3, respectively.

amount of sample the electropherograms were much cleaner and greater separation efficiency was achieved. The recoveries were similar for all the analytes and hence 0.5 g in 15 mL of UHQ water was chosen as the optimum amount of sample.

Working under these conditions on some occasions delays in the migration times of the analytes and slight losses of the sensitivity of the electrophoretic signals were observed. In order to obtain reproducible signals in the migration times and in sensitivity, different studies were conducted, observing that the increase in the intensity of the centrifugal force (from 1680 to 2800 × *g*) in the first centrifugation of the method and the incorporation of a previous conditioning of the CUF device with 5 mL of ultrapure water (15 min at 2800 × *g*) afforded precise electrophoretic signals and more reproducible quantifications with CE-ESI-MS.

The extraction recovery of the proposed CUF treatment was calculated as expressed in Eq. (1) [26] employing IF1 samples:

$$\text{Extraction Recovery (ER)} = \frac{a}{b} \quad (1)$$

where "*a*" refers to samples spiked before the ultrafiltration treatment had been applied to IF and "*b*" stands for the analytical signal provided by samples spiked after the ultrafiltration treatment had been applied to IF. The signal of CMP, the only analyte present in the sample, was corrected by subtracting the signal found in the unspiked sample. Three replicates were collected, obtaining extraction recovery values between 98% for GMP and 122% for IMP. Thus, the proposed sample treatment based on ultrafiltration can be seen as an efficient procedure for nucleotide extraction.

Table 1. Validation parameters for the proposed CUF prior to CE-ESI-MS method

Validation[a)]	AMP	CMP	GMP	UMP	IMP
Calibration parameters					
Intercept (10^2 UA)	−4 ± 4	2 ± 7	−2 ± 1	10 ± 10	0.2 ± 3.0
Slope (10^2 UA g/μg)	2.63 ± 0.07	3.6 ± 0.1	1.89 ± 0.03	2.3 ± 0.1	1.83 ± 0.07
LOD[b)] (μg/g)	1.7	0.8	1.8	1.4	1.4
CC_α[c)] (μg/g)	1.6	2.1	0.8	3.6	1.7
CC_β[d)] (μg/g)	2.8	3.5	1.3	6.1	3.0
Repeatability[e)] and (reproducibility) as RSD (%)					
6 μg/g	9 (16)	11 (12)	10 (11)	4 (16)[f)]	12 (16)
30 μg/g	7 (15)	6 (12)	5 (18)	11 (12)	6 (18)
90 μg/g	6 (12)	7 (13)	5 (17)	8 (12)	8 (12)
Recovery[g)] (%)					
6 μg/g	105 ± 15	95 ± 10	100 ± 15	100 ± 2[f)]	105 ± 15
30 μg/g	102 ± 3	90 ± 10	100 ± 3	90 ± 10	93 ± 3
90 μg/g	106 ± 1	103 ± 3	106 ± 1	103 ± 3	102 ± 2

a) According to the 657/2002/EC decision.
b) LOD: Limits of detection calculated for a S/N of 3.
c) CC_α: Decision limits calculated according to 2002/657/EC decision.
d) CC_β: Detection capability calculated according to 2002/657/EC decision.
e) Repeatability calculated as intraday precisions (6 injections per level). Reproducibility: calculated as interday precisions (3 different days, six injections per level each day).
f) Calculated at 15 μg/g.
g) Recoveries calculated as the average of three injections per concentration level.

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Table 2. Comparison between the values found in the analysis of ribonucleotides in different commercial infant formulas (IF2–IF5) and the values indicated by the manufacturer

	IF 2			IF 3			IF 4			IF 5		
	Found[a) (μg/g)	Labeled[b) (μg/g)	Ratio[c) (%)	Found[a) (μg/g)	Labeled[b) (μg/g)	Ratio[c) (%)	Found[a) (μg/g)	Labeled[b) (μg/g)	Ratio[c) (%)	Found[a) (μg/g)	Labeled[b) (μg/g)	Ratio[c) (%)
AMP	35 ± 2	37	95 ± 5	43 ± 2	34	127 ± 7	21 ± 2	33	64 ± 4	21 ± 6	28	74 ± 21
CMP	105 ± 3	120	88 ± 2	111 ± 3	116	96 ± 3	102 ± 21	110	93 ± 19	81 ± 9	77	106 ± 12
GMP	13 ± 1	23	57 ± 3	19 ± 2	19	98 ± 9	17 ± 2	20	82 ± 9	11 ± 1	18	63 ± 5
UMP	43 ± 1	51	84 ± 2	57 ± 3	48	119 ± 6	39 ± 3	47	83 ± 6	24 ± 2	62	40 ± 4
IMP	7 ± 1	17	39 ± 7	17 ± 2	15	112 ± 12	15 ± 1	15	98 ± 10	17 ± 1	18	97 ± 8

a) Concentration, in μg/g, in powder infant formula calculated using the standard addition method.
b) Concentration value, in μg/g, labeled by the manufacturer.
c) Ratio in percentage between found and labeled concentrations.

3.4 Validation of the proposed method

To validate the proposed method (CUF prior to CE-ESI-MS) the IF1 was used. Validation was accomplished following European Directive 2002/657/EC, which sets the validation criteria for analytical methods in foods of animal origin.

The matrix-matched calibration was performed in a concentration range from 6 to 90 μg/g, this being linear within the range studied and for all the nucleotides (Table 1). The LODs were calculated as the concentration for which a S/N of 3 would be obtained. Values between 0.8 and 1.8 μg/g were found. Moreover, the legislation recommends the study of two different analytical limits; the limit of decision (CC_{α}), which is defined as "the minimum concentration of analyte that can be detected in a sample with a 1% probability of obtaining a false-positive", and the detection capacity (CC_{β}), which is "the minimum concentration of analyte that can be detected with a 5% probability of obtaining a false-negative". Values ranging between 0.8 and 3.6 μg/g for CC_{α}, and between 1.3 and 6.1 μg/g for CC_{β} were obtained.

To calculate the repeatability and within-laboratory reproducibility of the method, samples of IF1 spiked at three concentration levels (6, 30, and 90 μg/g) were used. Repeatability was calculated as the precision obtained in analyses performed on the same day (intraday) of 6 consecutive replicas of the samples of spiked IF. By contrast, within-laboratory reproducibility was calculated as the precision obtained in the analyses of samples performed on three different days (interday), analyzing six replicas on each day. In all cases values lower than 20% were observed, as reflected in Table 1.

To estimate the accuracy of the method, the recovery was determined after analyzing samples spiked according to the described procedure. For this, IF samples spiked at three different concentrations, 6, 30, and 90 μg/g, were used. The signal obtained in each case was introduced into the calibration obtained previously (Table 1), thus obtaining the corresponding concentration value with its interval of confidence. As may also be seen in Table 1, according to the Codex Guidelines (CAC/GL 71-2009) satisfactory results were obtained: recoveries between 90 and 106%, with SDs lower than 15%.

3.5 Application to real samples

In order to check the applicability of the method developed, four different IF samples containing the mononucleotides and that had not been used previously were analyzed. The nature of the IF samples used is described in Section 2.5. The labeled content of ribonucleotide 5′-monophosphates of these samples is shown in Table 2.

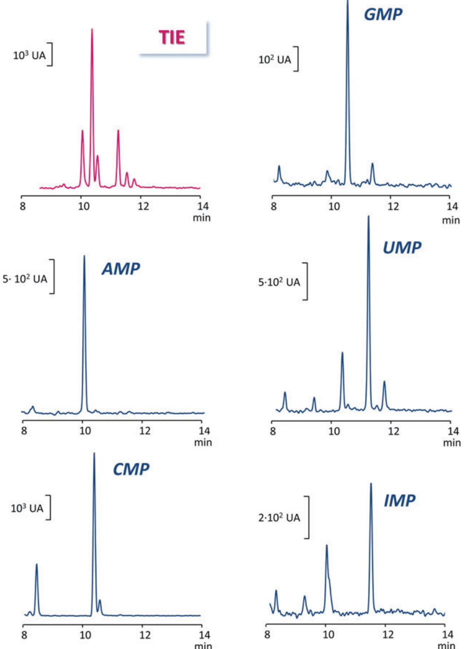

Figure 5. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) of a sample of powdered infant formula (IF 3) analyzed with the proposed method.

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Only three content values proved to be abnormally low. Bearing in mind that quantification was made by the standard additions method, these results cannot be attributed to ion suppression. Therefore, they may be attributed to the possible enzymatic degradation of nucleotides added during the manufacturing process [5] or during the storage. Thorell et al. [3] have reported the partial transformation of CMP and UMP to cytidine and uridine and GMP and AMP to guanine and uric acid in human milk samples. In other cases, satisfactory contents were obtained, which points to the applicability of the method in the analysis of nucleotides in quite different IF samples.

Figure 5 shows the total ion electropherogram and the extracted ion electropherograms for an IF sample (IF 3) analyzed with the proposed method. It can be seen the good resolution and sensitivity obtained with the coupled mass analyzer achieve for the five analytes. These results show that the method developed is a satisfactory approach that could be used as a control method for evaluating the nucleotide content in infant products.

4 Concluding remarks

A simple and highly efficient CE-ESI-MS method for the determination of five ribonucleotide monophosphates in pediatric formulas has been established for the first time. The sample treatment, based on CUF, is fairly simple, compatible with electrophoretic separation, and does not require the use of organic extractants.

The entire method was validated according to the 2002/657/EC decision, affording a reliable and rugged method for the determination of these compounds in IF samples of different commercial brands. The results obtained here indicate that CE coupled to MS offers an alternative to other chromatographic methods for the analysis of nucleotides and related compounds in samples of this kind.

The Ministerio de Ciencia e Innovación, Spain (Project CTQ 2011-24075) is gratefully acknowledged for financial support of this work. M.M.V. and D.G.G. gratefully acknowledge financial assistance from the Ministerio de Educación, Spain (FPU grants).

The authors have declared no conflict of interest.

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ANÁLISIS DE NUCLEÓTIDOS LIBRES EN LECHE MATERNA Y EFECTO DE LA PASTEURIZACIÓN Y DEL PROCESADO CON ALTAS PRESIONES EN EL CONTENIDO DE LOS MISMOS

I.3

ARTÍCULO DE INVESTIGACIÓN I.3

RESUMEN

I.3. Análisis de nucleótidos libres en leche materna y efecto de la pasteurización y del procesado con altas presiones en el contenido de los mismos

Los nucleótidos son nutrientes semi-esenciales que están presentes, de forma natural, en la leche de los mamíferos. Debido a su influencia en la regulación de la respuesta inmune, estos compuestos son especialmente importantes en etapas de crecimiento en bebés; siendo la leche materna la mejor fuente de nucleótidos en la alimentación infantil. Los nucleótidos libres que se encuentran en mayor concentración en la leche materna son los nucleótidos monofosfato: adenosina 5'-monofosfato (AMP), citidina 5'-monofosfato (CMP), guanosina 5'-monofosfato (GMP), inosina 5'-monofosfato (IMP) y uridina 5'-monofosfato (UMP).

Sin embargo, además de encontrarse como nucleótidos libres también pueden estar presentes en otras formas disponibles nutricionalmente: ácidos ribonucleicos poliméricos, nucleósidos y aductos que contienen nucleótidos. De acuerdo con esto, el contenido total de nucleótidos se expresa por las siglas inglesas TPAN (*Total Potentially Available Nucleotide*). Así, para su determinación en muestras de leche es habitual seguir un protocolo que incluye diferentes digestiones enzimáticas con el fin de transformar los nucleótidos, en sus diferentes formas, en nucleósidos. De esta forma es posible determinar los nucleósidos libres y los liberados enzimáticamente mediante cromatografía líquida. Se ha descrito que más del 80 % del contenido total de nucleótidos está presente como nucleótidos poliméricos (43.3 %) y libres (39.9 %). El resto se encuentran como nucleósidos y aductos de nucleótidos.

Según la revisión bibliográfica realizada previamente, la técnica de análisis aplicada más frecuentemente a la determinación de los nucleótidos en muestras de leche es la cromatografía líquida en diferentes modalidades. La utilización de electroforesis capilar (CE) sigue siendo muy escasa, y sólo ha sido propuesta anteriormente para la determinación de nucleótidos en leche materna utilizando detección espectrofotométrica, pero no acoplada a espectrometría de masas (CE-MS).

En este trabajo se utilizó el método CE-ESI-MS desarrollado previamente por nuestro grupo de investigación para el análisis de fórmulas infantiles, pero adaptándolo a su aplicación en leches maternas. Es necesario tener en cuenta que la determinación cuantitativa de los nucleótidos monofosfato en leche materna es más complicada debido a la presencia endógena de los analitos.

Como objetivo de este trabajo se planteó su aplicación para la evaluación del contenido de nucleótidos libres en muestras de leche materna procedentes de un banco de leche humana y que han sido sometidas a diferentes métodos de preservación, como la pasteurización a baja temperatura o el procesado con alta presión. El objetivo final es conocer el efecto de ambos procesos sobre el contenido total de estos compuestos.

La pasteurización a baja temperatura, también conocida como pasteurización Holder (HoP) es el tratamiento más utilizado en los bancos de leche materna para inactivar los microorganismos patógenos y asegurar la calidad de la leche que allí se deposita. Es un proceso de fácil aplicación (calentamiento a 62.5 °C en un baño de agua durante 30 minutos) pero puede ocasionar una pérdida variable de ciertos

componentes. En los últimos años, el procesado con alta presión (HPP) ha adquirido relevancia como método de preservación de alimentos. HPP es una tecnología con un tratamiento mínimo y puede considerarse como un método de pasteurización fría que no altera las características nutricionales y sensoriales de los alimentos.

La metodología seguida en las etapas de separación-detección mediante CE-ESI-MS es la misma descrita anteriormente para fórmulas infantiles, pero fue necesaria la adaptación del tratamiento de la muestra al análisis de leche materna. Se estudió la cantidad de muestra necesaria y su posterior dilución con el objetivo de minimizar la supresión iónica y obtener una buena sensibilidad. Se consiguieron los mejores resultados diluyendo 1 mL de leche materna con 4 mL de agua, realizando una centrifugación inicial para separar la mayor cantidad de grasa posible y llevando a cabo finalmente la etapa de ultrafiltración, que proporcionó extractos muy limpios. En estas condiciones, el efecto de matriz es despreciable, por lo que la cuantificación de los nucleótidos en leche materna con la metodología propuesta se puede realizar por calibración con estándares preparados en agua.

Para la validación del método propuesto se utilizó una leche materna procedente del banco de leche que no había sido sometida previamente a ningún tratamiento de preservación. Se encontró que el nucleótido presente en mayor concentración en esta muestra fue el CMP, seguido de UMP y AMP. Las concentraciones de los otros compuestos, GMP e IMP, fueron superiores a los límites de detección pero inferiores a los límites de cuantificación. Se obtuvieron valores de repetibilidad y reproducibilidad inferiores al 10 %, y recuperaciones, determinadas al analizar la muestra dopada, entre el 87 y el 112 %. La

exactitud del método fue evaluada analizando el contenido de nucleótidos en la muestra sin dopar, mediante adición estándar y por calibración con patrón externo. Las concentraciones obtenidas por ambos métodos, utilizando la adición estándar como método de control, no mostraron diferencias significativas.

Una vez validado, el método propuesto fue aplicado a la determinación de nucleótidos en una muestra de leche procedente de una donante voluntaria en su primer mes de lactancia y a muestras de leche materna, procedentes del banco de leche, sin tratar y tratadas por diferentes métodos, pasteurización (HoP) o alta presión (HPP). En la muestra de leche de un mes de lactancia fue posible cuantificar AMP, CMP, UMP y GMP, y detectar la presencia de IMP; lo cual es consistente con los resultados obtenidos por otros autores, que indican que IMP sólo se detecta en muestras de calostro a muy bajos niveles. También se evaluó el efecto del tiempo transcurrido desde la recolección de las muestras hasta su análisis, por lo que las muestras fueron congeladas y analizadas de nuevo tres meses después. Se comprobó que los contenidos de todos los nucleótidos aumentaron significativamente después del periodo de congelación, lo que puede atribuirse a la transformación enzimática de los nucleótidos poliméricos o de los aductos de nucleótidos en nucleótidos libres.

Finalmente, se analizaron muestras de leche materna, procedentes del banco de leche, sin tratar y tratadas por diferentes métodos, pasteurización (HoP) o alta presión (HPP) con el objetivo de evaluar el efecto de estos tratamientos sobre el contenido de nucleótidos libres. En todas las muestras el CMP fue el nucleótido encontrado en mayor concentración, seguido de UMP y AMP; IMP y GMP

no pudieron ser cuantificados. Si se compara el contenido de nucleótidos de la leche sin tratar del banco de leche con el de la muestra de un mes de lactancia, se comprueba que los niveles son inferiores en la muestra del banco. Esto se debe al hecho de que el contenido de nucleótidos disminuye a medida que progresa la etapa de lactancia. El contenido de nucleótidos en las muestras tratadas con HPP fue significativamente igual al encontrado en la muestra sin tratar, mientras que las muestras tratadas con HoP mostraron un incremento en el contenido de AMP, CMP y UMP. Estos resultados pueden ser debidos a que el incremento de temperatura durante la etapa de pasteurización puede generar nucleótidos libres a partir de los poliméricos y de los aductos.

Las muestras procedentes del banco también fueron analizadas después de un periodo de congelación de seis meses. El comportamiento de la muestra sin tratar fue similar al de la muestra de un mes de lactancia, observándose un incremento en el contenido de nucleótidos. En el caso de las muestras tratadas con HoP y HPP no se encontraron diferencias significativas entre las concentraciones obtenidas antes y después del periodo de congelación. Este hecho se debe a que los tratamientos con HoP y HPP disminuyen la actividad enzimática y por ello el contenido de nucleótidos libres no varía significativamente durante los seis meses de congelación. Por ello, valorando el contenido de nucleótidos, se puede concluir que HPP es una alternativa adecuada al tratamiento tradicional de HoP para la preservación de la leche materna en los bancos de leche.

ARTÍCULO DE INVESTIGACIÓN I.3

Food Chemistry, 174 (2015) 348-355

Food Chemistry 174 (2015) 348–355

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Analysis of free nucleotide monophosphates in human milk and effect of pasteurisation or high-pressure processing on their contents by capillary electrophoresis coupled to mass spectrometry

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ARTICLE INFO

Article history:
Received 7 July 2014
Received in revised form 3 November 2014
Accepted 8 November 2014
Available online 14 November 2014

Keywords:
Capillary electrophoresis–electrospray ionisation-mass spectrometry
Centrifugal ultrafiltration
Human milk
Nucleotide monophosphates
Processing
Freezing time

ABSTRACT

A simple, efficient and green analytical method for the determination of free nucleotide monophosphates in human milk is proposed. It involves centrifugal ultrafiltration (CUF) as sample treatment and capillary electrophoresis–electrospray mass spectrometry (CE–ESI-MS) for separation and simultaneous quantification.

The optimised method, applied to the analysis of human milk samples, included their dilution (1:5) with water followed by CUF treatment. No matrix effects were found. The method provided limits of detection between 0.08 and 0.13 μg mL^{-1} and limits of quantification between 0.26 and 0.43 μg mL^{-1}. The intralaboratory repeatability and reproducibility afforded relative standard deviation values lower than 10%.

The method was applied to the study of the effects of Holder pasteurisation and high-pressure processing on the nucleotide contents in samples from a human milk bank. The results showed concentration values between 0.5 and 10 μg mL^{-1}, with higher concentrations for the samples treated by pasteurisation. The effect of freezing time on the content of nucleotides was also assessed.

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1. Introduction

Nucleotides play a significant role in many physiological functions, ranging from the encoding of genetic information to signal transduction. They are semi-essential dietary nutrients and are present in the milk of mammals. It is well known that nourishment with human milk increases the immune response in infants. Human milk is the best source of nucleotides for infants and hence the supplementation of infant formulas with concentrations within the ranges found in human milk has been recommended by the Scientific Committee for Food of the European Community. The free nucleotides found at the highest concentrations in human milk are mononucleotides: cytidine monophosphate (CMP), uridine monophosphate (UMP), adenosine monophosphate (AMP), guanosine monophosphate (GMP) and inosine-monophosphate (IMP) (Sugawara, Sato, Nakano, Idota, & Nakajima, 1995).

As well as the free forms, nucleotides are also found in other forms that are available nutritionally to infants: polymeric ribonucleic acids, nucleosides, and nucleotide-containing adducts (NAD) (Aggett, Leach, Rueda, & MacLean, 2003). Accordingly, the total nucleotide content is expressed as total potentially available nucleotide (TPAN) (Gill, Indyk, & Manley-Harris, 2011, 2012; Leach, Baxter, Molitor, Ramstack, & Masor, 1995). Its determination in milk samples is accomplished following a protocol that includes different enzymatic digestions aimed at transforming the different forms of nucleotide into the corresponding nucleosides. Then, HPLC is implemented to determine the free and enzymatically released nucleosides. The distribution of the four major forms of nucleotides depends on the stage of lactation and the geographic location of sample collection. More than 80% of the total nucleotide content in human milk is present at approximately similar proportions of polymeric (43.3% of TPAN) and free nucleotides (39.9% of TPAN). The rest are present at similar proportions as nucleosides and nucleotide-adducts (Gill et al., 2011; Leach et al., 1995).

For the determination of free nucleotides in water-reconstituted infant formulas and the milk of mammals, the treatments most widely used include the addition of acid followed by centrifugation

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http://dx.doi.org/10.1016/j.foodchem.2014.11.051
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of precipitated proteins (Gill & Indyk, 2007; Perrin, Meyer, Mujahid, & Blake, 2001; Viñas et al., 2010). Other treatments include centrifugal ultrafiltration (CUF) (Inoue, Obara, Akiba, Hino, & Oka, 2008, 2010; Rodríguez-Gonzalo, Domínguez-Álvarez, Mateos-Vivas, García-Gómez, & Carabias-Martínez, 2014) or solid phase extraction (SPE) based on anion exchange (Gill, Indyk, Kumar, & Sievwright, 2010). Liquid chromatography (LC) (Lorenzetti, Lilla, Donato, & de Nucci, 2007) is generally used for the determination of nucleotides in these samples, after sample extraction/cleanup. Owing to the polar nature of the compounds, the LC modes most widely used include reversed-phase liquid chromatography (RPLC) (Lin, Li, & Dai, 2007), ion-pair liquid chromatography (IP-LC) (Viñas et al., 2010), ion-exchange liquid chromatography (IE-LC) (Viñas et al., 2009) and hydrophilic interaction liquid chromatography (HILIC) (García-Gómez, Rodríguez-Gonzalo, & Carabias-Martínez, 2013; Ikegami, Tomomatsu, Takubo, Horie, & Tanaka, 2008; Inoue & Dowell, 2012; Inoue et al., 2010).

Capillary electrophoresis (CE) offers another approach for the separation of highly polar compounds (Smyth & Rodriguez, 2007). CE has been used to separate mixtures of nucleotide standards (Cornelius, Wörth, Kliem, Wiessler, & Schemeiser, 2005; McKeown, Shaw, & Barrett, 2001), in the analysis of biological samples (Willems, Deforce, Van Peteghem, & Van Bocxlaer, 2005) and in the analysis of different foods (Xiao-Jia, Feng-Qing, Yi-Tao, & Shao-Ping, 2010). We have recently proposed a method for the determination of nucleotide monophosphates in infant formulas by CE–ESI-MS (Rodríguez-Gonzalo et al., 2014). In human milk samples, a CE method has been proposed for the determination of nucleotides but is based on UV spectrophotometric detection (Cubero et al., 2007).

Human milk banks are institutions that collect and store milk from donor mothers and are responsible for ensuring the safety and quality of the human milk deposited there. They must perform different treatments to inactivate any pathogenic microorganisms and part of the commensal flora that are potentially present in milk from donors. The most commonly used pasteurisation method in human milk banks is low-temperature long-time pasteurisation, also known as Holder pasteurisation (HoP), in which the milk is heated to 62.5 °C in a water bath for 30 min. However, this process results in a variable loss of certain components. In recent years, high-pressure processing (HPP) has received much attention as a novel food preservation method. HPP is a minimal processing technology and serves as a cold-pasteurisation method that does not impair the nutritional and sensory characteristics of foodstuffs (Contador, Delgado-Adámez, Delgado, Cava, & Ramírez, 2013; Moltó-Puigmartí, Permanyer, Castellote, & López-Sabater, 2011; Permanyer et al., 2010; Rastogi, Raghavaro, Balasubramaniam, Niranjan, & Knorr, 2007).

Quantitative determination of nucleotide monophosphates in human milk is complicated by the endogenous presence of these analytes. Accordingly, the first objective of this work was to adapt a previous method developed for the determination of nucleotide monophosphates supplemented in infant formulas (Rodríguez-Gonzalo et al., 2014) to human milk, where they are present naturally. For the analysis of human milk a fairly simple sample treatment based on centrifugal ultrafiltration (CUF) compatible with CE–ESI–MS was used. In addition the method was validated as a quantitative method for determination of nucleotide monophosphates according to the European Union Decision 2002/657/EC.

The method validated here was applied to the evaluation of the nucleotide content in samples subjected to different preservation methods at a human milk bank. A further aim of the work was to determine the possibilities of CE–ESI-MS in the assessment and control of the different processes used in human milk banks for the preservation of the samples.

2. Materials and methods

2.1. CE–ESI-MS method

The method used in the separation-detection by CE–ESI-MS has been described in a previous paper (Rodríguez-Gonzalo et al., 2014).

2.2. Human milk samples

Seven samples of maternal milk were assayed (Table S1, Supplementary material). One of them (MB.0) was provided by a Human Milk Bank in Extremadura (Mérida, Spain) and corresponded to a donor mother who had been breastfeeding for 1 year. This sample was used for the method validation step. The second sample (NMB) was donated by a 27-year-old mother in her first month of breastfeeding. Finally, another five samples (MB.1-MB.5) were provided by the Human Milk Bank after being subjected to different preservation processes (Fig. S1, Supplementary material). All samples were analysed within a window of 5–20 days since their arrival at the laboratory, where they were frozen at −18 °C until further analysis.

The Milk Bank samples were donated by healthy mothers between 29–45 years old and 2–6 months after giving birth. The normal protocol of the Milk Bank involves collecting the milk at home (100–150 mL) and freezing it in a domestic refrigerator until the sample is taken to the Milk Bank, where it is stored at −40 °C. All milk donations were thawed and pooled before processing under sterile conditions, achieving a final volume of 800 mL. The milk was mixed for better evaluation of the effect of the preservation processes applied. It was then vacuum-packed in 40-mL polyethylene bags (9.3 mL O_2 m^{-2} 24 h^{-1} at 0 °C) for HPP or placed in glass tubes for application of the HoP thermal treatment. The use of plastic packaging was necessary for the application of high pressure processing. Control samples (untreated) were also vacuum-packaged in the same type of plastic bags.

2.3. Thermal pasteurisation (HoP) and high-pressure processing (HPP)

HoP thermal processing was applied in sterile glass tubes using 10 mL of milk. These were heated at 62.5 °C for 30 min in a water bath under constant agitation. The tubes were submerged in the water above the level of milk. Three tubes with breast milk were used to monitor the temperature of the milk during heat processing (two tubes were placed in the extremes of the bath and the other was in the middle). When the temperature reached 62.5 °C, the process was allowed to continue for 30 min. After treatment, the tubes were submerged in cold water to reduce the temperature rapidly.

For HPP, the milk was pressurised in a semi-industrial discontinuous hydrostatic pressure unit of 55-L capacity; NC Hyperbaric Wave 6000/55 (Hyperbaric, Burgos, Spain) with a maximum working pressure of 600 MPa and a working volume of 55 L. Cooled water (10 °C) without additives was used as the pressure-transmitting fluid. Samples were treated at 400, 500 or 600 MPa for 5 min. The times required to reach pressures of 400, 500 and 600 MPa was 174, 202 and 230 s, respectively; the pressure-release time was a few seconds. This equipment does not have temperature control inside the vessel and hence only the initial water temperature could be monitored.

2.4. Sample treatment

To implement the proposed method, 1 mL of human milk was placed in a centrifuge tube (50 mL) and 4 mL of ultrapure water

(UHQ) was added. The mixture was shaken manually and then centrifuged for 15 min (2800*g*). A 5-mL aliquot of the supernatant was collected, attempting not to include the fat layer present at the top, and introduced into the CUF device to accomplish ultrafiltration (30 min, 2800*g*); this device was previously conditioned with 5 mL of UHQ water (15 min, 2800*g*). The filtrate was injected directly into the CE–ESI-MS system.

2.5. Quantification

Stock solutions at 500 mg L^{-1} of standard of nucleotides were prepared by dissolving the compounds in acidified MeOH (10 mM $HClO_4$). All solutions were kept at −18 °C in amber glass bottles. Working solutions containing the analytes at variable concentrations were prepared daily by mixing appropriate amounts of the stock solutions and diluting in UHQ water. The stability of the stock solutions was checked and it was observed that they remained stable for at least 18 months since their preparation.

Analyte quantification by MS was carried out in SIM acquisition mode, using the m/z ratio corresponding to the $[M-H]^-$ ions. The quantitative variable was the peak area generated by the respective ion expressed in arbitrary peak area units. Quantification of milk samples was performed by external calibration in UHQ water and by the standard additions method. For milk samples from the human milk bank (MB.1–MB.5), two samples from each of the treatments studied were analysed, quantifying each of them by both calibration procedures.

The standard additions method used was as follows: the milk samples were fortified after the CUF treatment by the addition of an adequate volume (20–60 μL) of standard solutions of the mononucleotides to 200-μL aliquots of the CUF extract. Samples were fortified at six levels ranging from 0.1 to 2 μg mL^{-1}. Both unspiked and spiked samples were analysed by CE–ESI-MS, obtaining three replicas per sample.

2.6. Matrix effect

The matrix effect was determined with the following equation:

$$\text{Matrix effect } (\%) = [1 - ((S_{\text{matrix}} - S_{\text{blank}})/S_{\text{solvent}})]\ 100$$

where S_{matrix} refers to the signal of the human milk sample spiked after the CUF treatment; S_{blank} is the signal of the unspiked sample and S_{solvent} is the signal of the standards at the same concentration at which the CUF extract was spiked.

2.7. Method validation

The method was validated with a milk bank sample (MB.0) evaluating the following parameters: external calibration curves, limits of detection and quantification; repeatability and intralaboratory reproducibility at three concentration levels, accuracy and recovery at three concentration levels.

2.8. Statistical analysis

In order to compare concentrations of NMPs from different groups (i.e. standard additions method vs. calibration in water, short vs. long storage and untreated vs. treated milks) p-values were calculated by means of a two-tailed paired t-test. Values of $p < 0.05$ were interpreted as a rejection of the null hypothesis, thus rejecting equal concentrations between the two groups.

3. Results and discussion

3.1. Electrophoretic separation–mass spectrometric detection (CE–ESI-MS)

Separation and detection were performed following the procedure proposed in a previous work (Rodríguez-Gonzalo et al., 2014). The nucleotide 5′-monophosphates (NMPs) studied were: adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP) and inosine 5′-monophosphate (IMP), i.e. those found at highest frequencies in analyses of human milk (Sugawara et al., 1995).

Before applying the method to the analysis of human milk samples, the instrumental quality parameters were determined. Linear calibration curves were obtained for all compounds in the range tested (100–2000 ng mL^{-1}). Isotopically Labelled Internal Standards (ILIS) of AMP and UMP were used at a concentration level of 1000 ng mL^{-1}. The ions selected for these two compounds were the most abundant ones $[M-H]^-$ the m/z selected being 361 and 334 respectively. However, the results obtained using calibration with ILIS did not significantly improve those obtained with external calibration. Accordingly, we did not work with ILIS in the quantification.

The instrumental limits of detection, based on a signal-to-noise (S/N) ratio of 3, ranged from 15 ng mL^{-1} for CMP to 26 ng mL^{-1} for UMP. The limits of quantification, based on an S/N of 10, ranged from 50 to 87 ng mL^{-1}.

3.2. Human milk pretreatment

Quantitative determination using CE–ESI-MS may be affected by the ion suppression that occurs in the electrospray device, mainly due to the co-migration of matrix interferents with the analytes (Kohler, Schappler, & Rudaz, 2013; Xiu-Qin, Zong, Qing-He, & Hong-Mei, 2014). This effect may change the response of the analytes by increasing or reducing ionisation (ion enhancement/suppression) at the mass spectrometer ionisation source. Several strategies have been suggested to minimise the interferences of matrix components such as sample dilution, use of pretreatment steps or another ionisation source. Alternatively, an appropriate calibration technique to compensate for the signal alterations must be used. In this sense, the standard addition method represents the most-effective way to compensate for the adverse influence of the matrix (Stuber & Reemtsma, 2004).

Initially, we applied a solid-phase extraction step to a milk sample to check the reduction in the number of interfering compounds. Sorbents of different nature (i.e. electrostatic, hydrophilic/hydrophobic and affinity interactions) were assayed, unsatisfactory results being obtained in all cases (Data not shown). Then another approach was studied, a matrix dilution followed by CUF, in order to reduce the presence of interfering compounds in the final extract (Ortelli, Cognard, Jan, & Edder, 2009) that may cause ion suppression (Lankova, Lacina, Pulkrabova, & Hajslova, 2013).

Prior assays were performed to determine whether it was necessary to carry out centrifugation before CUF. By performing a previous centrifugation it is possible to separate most of the fat and later centrifugal ultrafiltration generates very clean extracts. In order to minimise possible ion suppression, we optimised the amount of sample to be used and its later dilution. Human milk (MB.0) was diluted with electrophoretic background electrolyte (BGE) or with UHQ water at proportions of 1:5 (v/v). In the electropherograms obtained, a better resolution was found when the sample was diluted with UHQ water. Fig. 1a shows the extracted ion electropherogram (XIE) of a real unspiked sample. It may be

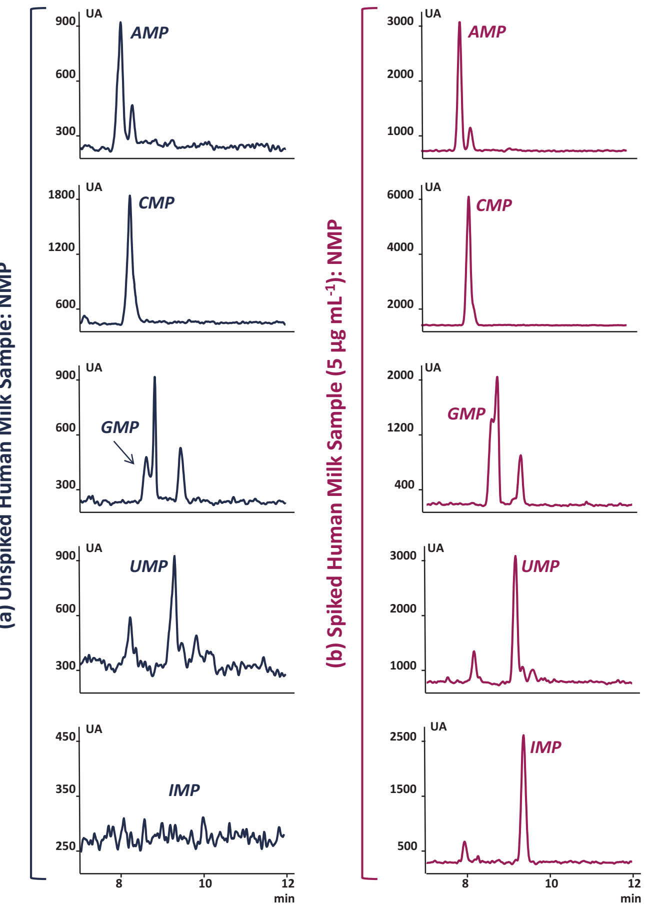

Fig. 1. (a) Extracted ion electropherograms (XIE) of an unspiked human milk sample (MB.0) and (b) extracted ion electropherograms of a human milk sample (MB.0) spiked with five nucleotide 5′-monophosphates (NMPs) at 5 μg mL^{-1}.

seen that the nucleotide present at the highest concentration was CMP, followed by UMP and AMP (Table 1). The concentrations of GMP and IMP were higher than the limit of detection and lower than the limit of quantification. These findings are in good agreement with what has been reported by other authors, who also found that CMP was the nucleotide present at the highest concentration in all samples of milk from mammals analysed (Gil & Sánchez-Medina, 1982; Leach et al., 1995; Sugawara et al., 1995; Tressler et al., 2003).

With a view to increasing the sensitivity of the method, the volume of human milk treated was modified, varying it between 1.0 and 5.0 mL. Upon increasing the volume, the signals did not increase proportionally to the sample volume. Furthermore, working with a volume of 5.0 mL affected the shape of the peaks adversely, these peaks showing a broadening and splitting in their electrophoretic signal, which hindered their quantification. Accordingly, in later experiments we worked with a sample volume of 1.0 mL.

The matrix effect (ME) was determined using milk diluted with UHQ water at a proportion of 1:5 (v/v). The human milk samples were spiked with target analytes at 1.0 and 5.0 μg mL^{-1}, after the ultrafiltration treatment, and we observed that the mean value for MEs ($n = 4$) ranged between +11% for CMP and −17% for GMP, which RSDs lower than 11%. Fig. 1b shows the XIE of a human milk spiked at 5.0 μg mL^{-1}. In light of these results, it may be concluded that there is no clear matrix effect and that the quantification of the analytes in human milk samples with the proposed methodology can be performed by calibration with standards prepared in UHQ water.

3.3. Validation of the method: CUF prior to CE–ESI-MS

The method was validated according to the requirements set by the Commission Decision 2002/657/EC. The untreated human milk sample (MB.0) was used for that purpose. The limits of detection and quantification for NMPs are shown in Table 1. The limits of

Table 1
Validation parameters for the proposed CUF prior to CE–ESI-MS method.

		AMP	CMP	GMP	UMP	IMP
Analytical limits	LOD[a] (μg mL^{-1})	0.10	0.08	0.11	0.13	0.12
	LOQ[b] (μg mL^{-1})	0.33	0.26	0.36	0.43	0.40
Recoveries	Unspiked sample[c] (μg mL^{-1})	0.45 ± 0.02	3.5 ± 0.3	<LOQ	0.70 ± 0.15	<LOQ
	Spiked sample[d] (μg mL^{-1})	5.55 ± 0.30	9.5 ± 0.5	5.0 ± 0.3	5.5 ± 0.5	4.7 ± 0.2
	R[e] (%)	102	112	94	96	87
Precision	Repeatability[f] (%)	4	3	<LOQ	8	<LOQ
	Reproducibility[g] (%)	8	9	<LOQ	9	<LOQ
Accuracy	St. Add.[h] (μg mL^{-1})	0.45 ± 0.15	2.9 ± 0.3	<LOQ	0.45 ± 0.10	<LOQ
	Aq. Cal.[i] (μg mL^{-1})	0.35 ± 0.15	3.6 ± 0.4	<LOQ	0.50 ± 0.15	<LOQ
	Acc.[j] (%)	78	124	–	111	–

[a] LOD: limits of detection calculated for a signal to noise ratio of 3.
[b] LOQ: limits of quantification calculated for a signal to noise ratio of 10.
[c] Unspiked milk sample. Concentrations obtained by the standard addition method.
[d] Milk sample spiked at 5.0 μg mL^{-1}. Concentrations obtained by the standard addition method.
[e] Recoveries calculated as: ([spiked sample*] – [unspiked sample*])/[added concentration] × 100. [Spiked sample*] and [unspiked sample*]: concentrations found by the standard addition method.
[f] Repeatability calculated as intraday precision (6 injections) for the analysis of unspiked milk sample.
[g] Reproducibility calculated as interday precision (3 different days, 6 injections for the analysis of 3 different unspiked milk samples).
[h] Concentration in μg mL^{-1} obtained by the standard addition method.
[i] Concentration in μg mL^{-1} obtained by calibration in UHQ water.
[j] Accuracy calculated using the standard addition method as quality control.

Table 2
Nucleotide monophosphate contents in a sample of human milk collected at 1 month of lactation (NMB). Comparison of two quantification methods (standard additions and external calibration in UHQ water) and the influence of freezing time.

	Analysis within 15 days since arrival			Analysis after frozen −18°C, 3 months		
	St. Add.[a] (μg mL^{-1})	Aq. Cal.[b] (μg mL^{-1})	P[c]	St. Add.[a] (μg mL^{-1})	Aq. Cal.[b] (μg mL^{-1})	P[c]
AMP	1.6 ± 0.1	1.2 ± 0.1	0.06	3.1 ± 0.3	3.0 ± 0.2	0.73
CMP	6.5 ± 1.3	4.2 ± 0.2	0.13	4.9 ± 0.4	5.5 ± 0.4	0.27
GMP	0.8 ± 0.1	0.6 ± 0.1	0.18	2.2 ± 0.4	2.0 ± 0.2	0.59
UMP	2.1 ± 0.2	1.9 ± 0.2	0.42	4.4 ± 0.3	5.0 ± 0.5	0.28
IMP	<LOQ	<LOQ	–	<LOQ	<LOQ	–

[a] Concentration in μg mL^{-1} obtained by the standard addition method.
[b] Concentration in μg mL^{-1} obtained by calibration in UHQ water.
[c] p Values obtained applying a two-tailed t test. Values above 0.05 indicate that the results obtained with both methods were not significantly different.

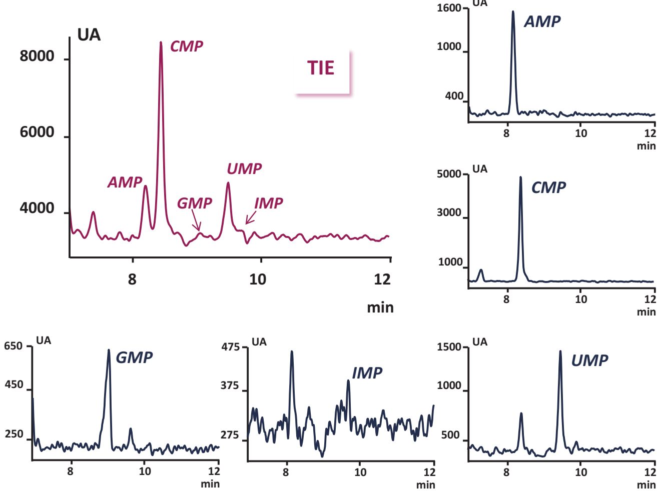

Fig. 2. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) of a human milk sample at month 1 of lactation (NMB).

quantification ranged between 0.26 µg mL^{-1} for CMP and 0.43 µg mL^{-1} for UMP.

In order to determine the repeatability and intralaboratory reproducibility of the proposed method, the relative standard deviations (RSDs) were evaluated (Table 1). For reproducibility (3 days, 6 injections per day for the analysis of 3 different unspiked milk samples) values ranged between 8% for AMP and 9% for CMP and UMP were found.

In view of the lack of certified samples of nucleotides in human milk, the method was validated by determining the recoveries using the sample (MB.0) spiked with NMPs at a level of 5.0 µg mL^{-1}. Fortification was performed directly on the MB.0 human milk sample, a period of about 12 h was allowed to elapse before continuing with any of the extraction processes in order to favour the interaction between the analytes and the matrix components. The results obtained, as percentages of analyte recovered, were between 87% and 112% (Table 1). These recoveries were in the accepted range (70–120%) by the Codex Guidelines (CAC/GL 71-2009).

Furthermore, accuracy was checked by analysing the content of NMPs in sample MB.0 by the standard additions method and comparing the results with those obtained by calibration in UHQ water. The concentrations obtained did not reveal significant differences between the two methods (Table 1).

3.4. Application to the determination of nucleotide monophosphates (NMPs) in human milk samples

The proposed methodology was applied to the determination of nucleotides in different milk samples: one of them from a volunteer woman in her first month of lactation (NMB) and other five samples provided by a human milk bank (MB.1–MB.5). The characteristics of these samples and the treatment to which they were subjected are indicated in the experimental part of this work (see Section 2.2) and Table S1 (Supplementary information).

The sample of milk NMB (1 month lactation) was analysed in duplicate using external calibration obtained with standards in UHQ water. To check the accuracy of the results obtained, quantification was also performed using the standard additions method. The concentrations obtained by standard additions and by calibration in UHQ water were compared for each nucleotide by means of a two-tailed *t*-test. In all cases *p* values >0.05 were found, suggesting that there were no significant differences between the two methods (Table 2). Fig. 2 shows the total ion electropherograms (TIE) and the extracted ion electropherograms (XIE) obtained on monitoring $[M-H]^-$ ions of the free nucleotides. In the NMB milk sample it was possible to quantify AMP, CMP, UMP and GMP and to detect the presence of IMP (Fig. 2). The low level of IMP in the sample analysed is consistent with the findings of Leach et al. (1995) and Tressler et al. (2003). The latter authors, analysing different human milk samples, indicated that IMP is only detected in colostrums samples at very low levels. Other authors have described that the presence of this nucleotide in milk samples is due to the conversion of AMP into IMP by adenosine deaminase (Thorell, Sjoberg, & Hernell, 1996).

To evaluate the effect of the time elapsed from sample collection to analysis, several aliquots of the NMB sample were frozen and analysed again after 3 months. On comparing the results with those obtained 3 months before, it was observed that the contents of most of the free nucleotides increased significantly (Table 2). This type of behaviour must be due to the enzymatic transformation of the polymeric nucleotides or nucleotide adducts into free nucleotides.

In order to explore the effects of HoP and HPP on the nucleotide content, these compounds were analysed in untreated (MB.1) and treated (MB.2–MB.5) samples provided by the milk bank (Fig. 3A). In all samples, the most abundant nucleotide was CMP, followed by UMP and AMP; but GMP and IMP could not be detected. These results are consistent with those reported by other authors (Gil & Sánchez-Medina, 1982; Leach et al., 1995; Sugawara et al., 1995; Tressler et al., 2003). On comparing the results for the sample MB.1 (untreated sample from the milk bank, at 12 months of breast-feeding) with those obtained for the sample NMB (untreated milk at 1 month of breast-feeding, not provided by the milk bank) it may be seen that in MB.1 the nucleotide levels were lower. These results were in agreement with those reported in the literature and it appears that they are due to the fact that the nucleotide content decreases as the stage of lactation progresses (Gil & Sánchez-Medina, 1982; Gill et al., 2011).

In the milk samples subjected to HPP (MB.2–MB.4) the contents of nucleotides were significantly equal to those of the untreated samples (MB.1). Nevertheless, the samples treated using HoP (MB.5) showed an increase in their AMP, CMP and UMP contents (Fig. 3A). These results may have been due to the fact that on increasing the temperature during the pasteurisation step, free nucleotides could have been generated from the polymeric nucleotides and/or nucleotide adducts.

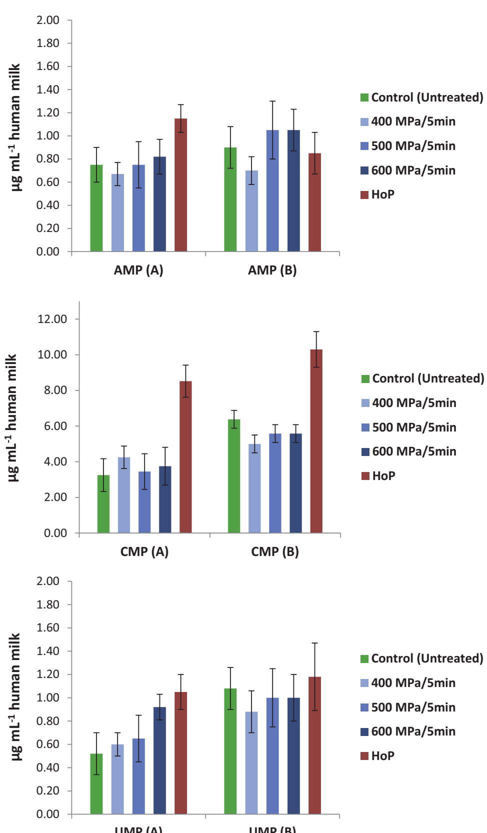

Fig. 3. Variation in the content of AMP, CMP and UMP of human milk samples: untreated (MB.1) and treated at high pressure (MB.2-MB.4) and by pasteurisation (MB.5). (A) Samples analysed within 5 days after their arrival at the laboratory. (B) Samples analysed after storage at −18 °C for 6 months.

Finally, we also evaluated the influence of the time elapsed since arrival of the samples at the laboratory in the samples MB.1–MB.5. During the 6-month period of freezing at −18 °C no decrease occurred in the nucleotide content of any of the samples studied (Fig. 3B). The untreated sample from the milk bank (MB.1), showed an increase in CMP and UMP. In the case of the samples treated with HPP and HoP, the concentrations obtained before and after 6 months of freezing were compared through a two-tailed *t*-test. In all cases values of $p > 0.05$ were found, suggesting that there were no significant differences. The behaviour of the untreated MB.1 sample was similar to that observed for the other untreated sample (NMB), donated by a mother during her first month of breast feeding. In both cases, an increase was observed in the content of most of the nucleotides after frozen storage (Fig. 3B and Table 2). Also, on comparing the results obtained with HPP and HoP and those obtained in the analysis of untreated milk (MB.1 and NMB), it may be concluded that treatment with HPP and HoP may have decreased the enzymatic activity and hence the free nucleotide content was not modified significantly during the 6 months of freezing.

4. Conclusions

A validated method for the determination of nucleotide monophosphates in human milk using CE–ESI-MS has been reported for the first time. The sample treatment based on centrifugal ultrafiltration is quite simple, avoids the use of organic extractants and is compatible with the electrophoretic separation.

The results obtained here indicate that capillary electrophoresis coupled to mass spectrometry (CE–ESI-MS) offers an alternative to chromatographic methods and could potentially be used for the evaluation of nucleotides in human milk.

The method was successfully applied to the analysis of nucleotides in milk samples from a human milk bank, untreated or treated by pasteurisation (HoP) or high pressure (HPP). The application of HoP increased the content of nucleotide monophosphates, whereas HPP did not cause significant variations. However, the samples treated with HPP and HoP did not show any significant variation in their nucleotide contents after a 6-month period of freezing. HPP could be a suitable alternative to traditional HoP in the treatment of human milk, because the HPP technique did not reveal any modification in the nucleotide content; thus the level of free nucleotides remained the same as those for the untreated milk samples, even after 6 months of freezing.

Acknowledgements

The Ministerio de Ciencia e Innovación, Spain (Project CTQ 2011-24075) is gratefully acknowledged for financial support of this work. M.M.V. gratefully acknowledges financial assistance from the Ministerio de Educación, Spain (FPU grant). The authors are especially grateful to Esmeralda Díaz Martín and to the Milk Bank of Extremadura (SES, Mérida, Spain) for providing samples of human breast milk for this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.11.051.

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Electronic Supplementary Material

Analysis of free nucleotide monophosphates in human milk and effect of pasteurisation or high-pressure processing on their contents by capillary electrophoresis coupled to mass spectrometry.

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Table S1. Description of human milk samples used for this study.

Human Milk	Origin	Time from parturition	Treatment
MB.0	Milk Bank	12 months	Untreated
NMB	Volunteer	1 months	Untreated
MB.1	Milk Bank	2-6 months	Untreated
MB.2	Milk Bank	2-6 months	HPP 400 MPa / 5 min
MB.3	Milk Bank	2-6 months	HPP 500 MPa / 5 min
MB.4	Milk Bank	2-6 months	HPP 600 MPa / 5 min
MB.5	Milk Bank	2-6 months	HoP / 62.5 °C for 30 min

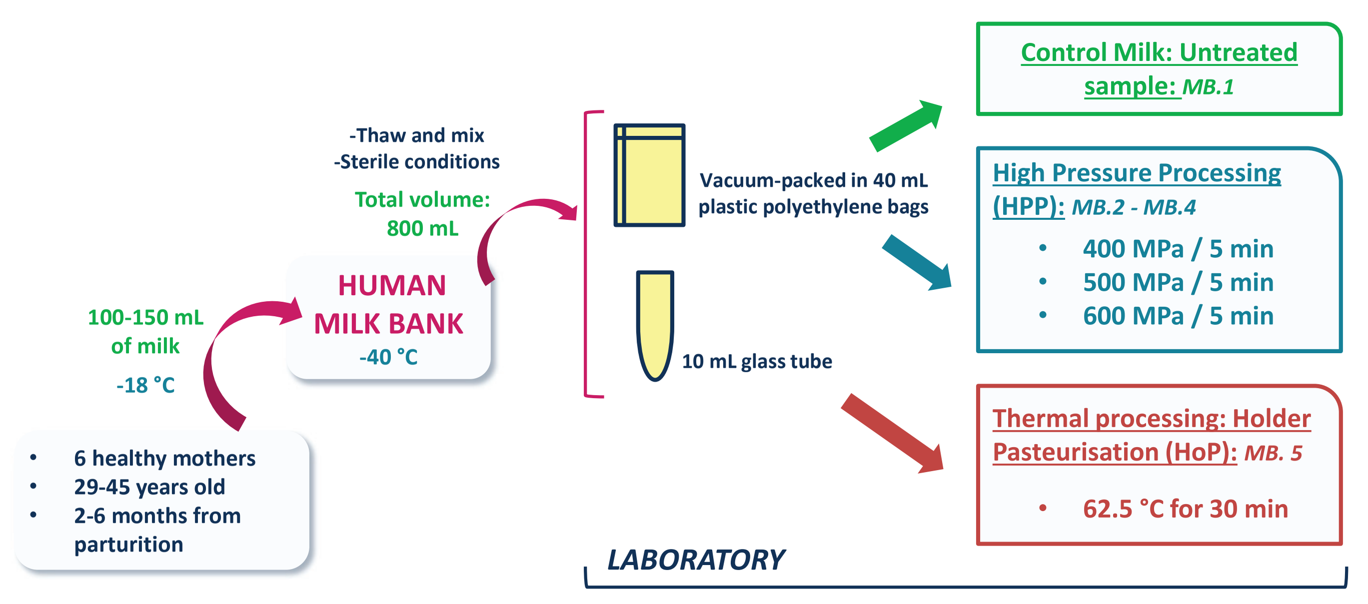

Figure S1. Scheme of sampling of human bank milk (MB.1-MB.5).

Capítulo II

Nuevas Tendencias en Cromatografía Líquida

Determinación de Nucleótidos con Nano-Cromatografía Líquida

II.1

ARTÍCULO DE INVESTIGACIÓN II.1

RESUMEN

II.1. Determinación de nucleótidos con nano-cromatografía líquida

En los últimos años, la elevada demanda de herramientas analíticas capaces de ofrecer metodologías rápidas y fiables ha fomentado la investigación analítica en el campo de las técnicas miniaturizadas de separación. Entre este tipo de técnicas merece especial atención la nano-cromatografía líquida (nano-LC), que es aquella según se acepta por definición, en la que la separación cromatográfica se realiza en columnas capilares con un margen de diámetro interno entre 10 y 100 µm. La nano-LC se ha convertido en una alternativa importante a la LC convencional debido a las ventajas únicas que ofrece, como el bajo consumo de fases móviles y de fases estacionarias, que conlleva por una parte la reducción de residuos, favoreciendo la seguridad medioambiental, y por otra parte, este bajo consumo implica un menor gasto tanto de reactivos como de fases estacionarias para empaquetar, que suelen ser caras, originando una técnica menos costosa que la convencional. Además, es necesario destacar que en esta técnica se utilizan volúmenes bajos de muestra, se consigue una alta eficiencia en la separación con tiempos de análisis cortos, y presenta una alta compatibilidad con espectrometría de masas (MS) debido al uso de flujos cromatográficos relativamente bajos (40-600 nL min^{-1}) que permiten la transferencia de todo el eluyente desde la columna al detector.

A pesar de todas estas características y de los buenos resultados obtenidos en diversas áreas de aplicación, la nano-LC no ha sido muy utilizada en el análisis de alimentos. En el caso concreto de la alimentación infantil, sólo hay propuestos dos trabajos referentes al análisis de diferentes compuestos en fórmulas lácteas de iniciación. En

nuestro conocimiento, no existían antecedentes para la determinación de nucleótidos en este tipo de muestras utilizando esta técnica miniaturizada.

Por ello, el objetivo principal de este trabajo fue el desarrollo de un método rápido con nano-LC para analizar nucleótidos monofosfato en fórmulas infantiles. Para la optimización del método se evaluaron varios parámetros como la selección de la fase estacionaria, la composición de la fase móvil o el volumen de inyección de la muestra. La experimentación se llevó a cabo utilizando capilares de sílice fundida de 75 µm de diámetro interno, empaquetados en el propio laboratorio con las diferentes fases estacionarias que se estudiaron. Este proceso resulta de especial importancia, ya que es necesario evitar volúmenes muertos y obtener un empaquetado estable y fijo, lo cual depende de parámetros como el tipo y tamaño de la fase estacionaria, el disolvente orgánico usado en la preparación o la temperatura. Las partículas son retenidas dentro del capilar a través de dos fritas mecánicas preparadas sinterizando el material a elevada temperatura, lo cual supone una etapa crítica del proceso. En este trabajo se estudiaron diferentes fases estacionarias, desde las convencionales porosas utilizadas habitualmente en fase inversa hasta nuevos materiales con tamaños de partícula inferiores a 2 µm, con el objetivo de conseguir columnas más eficientes y separaciones más rápidas; y también materiales compuestos de partículas *core-shell* para reducir la resistencia de la transferencia de masa, obtener un perfil de flujo más plano y con ello una mayor eficiencia en la separación. Los mejores resultados se consiguieron con esta última, una fase estacionaria tipo *core-shell* Kinetex XB-C_{18} (3.6 µm), que fue la seleccionada para los estudios posteriores.

Respecto a la composición de la fase móvil, se evaluó en primer lugar la posibilidad de llevar a cabo la separación de los nucleótidos utilizando fases móviles formadas por mezclas de acetonitrilo/agua en diferentes proporciones y medios tamponados a diferentes pHs entre 3 y 9. Debido a la naturaleza extremadamente polar de los analitos, no se consiguió la retención de los mismos en ninguna de estas condiciones cromatográficas. Por ello, se planteó la utilización de reactivos formadores de pares iónicos, entre los que se probaron: dibutilamina (DBA), trietilamina (TEA) e hidróxido de tetrabutilamonio (TBAOH), obteniéndose los mejores resultados con TBAOH en cuanto a separación y eficiencia de pico. Con el objetivo de mejorar la eficiencia de la separación, sensibilidad, forma de pico y tiempo de análisis, se estudiaron los siguientes parámetros: concentración del reactivo formador de par iónico; naturaleza, concentración y pH del tampón utilizado en la fase móvil; y naturaleza y porcentaje del disolvente orgánico que forma parte de la misma. Finalmente, con una fase móvil compuesta por 5 % (v/v) de metanol y 95 % (v/v) de formiato amónico 100 mM a pH 8 conteniendo TBAOH 20 mM, y utilizando un flujo cromatográfico de 500 nL/min, se consiguió la separación de los cinco nucleótidos monofosfato en menos de 5 minutos. Se llevó a cabo la detección espectrofotométrica a una longitud de onda de 260 nm.

Para la inyección de la muestra se ensayaron tiempos de inyección entre 20 y 80 segundos, obteniéndose una respuesta lineal, con respecto a la altura de pico, hasta un tiempo de 60 segundos (450 nL de volumen). Los límites de detección obtenidos, aproximadamente 0.25 $\mu g\ mL^{-1}$ para todos los compuestos, resultan adecuados para el análisis de nucleótidos monofosfato en muestras de fórmulas infantiles.

En las condiciones óptimas encontradas se realizó la validación del método analítico. Se obtuvieron buenos valores de precisión *intra-* e *interday*, tanto para los tiempos de retención como para las áreas de las señales cromatográficas, estando en todo caso por debajo del 5 %. Además se evaluó la reproducibilidad entre columnas realizando el análisis de los nucleótidos en tres columnas capilares diferentes empaquetadas siguiendo el mismo procedimiento. Se obtuvieron resultados satisfactorios con precisiones inferiores al 4 % en tiempos de retención e inferiores al 6 % en áreas de pico.

Finalmente, con el fin de comprobar la aplicabilidad del método propuesto, se analizaron los nucleótidos monofosfato en tres fórmulas infantiles suplementadas con dichos analitos. Como tratamiento previo, la muestra se diluyó con agua, se centrifugó para separar la mayor cantidad posible de grasa, y posteriormente se realizó una etapa mediante ultrafiltración asistida por centrifugación (CUF) del sobrenadante obtenido con el objetivo de separar las especies macromoleculares. El extracto resultante, en medio acuoso, es totalmente compatible con la inyección en el sistema nano-LC. Trabajando con muestras dopadas, se obtuvieron valores de recuperación entre 91 y 103 %, con desviaciones inferiores al 8 %. Los contenidos de nucleótidos encontrados en las fórmulas infantiles estaban en consonancia con los etiquetados por el fabricante.

Como conclusión se puede decir que se ha conseguido el objetivo propuesto y se ha desarrollado un método rápido y eficiente basado en nano-LC, para la determinación de nucleótidos monofosfato en fórmulas infantiles de iniciación. Los buenos resultados alcanzados en este trabajo ponen de manifiesto las posibilidades de esta técnica

miniaturizada, nano-LC, como método alternativo o complementario a la cromatografía líquida convencional.

Trabajo desarrollado en: *Istituto di Metodologie Chimiche, Consiglio Nazionale delle Ricerche (CNR), Monterotondo (Rome), Italy.*

ARTÍCULO DE INVESTIGACIÓN II.1

Electrophoresis, doi: 10.1002/elps.201500526

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Received November 16, 2015
Revised December 23, 2015
Accepted January 11, 2016

Research Article

Rapid determination of nucleotides in infant formula by means of nano-liquid chromatography

A rapid method for the quantification of five ribonucleotides 5′- monophophates (adenosine, cytidine, guanosine, inosine, uridine, 5′-monophosphate), in infant formula, has been proposed using nano-LC. To separate the studied compounds, capillary columns packed with different C18-based stationary phases were investigated. All the columns tested were laboratory prepared. The experiments were performed in ion-pairing RP chromatographic mode using tetrabutylammonium hydroxide as ion-pairing reagent. The method was developed using a core-shell XB-C_{18} capillary column with a mobile phase consisting of 5% v/v methanol and 95% v/v 100 mM ammonium formate, pH 8, containing 20 mM tetrabutylammonium hydroxide. All compounds were baseline resolved in less than 5 min with a flow rate of 500 nL/min in isocratic elution mode. Nucleotides were detected at 260 nm. Analytical validation parameters were evaluated. The RSD values for intraday and interday repeatability for retention time and peak area were <2.4 and 4.2%, respectively. The method linearity was good (R^2 < 0.9995) for the studied compounds. LOD and limit of quantitation were 0.25 and 0.50 μg/mL, respectively. The method was applied to the determination of nucleotides in infant formula, subjected to a centrifugal ultrafiltration process, prior their analysis. The amounts found were in agreement to the labeled contents.

Keywords: Infant formula / Ion pair reversed-phase chromatography / Nano-LC / Nucleotide 5′-monophosphates
DOI 10.1002/elps.201500526

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1 Introduction

Nucleotides have numerous essential roles in many biological processes. They can be synthesized endogenously and thus are not essential nutrients. However, they become fundamental when the endogenous supply is not appropriate especially during pediatric growth or after their injury. Breast milk, with its unique composition, is considered to be the best source of nucleotides that have beneficial effects in infants, upon the immune system, small intestinal growth and development, lipid metabolism, and hepatic function [1–4]. Although not essential dietary nutrients, it has been demonstrated that supplementation of pediatric formulas with nucleotides is of benefit in neonatal nutrition for improving the immuno and protective responses [5–10]. According to the European regulations, five ribonucleotides 5′-monophophates including adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), and uridine 5′-monophosphate (UMP) are permitted as supplements at a maximum concentration of 5 mg/100 kcal in food products. Taking into account the importance of nucleotides for infant health, to control and monitor the quality of baby foods, appropriate analytical methods for their quantification are needed.

Several protocols for the analysis of free nucleotides in infant formula (IF) have been optimized. Among them is the acidic precipitation of casein proteins with subsequent centrifugation [11, 12]. The extraction of nucleotides from food infant products can be also performed using ion exchange SPE [13], or employing centrifugal ultrafiltration (CUF), an alternative and simple approach to remove proteins from sample matrix [14, 15]. So far, various analytical methods for the analysis of nucleotides in pediatric or in biological matrices,

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Abbreviations: **AMP**, adenosine 5′-monophosphate; **CMP**, cytidine 5′-monophosphate; **CUF**, centrifugal ultrafiltration; **DBA**, dibutylamine; **GMP**, guanosine 5′-monophosphate; **IF**, infant formula; **IMP**, inosine 5′-monophosphate; **MeOH**, methanol; **TBAOH**, tetrabutylammonium hydroxide; **TEA**, triethylamine; **UMP**, uridine 5′-monophosphate

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such as enzymatic assays, TLC, paper chromatography, and HPLC, have been proposed [16].

In particular, RPLC, ion-pair RPLC, and ion-exchange LC are the three main modes of LC employed for the analysis of these compounds. RPLC exhibited some drawbacks such as low retention and peak tailing as a consequence of the adsorption of the phosphate groups of nucleotides with residual silanol groups of the stationary phase [17]. Recently, novel polar embedded, polar end-capped, or polymer grafted C18 stationary phases, characterized by higher selectivity and good pH stability, have been used showing better peak efficiency [13, 18].

The analysis of nucleotides is usually performed by ion-pairing RP and anion-exchange LC employing cationic ion-pair reagents in the mobile phase and anion-exchange stationary phases, respectively, allowing stronger interactions between the analytes and the stationary phases with consequent resolution and selectivity improvements [14, 19–22]. Recently, hydrophilic interaction LC technique, suitable for the separation of highly polar substances, has been well proposed for the analysis of nucleotides [23, 24].

For the analysis of those highly polar compounds, CE has reported as a valuable alternative separation technique to HPLC, offering the advantage to determine nucleotides in a wide range of pH, in short analysis time, and with high efficiency [25–29].

In recent years, the high demand for analytical tools able to offer reliable and fast methodologies has encouraged the analytical research toward miniaturized separation techniques that provide reduced waste production and are cost-effective. Among them, capillary and nano-LC have become an important alternative to conventional LC offering unique advantages as the large decrease in mobile and stationary phase consumption, including toxic reagents minimizing waste generation; small sample volume needed; high separation efficiency; short analysis time; and easy coupling to MS [30, 31].

Despite all the above-mentioned features and good performance shown in other application fields, the use of nano-LC for food analysis has not been widely exploited [32–34]. In particular, only two papers concerning the analysis of different compounds in IF utilizing the chromatographic miniaturized technique have been proposed [35, 36]. To the best of our knowledge, no study on the analysis of nucleotides in IF by nano-LC has been reported.

Therefore, the aim of this work was the development and validation of a rapid nano-LC-UV method to analyze nucleotides in IF. For method optimization, several parameters including the selection of the stationary phase, mobile phase composition, and sample volume injection were evaluated. The method was applied to the determination of nucleotides in pediatric samples after an extraction CUF procedure.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and were used without further purification. Formic and acetic acids, ACN, and methanol (MeOH) were purchased from Carlo Erba (Milan, Italy). Dibutylamine (DBA), triethylamine (TEA), and tetrabutylammonium hydroxide (TBAOH) were acquired from Sigma-Aldrich (Milan, Italy). DI water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). 5′-monophosphate nucleotides, AMP, CMP, GMP, IMP, and UMP disodium salts, were purchased from Sigma-Aldrich (Stenheim, Germany) (for their chemical structures, see Fig. 1). CUF devices (CUF; Spin-X UF 20, 5 k molecular weight cut-off, polyethersulfone, nonsterile, 20 mL) were purchased from Corning (NY, USA).

Stock solutions (1 mg/mL) were prepared dissolving each analyte in water and stored at −20°C. To perform analyses, the samples mixture was prepared diluting stock standard solutions with water at the desired concentration. Mobile phases used for nano-LC experiments were daily prepared by mixing proper volumes of organic modifiers, water, and proper additives. Each mobile phase was sonicated before use.

2.2 Instrumentation

A Perkin-Elmer series 10 LC-pump (Palo Alto, CA, USA) was used for packing capillary columns. Nano-LC experiments were carried out by means of a Spectra System P2000 conventional gradient HPLC pump, equipped with a Spectra System SCM1000 vacuum membrane degasser and a UV–vis on-column Spectra System UV 1000 detector (all purchased from Thermo Separation Products, San José, CA, USA). Detection wavelength was selected at 260 nm and data were collected by Spectra System Software PC1000 (Fremont, CA, USA). To reduce the flow rate from μL/min to nL/min, a split-flow system was assembled in our laboratory. Briefly, the pump was connected to a stainless steel tee (VICI Valco, Houston, TX, USA) with a polyetheretherketone tube (50 cm × 130 μm id). The tee was further joined to the eluent reservoir of the pump through a fused silica capillary (50 cm × 50 μm id). In this way, part of the solvent delivered by the pump was recycled. The third exit of the tee was connected with a stainless steel tube (3 cm × 500 μm id) to a modified injection valve. At the optimized conditions, a flow rate of about 400 nL/min was estimated, connecting a micro-syringe (Hamilton, Reno, NV, USA) to the end of the capillary column through a Teflon tube and measuring the mobile phase volume after 5 min.

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Figure 1. Chemical structures of the analyzed ribonucleotides 5′- monophosphates.

Samples were introduced into the column by an EnantioSep modified six-port injection valve (Munster, Germany), in accordance with the following scheme:(i) after loading, the sample was introduced into the column for a certain time, corresponding to a certain injection volume; (ii) subsequently, with the injector in load position, the excess of sample was removed from the loop (about 50 μL) washing it with mobile phase, by the introduction of a high volume of mobile phase (500 μL); (iii) With the loop filled with the mobile phase (and in this way used as mobile phase reservoir to perform experiments), the analysis started immediately after positioning the injector device in the injection mode.

The loop was also used as mobile phase reservoir to perform the experiments. The injection valve was manually controlled. Following this procedure, the HPLC pump delivered MeOH to the splitting tee and then to the modified injection valve opportunely filled with the selected mobile phase at the desired time. The organic solvent had only the function to deliver the mobile phase present in the loop into the column. Since the loop was about 50 μL and the flow rate of the column was about 400 nL/min, the organic solvent could not enter into the column. When the mobile phase had to be changed, it was directly introduced into the valve [33, 34].

2.3 Column preparation and chromatographic conditions

Nano-LC experiments were performed in uncoated fused silica capillaries (75 μm id, 375 μm od, from Composite Metal Services, Hallow, UK), packed in our laboratory. For preliminary studies, four different stationary phases were considered, including: Lichrospher C18 (5 μm particle size) from Merck (Darmstadt, Germany); Type-C Silica Cogent^{TM} Bidentate C18 (4.2 μm particle size) from MicroSolv Technology Corporation (Eatontown, NJ, USA); PinnacleTM II Phenyl (3 μm particle size) from Restek Corporation (Bellefonte, PA, USA); Hydride-based RP C18 (sub-2 μm particle size) from MicroSolv Technology Corporation; Kinetex XB-C18 core-shell (3.6 μm particle size) from Phenomenex (Torrance, CA, USA).

Columns were laboratory made and prepared following the slurry packing procedure, already described in our previous work [37]. Briefly, about 20 mg of stationary phase was suspended in 1 mL of acetone and sonicated in order to avoid particle agglomeration. The slurry was pumped into the capillary under a maximum pressure of 35 MPa, using MeOH as packing solvent. Particles were retained into the capillary by means of a mechanical frit placed at the end of the capillary. Once capillary was packed, the slurry was removed and the precolumn filled with water. The column was flushed with water for 30 min, followed by a 5 mM NaCl solution for 10 min. Frits were fabricated by sintering the stationary phase for 6 s at about 700°C using a laboratory-made heated electrical wire. The Lichrospher C18, Cogent^{TM} Bidentate C18, PinnacleTM II Phenyl columns were packed for 25 cm with an effective length of 27 cm.

For Hydride-based RP C18 and Kinetex XB-C18 core-shell, column frits were prepared with an RP18 packing material of 5 μm particle size as described in a previous work [38]. These two columns were prepared with packed and effective lengths of 15 and 17 cm, respectively. Mobile phases utilized for isocratic elution of studied compounds were composed by mixtures of H_2O/MeOH and different buffers, additives, depending from the stationary phases.

2.4 Sample preparation

Three different brands of IF were purchased on the market and analyzed. The samples were subjected to a CUF-based protocol following a procedure previously described [14, 28].

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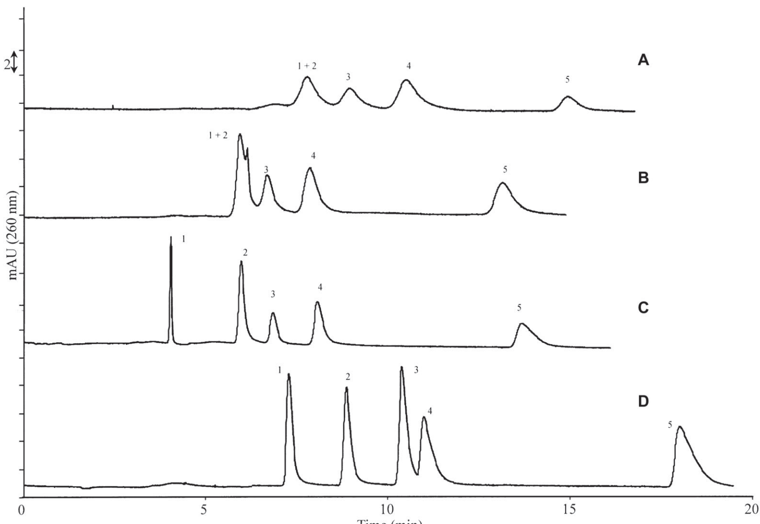

Figure 2. Nano-LC chromatograms of the separation of a standard mixture of nucleotides on different stationary phases: capillary columns packed with (A) LiChrospher C18 5 μm (75 μm × 25 cm), (B) Cogent Bidentate C18 4.2 μm (75 μm × 25 cm), (C) Hydride-based C18 sub-2 μm (75 μm × 15 cm), (D) Core-Shell XB-C18 3.6 μm (75 μm × 15 cm); mobile phase, 5% v/v methanol and 95% v/v 100 mM ammonium formate, pH 8, containing 30 mM TBAOH; column temperature, 25°C; UV detection, 260 nm; standard mixture concentration, 10 μg/mL; injection time, 20 s; flow rate, 300 nL/min. (1) CMP, (2) UMP, (3) GMP, (4) IMP, (5) AMP.

In short, samples were prepared by weighting 0.5 g of IF into a centrifuge tube (50 mL) adding water up to 15 g. The mixture was vortexed for 1 min, stored at 4°C for 15 min, and then centrifuged at 3000 rpm for 15 min. The supernatant was collected, avoiding to recover the layer of fat present on the surface. The sample solution was eluted through the CUF cartridge (3000 rpm × 30 min), previously conditioned with 5 mL of water (3000 rpm × 5 min) to reach ultrafiltration. The sample solution obtained was diluted ten times with water and injected into the nano-LC system.

3 Results and discussion

3.1 Nano-LC separation

The studied nucleotides are primary units of nucleic acids consisting of a cycling nitrogenous base (purine or pyrimidine), a pentose sugar, and at least one phosphate group [2]. They possess similar chemical structures differing in the purinic or pyrimidinic base or in several substituents, requiring a highly efficient and selective analytical method for their separation.

As reported in literature, RPLC was one of the principal mode of separating nucleotides using C18 nonpolar stationary phases. In our study, preliminary nano-LC experiments were performed studying three RP stationary phases as Lichrosphere C18, Bidentate C18, and PinnacleTM II Phenyl with mobile phases consisting of ACN/water at different ratios with a buffer medium at different pH values (3, 4, 7–9). In all of these chromatographic conditions, only a slight retention and no selectivity were obtained for the studied compounds. As expected, nucleotides, owing to their polar nature, showed a greater affinity for the mobile than the nonpolar stationary phases.

Ion-pair chromatography, generally providing important additional selectivity for charged compounds, was then selected to increase retention of nucleotides. For this purpose, different ion pairing reagents including DBA, TEA, and TBAOH were assayed.

To find the suitable reagent, as a starting point, experiments were carried out using a Lichrosphere C18 column. Taking into account the previous published data concerning the separation of nucleotides in ion-exchange LC [14], a mobile phase, consisting of 5% v/v MeOH and 95% v/v 100 mM ammonium formate, pH 5.5, containing 10 mM of the cationic ion-pair reagent, was chosen. With both DBA and TEA, a poor resolution of CMP and UMP was achieved, while GMP, IMP, and AMP were separated showing broaden peaks. The best results in terms of separation and peak efficiency were obtained with TBAOH.

With the aim to improve the column selectivity, besides the conventional porous stationary phases, Lichrospher C18 and Cogent™ Bidentate C18, innovative sorbents materials

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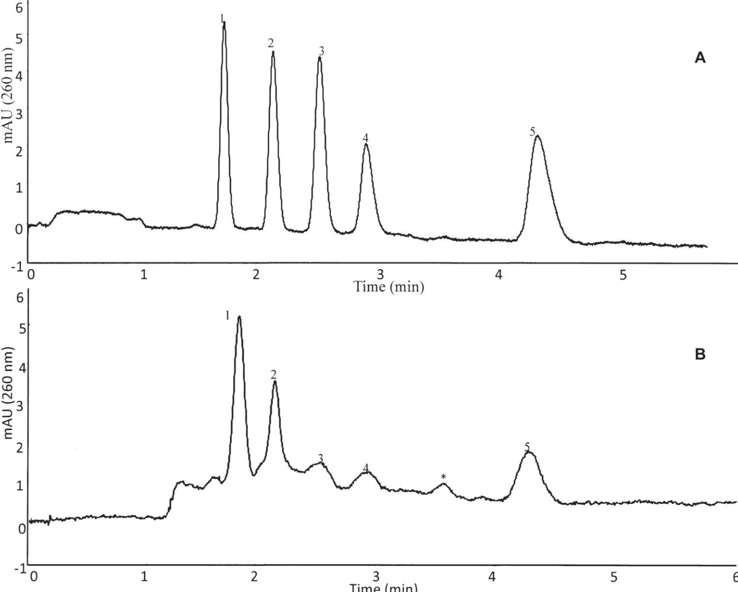

Figure 3. Nano-LC profiles of the five nucleotides standard mixture and one IF sample utilizing the optimum conditions: mobile phase, 5% v/v methanol and 95% v/v 100 mM ammonium formate, pH 8, containing 20 mM TBAOH; flow rate, 500 nL/min; injection time, 60 s. (A) Chromatogram of the standard mixture (concentration of each nucleotide, 5 μg/mL); (B) chromatogram of the IF sample after the CUF procedure.

such as Hydride-based RP C18 sub-2 μm and Kinetex XB-C18 core-shell 3.6 μm were investigated.

The last two stationary phases represent some of the recent developments in chromatographic supports for LC. The trend to reduce the particle size of the stationary phases in LC has been generated by the need to obtain more efficient columns and faster separations. The reduction of particles diameter causes a remarkable decrease of mass transfer resistance with a consequent flatter flow profile achieving optimum chromatographic performances leading to sharper peaks [39]. In our recent works on food analysis, core-shell and sub-2 μm particles stationary phases were successfully used for the separation of polyphenols [33, 34].

The standard mixture of the five nucleotides was analyzed with the four different columns using a mobile phase composed by 5% v/v MeOH and 95% v/v 100 mM ammonium formate, pH 8, containing 30 mM TBAOH, operating with a flow rate of 300 nL/min in an isocratic elution mode as shown in Fig. 2. As it can be observed, with Lichrospher C18 and Cogent™ Bidentate C18 columns, CMP and UMP eluted with the dead time providing only a partial resolution of the studied compounds. The standard mixture was then analyzed with Hydride-based RP C18 sub-2 μm, obtaining the separation of four nucleotides while CMP was still eluted with the dead volume.

Kinetex XB-C18 was the last stationary phase investigated for the nucleotides separation. This sorbent material differs from the Kinetex C18 column for di-isobutyl side chains, with a high hydrogen bond accepting capacity, which allows an increased retention of polar acidic compounds and a peak shape improvement for basic compounds (www.phenomenex.com/Info/WebDocumentServe/reversedguide.pdf). With respect to sub-2 μm particles, the core-shell technology enables to reach high peak efficiency without using high-pressure LC instrumentation.

Kinetex XB-C18 stationary phase allowed the complete separation of the five nucleotides maintaining the same elution order of the previous studied stationary phases. This last column was selected for further experiments. In order to improve the peak shape and reduce the analysis time, keeping the resolution of the studied compounds, the composition of mobile phase in terms of ion-pair reagent concentration, nature and pH of buffer, and nature of organic solvent was examined.

With the purpose to achieve the greatest separation, the retention (k) of the five nucleotides in function of TBAOH concentration in the range 5–30 mM was studied. The experiments were carried out using a mobile phase containing a mixture of 5% v/v MeOH and 95% v/v 100 mM ammonium formate, pH 8. It is clear that the concentration of the ion-pairing reagent had a relevant impact on resolution. With a concentration value of 5 mM, only GMP, IMP, and AMP were sufficiently retained. Increasing TBAOH concentration, stronger interactions between the analytes and the reagent with consequent retention and resolution were achieved (see Supporting Information Fig. 1).

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Table 1. Precision and calibration data of the nano-LC method

Analytes	Intraday precision ($n = 6$) RSD%		Interday precision (3 days, $n = 15$) RSD%		Column to column reproducibility (3 columns, $n = 9$) RSD%		LOD (μg/mL)	LOQ (μg/mL)	Linear range (μg/mL)	Slope ± SD	Intercept ± SD	R^2
	t_r	Peak area *A*	t_r	Peak area *A*	t_r	Peak area *A*						
CMP	1.2	2.3	1.8	2.8	2.4	3.1	0.25	0.50	0.5–50	$(70 \pm 1) \times 10^2$	$(-3 \pm 2) \times 10^2$	0.9995
UMP	1.8	2.5	1.9	2.9	2.5	3.8	0.25	0.50	0.5–50	$(69 \pm 1) \times 10^2$	$(3 \pm 2) \times 10^2$	0.9994
GMP	1.2	2.1	1.8	3.0	3.1	4.2	0.25	0.50	0.5–50	$(72 \pm 1) \times 10^2$	$(1 \pm 1) \times 10^2$	0.9992
IMP	1.7	2.9	2.0	3.5	3.5	4.9	0.25	0.50	0.5–50	$(64 \pm 1) \times 10^2$	$(2 \pm 1) \times 10^2$	0.9992
AMP	1.5	3.1	2.4	4.2	3.8	5.4	0.25	0.50	0.5–50	$(81 \pm 1) \times 10^2$	$(2 \pm 1) \times 10^2$	0.9988

Among the ion-pair reagent concentrations studied, a mobile phase with 20 mM of TBAOH provided the best compromise in terms of both analytes separation and analysis time. Considering the polar character of the studied solutes, the presence of a system buffer in the mobile phase is needed; therefore, nature, concentration, and pH of buffer were carefully evaluated.

Using the same mobile phase described previously, 100 mM of ammonium formate, 100 mM ammonium acetate, and 100 mM sodium phosphate, prepared at pH 8, were tested, obtaining the best peak shape of the compounds with ammonium formate buffer. The dependence of the retention with consequent separation in function of the buffer concentration was also evaluated. The nucleotides mixture was analyzed using a formate buffer, pH 8, at 50, 100, and 150 mM in the mobile phase. Lower buffer concentration produced asymmetric peaks. Although, only a partial separation of the compounds with 150 mM buffer was obtained, owing to the competing effect of the anionic buffer with the analytes for the cationic ion pairing agent (results not shown). A mobile phase with a 100 mM ammonium formate provided the complete resolution of the standard mixture.

Mobile phase buffer pH is another important parameter that influences nucleotides retention. Indeed, the ionic nature of the analytes, due to the presence of the dissociated phosphate groups, facilitates strong interactions with the cationic ion pair reagent at the appropriate pH value.

The effect of the pH in the range 4–8.5 on retention of the analytes was investigated. At pH 4, a very poor resolution was obtained. In the range from 5.5 to 8, a general increase of retention factors was observed as shown in Supporting Information Fig. 2; at pH 8.5, a decrease of the retention factors with a consequent loss of resolution mixture was achieved. Thus, a value of pH 8 was selected giving the best resolution and peak efficiency.

The retention of nucleotides was also investigated in function of the nature and content of the organic modifier. Mobile phases with a content of 5% v/v of MeOH, CAN, and 2-propanol were studied. 2-Propanol provided only a partial resolution with respect to MeOH. ACN, owing to its higher elution strength compared to MeOH, showed the worse separation results. The effect of the variations in MeOH content in the range 2.5–10% v/v on the resolution was also studied. For values lower than 5%, the compounds were strongly retained with consequent broaden peaks. An increase of the organic modifier content leads to a reduction in retention for all compounds with a resulting decrease in the resolution mixture.

In order to improve the method sensitivity, the nanoinjector with a fixed volume of 100 nL was replaced with a modified injection valve. This device allowed to introduce in the column increasing sample volumes by rising the time of injection. Analyses of the standard mixture with values of injection time in the range 20–80 s were carried out, plotting the injected volumes in function of the peak height. A linear trend was obtained up to 60 s (about 450 nL of volume injected), while broaden peaks were obtained with 80 s. The

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Table 2. Recovery and content of nucleotides in IF samples

Analytes	Added concentration (μg/mL)	Recovery (%) ± SD	RSD%	Content of nucleotides found (μg/g ± SD)		
				IF 1	IF2	IF3
CMP	0.5	95 ± 5	5.6	108 ± 6	111 ± 9	70 ± 6
	5	97 ± 2	3.2			
UMP	0.5	94 ± 6	4.9	48 ± 7	42 ± 7	53 ± 7
	5	101 ± 4	2.9			
GMP	0.5	93 ± 5	4.2	11 ± 8	13 ± 6	12 ± 8
	5	103 ± 4	3.5			
IMP	0.5	91 ± 7	5.7	11 ± 9	13 ± 7	13 ± 8
	5	103 ± 8	6.4			
AMP	0.5	92 ± 7	7.9	29 ± 7	27 ± 9	20 ± 9
	5	103 ± 6	6.1			

best injection conditions showed LODs of 0.25 μg/mL for all the studied compounds. The values found were higher than those published in some works performed with HPLC and CE-MS [14, 28] but they were in accord with the sensitivity requirements for nucleotides analysis in IF samples.

Figure 3A shows the nano-LC separation of the five nucleotides obtained in less than 5 min, employing a mobile phase composed by 5% v/v MeOH and 95% v/v 100 mM ammonium formate, pH 8, containing 20 mM TBAOH with a flow rate of 500 nL/min.

3.2 Nano-LC method validation

Under the optimal conditions, in order to perform the quantitative analysis of the studied nucleotides in IF samples, the nano-LC method was validated evaluating repeatability, column-to-column reproducibility, sensitivity, linearity range, and recovery. The analytical precision was performed by assessing a standard mixture at a concentration of 5 μg/mL for six consecutive runs in the same day and over three different days. In Table 1, intra- and interday precision data concerning retention time and peak areas expressed as the RSD percentage (RSD%) are reported. The RSD values for the retention time were in the range 1.2–1.5 and 1.8–2.4% for intraday and day-to-day precision, respectively. Good results were also achieved for peak areas with RSD < 3.1% and 4.2% for intraday and day-to-day experiments, respectively.

Column-to-column reproducibility was also evaluated performing the analysis of the nucleotides in three different capillary columns packed with the same procedure, obtaining satisfactory results with RSD values lower than 3.8 % for retention time and 5.4% for peak area. Method sensitivity was characterized by LODs and quantitation (LOQ), which were determined at an S/N of three and ten times, respectively. LODs and LOQs values of 0.25 μg/mL and 0.5 μg/mL, for all selected nucleotides, were achieved.

Method linearity was evaluated considering the concentration range between LOQ values and 50 μg/mL for all the analytes, assessing six concentration levels in triplicate runs for each analyte. The calibration curves were obtained plotting the peak areas as a function of analytes concentration. The regression analysis was performed by calculating the coefficient of determination (R^2), which ranges between 0.9988 and 0.9995 confirming a good linearity (Table 1).

3.3 Application to real samples

The developed nano-LC method was applied to the analysis of nucleotides in three different IF subjected to a CUF procedure as described in Section 2.4.

To assess the accuracy of the method, recovery experiments were carried out using milk samples, fortified with a standard mixture of nucleotides at two different concentration levels (0.5 and 5 μg/mL). The recovery of the analytes was obtained as the percentage ratio between the peak area from the spiked and unspiked samples. The analyses were performed in triplicate at each concentration level. Table 2 reports the mean recovery values, SD and RSD% of the selected nucleotides for both fortification levels. Recovery data were in the range between 91 and 103% with RSD% values lower than 7.9% showing that the CUF procedure was an efficient method for nucleotides extraction. In Table 2, the amounts found in IF samples are also reported. The values obtained are in agreement with the data declared and with those reported in [28]. Figure 3B shows the nano-LC profile of one IF sample.

4 Concluding remarks

A nano-LC method for the rapid determination of ribonucleotides 5′-monophophates in pediatric formula has been developed and validated. A laboratory-assembled nano-LC system was utilized for chromatographic experiments employing a capillary column packed for 15 cm with a novel core-shell XB-C18 (3.6 μm). The studied nucleotides were

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completely separated in less than 5 min in isocratic elution mode. A simple and fast sample treatment based on a CUF procedure, not requiring hazardous solvents, was applied. A good chromatographic performance, assessed in terms of repeatability, linearity, and sensitivity, was achieved. The applicability of the method was demonstrated analyzing the studied nucleotides in IF. The positive results reached in this study proved that the nano-LC method developed is a useful approach for analyzing nucleotides in pediatric samples and can be proposed as a complementary method to a conventional chromatographic one. In addition, a great advantage results from the use of a low flow rate with a corresponding minimal solvent consumption making this technology eco-friendly.

María Mateos-Vivasgratefully acknowledges financial assistance from the Ministerio de Educación, Spain (FPU grant), especially for the additional fellowship (EST13/00852) granted for the research stay at the CNR, Rome. Acknowledgments are due to MicroSolv Technology Corporation (Eatontown, NJ, USA) and to Phenomenex, Torrance, CA, USA) for donating us the Hydride-based RP C18 (sub-2 μm, particle size) and Kinetex XB-C18 core-shell (3.6 μm, particle size) stationary phases, respectively.

The authors have declared no conflict of interest.

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Supporting information: Rapid determination of nucleotides in infant formulas by means of nano-liquid chromatography

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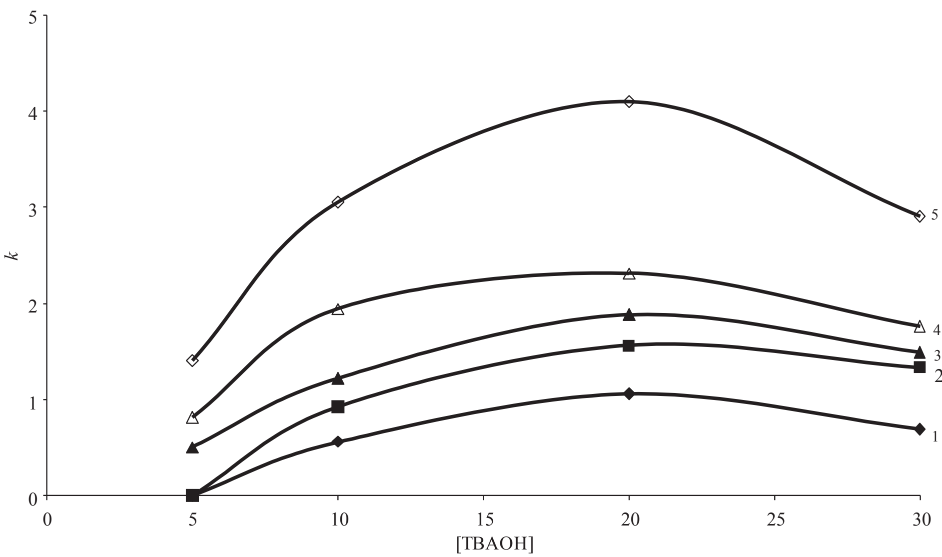

Figure 1S: Influence of the ion pairing reagent concentration present in the mobile phase on the retention factors *k* of the studied 5'-nucleotides. Capillary column: Core-Shell XB-C18 3.6 µm (75 µm x 15 cm); mobile phase, 5% (v/v) methanol and 95% (v/v) 100 mM ammonium formate pH 8, concentration of TBAOH varied in the range 5 – 30 mM, column temperature, 25 °C, UV detection, 260 nm; standard mixture concentration, 10 µg/mL, injection time 20 s, flow rate, 300 nL/min.

1) CMP, 2) UMP, 3) GMP, 4) IMP, 5) AMP.

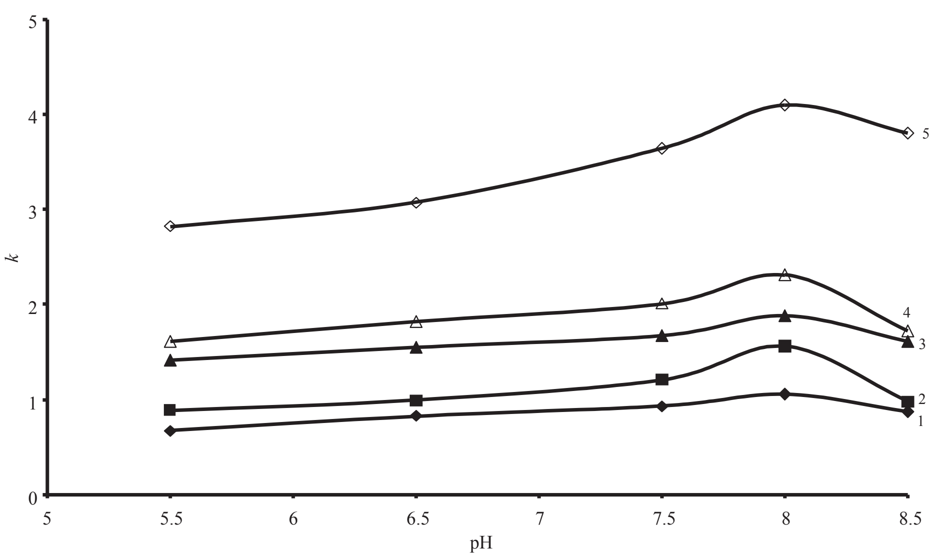

Figure 2S: Effect of pH buffer in the mobile phase on the retention factors *k*.

Mobile phases: 5% (v/v) methanol and 95% (v/v) 100 mM ammonium formate at different values of pH (5.5 – 8.5). For other conditions see Fig 1S.

Cromatografía de Interacciones Hidrofílicas acoplada a Espectrometría de Masas en Tándem, en Presencia de Reactivos Formadores de Pares Iónicos Hidrofílicos, para la Separación de Nucleósidos y Nucleótidos mono-, di- y trifosfato

II.2

ARTÍCULO DE INVESTIGACIÓN II.2

RESUMEN

II.2. Cromatografía de interacciones hidrofílicas acoplada a espectrometría de masas en tándem, en presencia de reactivos formadores de pares iónicos hidrofílicos, para la separación de nucleósidos y nucleótidos mono-, di- y trifosfato

Los nucleósidos y nucleótidos son compuestos relacionados con la regulación y modulación de numerosos procesos fisiológicos, por lo que su determinación resulta de considerable interés en diferentes áreas científicas como bioquímica, medicina, genética y análisis de alimentos. Sin embargo, la separación simultánea de estos compuestos mediante cromatografía líquida acoplada a espectrometría de masas (LC-MS) no es una tarea fácil debido a su elevada polaridad, que limita su retención en la modalidad de fase inversa. Otra dificultad adicional es a causa de la interacción de los compuestos fosforilados (como los nucleótidos mono-, di- y trifosfato) con partes específicas del sistema cromatográfico, que genera una falta de sensibilidad y precisión. Estas interacciones se producen a través de la adsorción de los analitos sobre los grupos silanol de las paredes internas de los capilares, cuando se trabaja en medio ácido, o bien a través de la formación de complejos metálicos entre los compuestos fosforilados y los metales, principalmente el hierro procedente del acero inoxidable de las diferentes partes que forman el sistema instrumental.

Los métodos cromatográficos desarrollados hasta la fecha están centrados en la separación bien de nucleósidos o bien de nucleótidos, de forma individual. Hay pocos métodos descritos en bibliografía enfocados a la separación conjunta de ambos grupos, puesto que ello implica elevados tiempos de análisis, lo que limita su aplicación.

El objetivo principal de este trabajo fue desarrollar un método rápido y eficiente para la separación conjunta de 20 nucleósidos y nucleótidos mono-, di- y trifosfato mediante cromatografía líquida de interacciones hidrofílicas (HILIC) acoplada a espectrometría de masas en tandem (MS/MS), capaz de solventar los diferentes problemas que presenta la determinación de estos compuestos.

Se estudió el comportamiento de los nucleósidos y nucleótidos en tres columnas HILIC diferentes (*XBridge-Amide, CoreShell y ZIC-HILIC*) utilizando fases móviles con diferentes porcentajes de acetonitrilo y agua. En el caso de los nucleósidos, se observa su retención incluso cuando el contenido de fase orgánica es bajo; esta retención sufre un ligero aumento hasta un 80 % (v/v) de acetonitrilo/agua, y después se produce un incremento importante de la misma. Este es un comportamiento compatible con un mecanismo de partición entre la fase móvil rica en componente orgánico y la capa de agua inmovilizada sobre la fase estacionaria HILIC.

En el caso de los nucleótidos, sin embargo, se observa que no se retienen en las fases estacionarias estudiadas cuando se trabaja con porcentajes de acetonitrilo inferiores al 80 % (v/v). Los nucleótidos son compuestos más polares que los nucleósidos por lo que su retención en HILIC debería ser mayor. Este comportamiento opuesto al esperado pudo ser explicado teniendo en cuenta que los nucleótidos, cargados negativamente, sufren repulsión debido a la carga negativa residual de la fase estacionaria, lo que impide su retención. Sin embargo, cuando la fase móvil es mayoritariamente orgánica, la interacción hidrofílica de los analitos es más fuerte que la repulsión electrostática de la fase estacionaria. Este comportamiento ha sido descrito por Alpert bajo el

término ERLIC (*Electrostatic Repulsion-Hydrophilic Interaction Chromatography*).

La concentración de sales y el pH de la fase móvil afectan de manera significativa a la separación en HILIC, ya que si los analitos se encuentras ionizados al pH de trabajo se producirá una competición con las sales por los sitios activos de la fase estacionaria. Se evaluaron fases móviles (acetonitrilo/agua) con diferentes sales y concentraciones de las mismas. Los nucleósidos mostraron señales bien definidas en todos los medios estudiados y su retención y sensibilidad no se vieron afectadas por la presencia de sales. En el caso de los nucleótidos se observaron comportamientos fuertemente dependientes de la composición y concentración de las sales. En ausencia de sales, o con una baja concentración, los nucleótidos no se retenían debido a la repulsión entre las cargas negativas de los grupos fosfato y la carga negativa residual de la fase estacionaria. Utilizando una mayor concentración de sales se evitaba dicha repulsión y se conseguía la retención de los nucleótidos, aunque se obtenían señales muy anchas debidas posiblemente a la interacción de los compuestos fosforilados con diversas partes del sistema cromatográfico. Se encontró una adecuada retención de los nucleótidos con señales bien definidas cuando se utilizaron fases móviles con dietilamina (DEA) 50 mM y en presencia de hexafluoroisopropanol (HFIP) 100 mM (${}_{w}^{w}pH$ 9-${}_{w}^{s}pH$ 10).

Manteniendo la relación HFIP-DEA (2:1) para no variar el pH de la fase móvil, se estudió el efecto de la concentración sobre la retención de los analitos. El tiempo de retención de los nucleósidos sólo se vio afectado ligeramente con el incremento en la concentración de sales, mientras que la retención de los nucleótidos aumentaba

significativamente. También se comprobó que sólo en el caso de utilizar concentraciones de HFIP-DEA superiores a 150-75 mM se observaba una disminución en la sensibilidad de la señal probablemente debido a la supresión iónica. No se producía dicha supresión cuando se utilizaban concentraciones inferiores debido a que la DEA y el HFIP tienen puntos de ebullición muy bajos, lo cual reduce la tensión superficial en el electrospray y resulta en un incremento general de la eficiencia de la ionización.

Además de la DEA, se estudió el efecto de otras especies básicas susceptibles de formar asociaciones iónicas con los nucleótidos: hidróxido de tetrabutilamonio (TBA), trietilamina (TEA) y amoniaco (NH_3). Se comprobó que la adición de cualquiera de ellas a la fase móvil incrementaba la retención de los nucleótidos sin afectar significativamente a la retención de los nucleósidos. Además, para un mismo nucleótido, la retención era mayor cuanto más polar era la base (NH_3>DEA>TEA>TBA). En el caso del TBA, el compuesto de asociación iónica formado era muy apolar y por tanto no se retenía en la fase estacionaria HILIC. El efecto de estas especies básicas en la sensibilidad de la detección en MS/MS depende de la volatilidad de la base utilizada (NH_3≈DEA>TEA>TBA). Aunque el efecto era similar cuando se utilizaban NH_3 o DEA, en presencia de NH_3 se obtuvieron señales más anchas tanto para nucleósidos como para nucleótidos. A la vista de los resultados obtenidos, la composición óptima de la fase móvil para la separación de los 20 analitos fue la formada por mezclas de acetonitrilo/agua con HFIP 100 mM y DEA 50 mM.

Una vez optimizada la fase móvil se realizó un estudio comparativo de las tres fases estacionarias para la separación por

grupos (nucleósidos y nucleótidos mono-, di- y trifosfato) y por familias. La columna *XBridge-Amide* es la que mejores resultados proporciona en cuanto a sensibilidad y eficiencia de la separación. En esta columna se consiguió la separación de los analitos en menos de 9 minutos con un gradiente acetonitrilo/agua en las siguientes condiciones: elución isocrática con acetonitrilo/agua 82:18% v/v (con HFIP 100 mM y DEA 50 mM) durante 3 minutos, y después, un aumento rápido de 18 a 22 % de agua. Excepto para un par de nucleósidos, Adenosina (A) e Inosina (I), se obtuvieron buenos valores de resolución, lo cual es importante teniendo en cuenta que en cada uno de los cuatro grupos de analitos (nucleósidos y nucleótidos mono-, di- y trifosfato) existen dos parejas de analitos que se diferencian sólo en una unidad m/z. En estas condiciones se obtuvieron límites de detección (LODs) entre 5 y 60 ng mL^{-1}.

El trabajo se completó con un estudio detallado del mecanismo de retención de los nucleósidos y nucleótidos en las condiciones óptimas de fase móvil y fase estacionaria. Para ello se evaluó el efecto de la temperatura sobre la retención y se relacionó a través de la ecuación de Van't Hoff. Se estudiaron seis temperaturas de columna diferentes (20 a 45 °C) y se comprobó que, en las condiciones optimizadas, los nucleótidos generan pares iónicos hidrofílicos que interaccionan con la fase estacionaria a través de un mecanismo de partición, sin que existan influencias significativas de otro tipo de interacciones. Estos resultados fueron confirmados con los obtenidos al correlacionar los factores de retención con el log D, y también estudiando la influencia del porcentaje de acetonitrilo sobre la retención, en una fase móvil que contenía HFIP 100 mM y DEA 50 mM. Los resultados obtenidos indican que los nucleósidos se retienen por un mecanismo de partición en las distintas

condiciones experimentales estudiadas; sin embargo, el comportamiento de los nucleótidos se ajusta a un mecanismo de partición sólo cuando la fase móvil contiene HFIP 100 mM y DEA 50 mM.

ARTÍCULO DE INVESTIGACIÓN II.2

Journal of Chromatography A, 1414 (2015) 129-137

Journal of Chromatography A, 1414 (2015) 129–137

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates

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ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form 14 August 2015
Accepted 20 August 2015
Available online 28 August 2015

Keywords:
Hydrophilic interaction chromatography (HILIC)
Hydrophilic ion-pairing
Nucleosides
Nucleotide mono-, di- and triphosphates

ABSTRACT

A fast and efficient method for the simultaneous separation of highly polar compounds, in this case nucleosides and nucleotide mono-, di- and triphosphates, using hydrophilic interaction chromatography coupled with tandem mass spectrometry (HILIC–MS/MS) is proposed. This new separation method revealed the possibilities of the formation of hydrophilic ion-pairing compounds.

Three stationary phases (HILIC XBridge-Amide, HILIC-CoreShell and ZIC-HILIC) were assayed for the separation of 20 target analytes, and a detailed study of the composition of the mobile phase was made using different salts at different concentrations in a organic-rich mobile phase. We report that in order to prevent the adsorption of nucleotides on the LC–MS setup and to enhance their retention on the HILIC stationary phase, a mobile phase containing hexafluoro-2-propanol and different cations should be used. Four cations were evaluated: ammonium, diethylammonium, triethylammonium and tetrabutylammonium. The results revealed the formation of an ionic-association compound between the phosphorylated analytes and the cationic ion-pairing reagents, whose retention increased with the polarity of the cationic ion-pairing reagent.

HILIC XBridge-Amide was found to be the most suitable column for the separation of these analytes, and the optimized mobile phase consisted of an ACN/UHQ water mixture (3 min of isocratic elution using 82:18%, v/v and then a fast gradient from 18% to 22% of water) with 100 mM hexafluoro-2-propanol and 50 mM diethylamine (${}_{w}{}^{w}$pH 9–${}_{w}{}^{s}$pH 10). In a total analysis time of 8 min, good results were achieved in terms of resolution. Under these optimum conditions, a further comprehensive study of the retention mechanism was carried out.

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1. Introduction

Nucleosides and nucleotides are a class of chemicals related to the regulation and modulation of several physiological processes [1], their determination being of considerable interest across a wide range of scientific areas such as biochemistry, medicine, genetics, metabolomics and food analysis [2,3]. Their concentration in cells provides information for understanding cellular energy metabolism, and their quantification in plasma samples can be used to assess oxidative stress. They are also useful for obtaining information about different pathologies, since it is well known that the concentration of some nucleosides and nucleotides is related to the immune response in oncologic patients [4–6]. Nucleosides and nucleotides are also found in foodstuffs. Their occurrence in these matrices helps the absorption of iron and stimulates damage repair in the gut and also enhances the immune response [7].

Owing to the high-polarity of these compounds, capillary zone electrophoresis (CZE) [8,9] and micellar electrokinetic chromatography (MEKC) [10,11] have been the techniques used for their determination in urine samples. Our group [12,13] has also developed a method for the determination of nucleotide monophosphates in infant formulas and in human milk by means of CZE–MS. Several different types of chromatographic separation have also been proposed based on reversed-phase liquid chromatography [14], ion-pair liquid chromatography [15], ion-exchange liquid chromatography [16] and hydrophilic interaction liquid chromatography (HILIC) [17–19].

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http://dx.doi.org/10.1016/j.chroma.2015.08.040
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It has been suggested that in the analysis of phosphorylated compounds (e.g. nucleotides mono- di- and tri-phosphates) by LC–MS these analytes may interact with specific parts of the LC–MS setup [20]. These interactions are produced through the adsorption of the analytes on silanol groups or through the complexing of metallic cations with their phosphorylated residues. This results in a decrease in the chromatographic signal and in peak tailing [21]. Different strategies have been proposed to prevent hydrogen bonding between the silanol groups on the inner-wall surface of the fused silica capillary and phosphorylated compounds, such as derivatization of the free silanol groups of the silica by endcapping [22], or deactivation of the fused silica tubing with dimethyldichlorosilane [21]. The interaction between phosphopeptides and metals (mainly iron from stainless steel or solvents) in HPLC–ESI-MS devices affects detection due to the formation of phosphopeptide–Fe(III) complexes [23]. In order to prevent this interaction, several strategies have been proposed, such as the substitution of stainless steel tubing by polyether ether ketone (PEEK), the use of a high-pH mobile phase [20], pre-treatment of the chromatographic system with phosphoric acid [24], and the use of mobile phases with carbonate anions [25] or chelating agents such as EDTA [23].

Although CE has been applied to the determination of nucleosides and nucleotides, to the best of our knowledge there is no CE method for the simultaneous separation of these compounds. Moreover, chromatographic methods have focused on the separation of nucleosides or nucleotides and the few methods that do allow simultaneous separation are limited by excessively long separation times [7,26–29]. It may be concluded that the simultaneous separation of these compounds is a highly complex issue owing to their chemical similarities, their high polarity, which limits their retention in reversed-phase chromatography, and also because of the adsorption of these phosphorylated compounds on the chromatographic system, resulting in a lack of sensitivity and precision.

The aim of this work was thus to solve these issues by developing a method that would allow the rapid and efficient chromatographic separation of a large number of nucleosides and nucleotides by means of HILIC coupled with tandem mass spectrometry. In order to achieve this, a detailed study of the variables affecting HILIC separation was carried out, involving three HILIC columns and organic-rich mobile phases in the presence of cationic ion-pairing reagents. The retention mechanism in the final conditions was also characterized.

2. Experimental

2.1. Chemicals

Analytical standards of *adenosine* (A), CAS RN [58-61-7]; *cytidine* (C), CAS RN [65-46-3]; *guanosine* (G), CAS RN [118-00-3]; *inosine* (I), CAS RN [58-63-9]; *uridine* (U), CAS RN [58-96-8]; *adenosine 5′-monophosphate* (AMP), CAS RN [4578-31-8]; *cytidine 5′-monophosphate* (CMP), CAS RN [63-37-6]; *disodium salt hydrate of guanosine 5′-monophosphate* (GMP), CAS RN [5550-12-9]; *disodium salt of inosine 5′-monophosphate* (IMP), CAS RN [352195-40-5]; *disodium salt of uridine 5′-monophosphate* (UMP), CAS RN [3387-36-8]; *sodium salt of adenosine 5′-diphosphate* (ADP), CAS RN [20398-34-9]; *sodium salt hydrate of cytidine 5′-diphosphate* (CDP), CAS RN [63016-64-8]; *sodium salt of guanosine 5′-diphosphate* (GDP), CAS RN [43139-22-6]; *sodium salt of inosine 5′-diphosphate* (IDP), CAS RN [81012-88-6]; *disodium salt hydrate of uridine 5′-diphosphate* (UDP), CAS RN [27821-45-0]; *disodium salt hydrate of adenosine 5′-triphosphate* (ATP), CAS RN [34369-07-8]; *disodium salt of cytidine 5′-triphosphate* (CTP), CAS RN [36051-68-0]; *sodium salt hydrate of guanosine 5′-triphosphate* (GTP), CAS RN [36051-31-7]; *trisodium salt of inosine triphosphate* (ITP), CAS RN [35908-31-7] and *trisodium salt dihydrate of uridine 5′-triphosphate* (UTP), CAS RN [116295-90-0] were purchased from Sigma–Aldrich (Steinheim, Germany). The physical properties and the MS/MS parameters of these analytes are shown in Table S1.

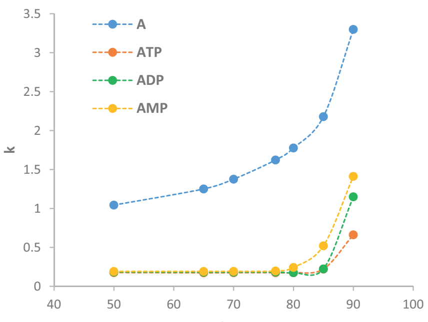

Fig. 1. Retention factors (*k*) for A, AMP, ADP and ATP, plotted versus the ACN percentage in the mobile phase, using the HILIC-CoreShell column and a mobile phase composed of only ACN and UHQ water.

The organic solvent, acetonitrile (ACN), was of HPLC grade (Merck, Darmstadt, Germany) and was used as received. Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noain, Spain) Ultramatic water purification system. The reagents used in the study of the mobile phase composition – hexafluoro-2-propanol (HFIP), diethylamine (DEA), triethylamine (TEA), tetrabutylammonium hydroxide (TBA), formic acid (HCOOH) and trifluoroacetic acid (TFA) – were provided by Sigma–Aldrich; reagent grade ammonia (NH_3) was purchased from Scharlau (Barcelona, Spain) and perchloric acid ($HClO_4$) was provided by Panreac (Barcelona, Spain).

2.2. Instrumentation

HPLC analyses were performed on an HP 1200 Series chromatograph from Agilent (Waldbronn, Germany). The 6410 Triple Quad mass spectrometer (Agilent, Waldbronn, Germany) was equipped with an electrospray ionization (ESI) source. The ESI settings were a capillary voltage of ±3500 V, a drying-gas flow of 12 L min^{-1} at a temperature of 350 °C, and a nebulizer pressure of 35 psi. The analytical columns were a HILIC XBridge Amide packed with 3.5-μm particles from Waters (Milford, MA, USA), a HILIC-CoreShell packed with 2.6-μm particles from Phenomenex (Torrance, CA, USA), and a ZIC-HILIC packed with 3.5-μm particles from Merck (Darmstadt, Germany). The characteristics of the chromatographic columns used are shown in Table S2. ${}_{w}^{w}pH$ and ${}_{w}^{s}pH$ values were determined with a Metrohm 691 pH Meter (Barcelona, Spain). Other mass spectrometry conditions were a fragmentor voltage of 100 V, collision energies optimized for each analyte and ranging from 5 to 10 eV for NUs to 25 eV for NMPs, NDPs, and NTPs; and a window of 1u for the isolation of the precursor ions, widened to 4u for the product ions. The dwell time for the analysis of the 20 analytes was 200 ms.

2.3. Preparation of standards

Initial stock solutions at 500 μg mL^{-1} were prepared in UHQ water. These stock solutions were stored at 4 °C in brown glass bottles. Working solutions were prepared daily at 5 μg mL^{-1} by mixing the appropriate amounts of the stock solution/s and diluting in ACN.

Table 1
Conditions used and results obtained for nucleotide monophosphates (NMPs) in the HILIC stationary phases (XBridge Amide, CoreShell and ZIC) using mobile phases (ACN/UHQ water: 82:18%, v/v with Amide and ZIC columns and 77:23%, v/v with CoreShell) with different salts and concentrations of them.

Acid	Base	pH[a]	Result	Acid	Base	pH[a]	Result
–	–	7	No retention (Fig. 2a)	HCOOH 15 mM	NH_3 50 mM	9	Retention with broad peak shapes
HFIP 100 mM	–	5.7	No retention (Fig. 2.b)	NH_4ClO_4 100 mM	NH_3 50 mM	9	Retention with broad peak shapes
HFIP 10 mM	DEA 5 mM	9	No retention	TFA 100 mM	NH_3 200 mM	9	Retention with broad peak shapes
HFIP 10 mM	NH_3 5 mM	9	No retention	HFIP 100 mM	NH_3 50 mM	9	Retention with **good** peak shapes
HCOOH 49 mM	DEA 50 mM	9	Retention with broad peak shapes (Fig. 2c)	HFIP 100 mM	DEA 50 mM	9	Retention with **good** peak shapes (Fig. 2d)

[a] pH measurements were performed before the addition of organic solvent ($_{w}{}^{w}$pH).

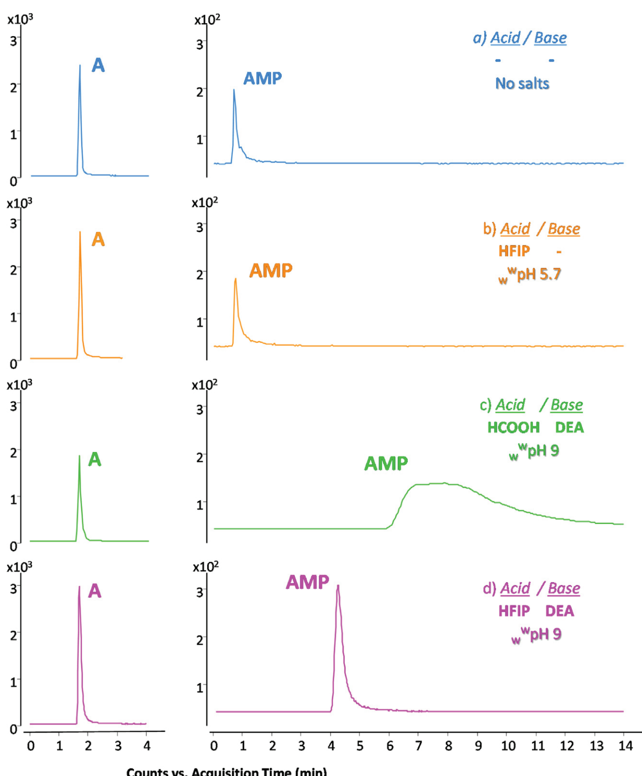

Fig. 2. Selected reaction monitoring (SRM) of A (left) and AMP (right) using four different mobile phases consisting mainly of ACN/UHQ water and the following additives: (a) without salts; (b) 100 mM HFIP $_{w}{}^{w}$pH = 5.7; (c) 49 mM HCOOH and 50 mM DEA $_{w}{}^{w}$pH 9 and (d) 100 mM HFIP + 50 mM DEA $_{w}{}^{w}$pH 9.

The mobile phases contained a mixture of organic solvent (ACN) and aqueous media at different pH values and with different concentrations of salts. To measure $_{w}{}^{w}$pH values, the pH meter was calibrated using aqueous buffers and pH measurements were performed before the addition of organic solvent. The apparent $_{w}{}^{s}$pH, after adding the organic solvent was also measured.

The mobile phase optimized for the simultaneous separation of Nus, NMPs, NDPs and NTPs consisted of 100 mM HFIP and 50 mM DEA in UHQ water, $_{w}{}^{w}$pH 9 (solvent A), and 100 mM HFIP and 50 mM DEA in ACN, $_{w}{}^{w}$pH 9–$_{w}{}^{s}$pH 10 (solvent B). The separation was carried out as follows: 3 min of isocratic elution using 18:82% (v/v) of solvents A:B, then a fast gradient from 18% to 22% of solvent A, another isocratic period of 7 min and finally back to the initial conditions. The chromatography flow rate was set at 0.35 mL min^{-1} and the analytical column was thermostatted at 20 °C.

2.4. Chromatographic parameters

The parameters evaluated are shown in Supplementary information.

3. Results and discussion

As mentioned above, the aim of this work was to develop a fast and efficient method for the simultaneous separation of nucleosides and nucleotide mono- di- and triphosphates by means of

HILIC–MS/MS. To accomplish this, three steps were carried out sequentially: (i) optimization of the composition of the mobile phase to improve separation; (ii) selection of the stationary phase and optimization of separation; and (iii) study of the retention mechanism.

3.1. Effect of the composition of the mobile phase on the retention of nucleosides and nucleotides in different HILIC stationary phases

3.1.1. Influence of the water-organic composition of the mobile phase

A study was made of the behaviour of all the nucleosides and nucleotides in the three columns described in Section 2, using mobile phases with different percentages of ACN and UHQ water. Fig. 1 shows the results obtained for the adenosine family with the HILIC-CoreShell column; they are similar for the other families and columns (data not shown). The nucleosides were retained in the stationary phase even when the content of the organic phase was low, and this retention increased slightly with an increase in the ACN percentage from 50% to 77%, after which it increased dramatically from 80% to 90%. This behaviour is compatible with a HILIC partition mechanism between the mobile phase, which is rich in organic components, and the layer of water immobilized on the stationary phase.

Since nucleotides are more polar than nucleosides, they should exhibit greater retention than the latter. However, for ACN percentages below 80%, nucleotides were not retained on the stationary phase and showed a slight degree of retention when the mobile phase contained ACN percentages higher than 80%. Although the increase in the organic percentages, between 80% and 90%, favoured the retention due to hydrophilic interactions reaching an optimum for the separation at 90%, the fact that the nucleotides showed less retention than nucleosides indicates that under these conditions other interactions must occur. This type of behaviour can be explained if it is taken into account that nucleotides (negatively charged) may undergo repulsion due to the residual negative charges of the stationary phase. In 2008, Alpert [30] reported similar findings and indicated that when the mobile phase is mostly organic nucleotides will be retained through hydrophilic interactions by means of a combination designated by Alpert et al. as ERLIC (electrostatic repulsion–hydrophilic interaction chromatography). Under ERLIC conditions, the hydrophilic interaction of the analytes is stronger than the electrostatic repulsion from the stationary phase.

3.1.2. Salt concentration in the mobile phase

The presence of salts and the pH value of the mobile phase may exert a strong influence on separation in HILIC. Depending on the pH, the analytes may become ionized, which makes them susceptible to undergoing other types of interaction. Such interactions are strongly dependent upon the concentration of salts owing to their competition for the active sites of the stationary phase, such that it may have a strong effect on HILIC separation [31]. The increase in the concentration of salts usually involves an increase in retention [32]. Although different authors have noted that in the presence of carbonate or EDTA nucleotides do not exhibit tailing peaks [23,25], in the present work it was not possible to study these salts because they are not soluble in media with high percentages of ACN, such as those used in HILIC. The reagents used here are shown in Table 1. The weak acid HFIP was used owing to its low boiling point (bp = 58.2 °C), a property that makes it compatible with coupling with mass spectrometry and that influences the improvement of peak symmetry [33–35].

Different previous studies were conducted using mobile phases (ACN/UHQ water) with different salts at different concentrations. Table 1 shows the conditions and the results obtained for the

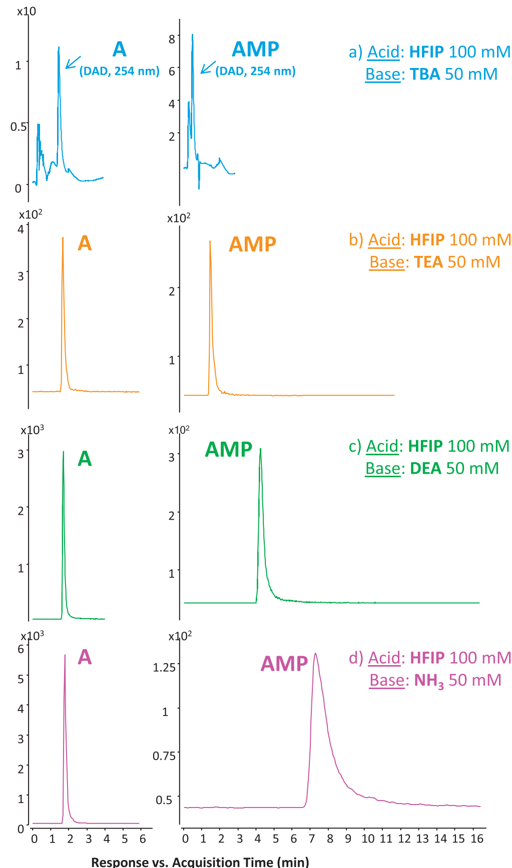

Fig. 3. Selected reaction monitoring (SRM) of A (left) and AMP (right) using four different mobile phases containing mainly ACN/UHQ water mixtures and 100 mM HFIP and the following ion-pair reagents: (a) 50 mM TBA (DAD, 254 nm); (b) 50 mM TEA; (c) 50 mM DEA and (d) 50 mM NH_3 ($_w{}^w$pH 9 in all cases). A HILIC XBridge-Amide column and a mobile phase containing 82% of ACN were used.

determination of the five nucleotide monophosphates (NMPs) with the three different columns studied in this work. Fig. 2 compares the behaviour of the nucleoside (A) and the nucleotide from the same family (AMP). In all media studied, the nucleosides (NUs) showed well-defined signals, their retention and sensitivity being independent of the salts present in the mobile phase (Fig. 2). However, the NMPs exhibited a behaviour that was strongly dependent on the salt composition and concentration. In the complete absence of salts, or when they were present at a very low concentration, the electrostatic repulsion between the negatively charged phosphate group and the residual negative charge of the stationary phase led to the non-retention of the NMPs and their elution in the injection front (Fig. 2a and b). Upon using a mobile phase containing a higher salt concentration (formate-DEA and formate, perchlorate and ammonium trifluoroacetate, all at $_w{}^w$pH 9–$_w{}^s$pH 10), the charge repulsion was abolished and NMPs were retained. However, it should be noted that a marked broadening of the NMP signals occurred, possibly due to the interaction of the phosphorylated

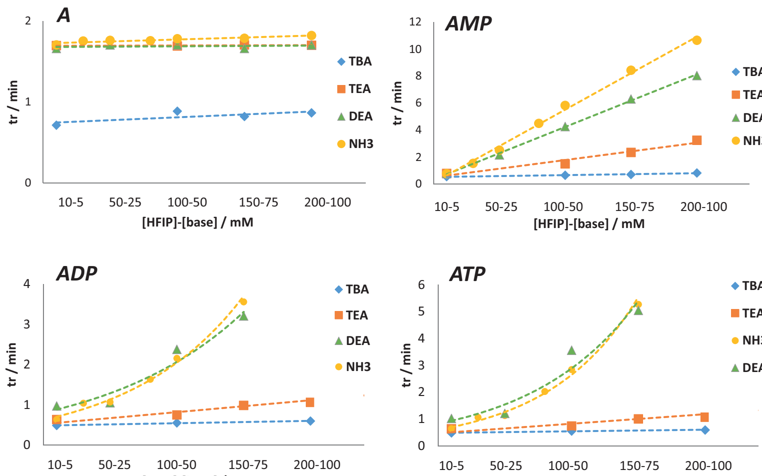

Fig. 4. Retention times for A, AMP, ADP and ATP, plotted versus HFIP-base concentrations, the bases being TBA, TEA, DEA and NH_3. A HILIC XBridge-Amide column and a mobile phase containing 82% of ACN in the case of A and AMP, and 75% of ACN for ADP and ATP were used.

compounds and some part of the chromatographic system (Fig. 2c). The signals of the NMPs were well defined and remained unaffected by peak-broadening phenomena or electrostatic repulsion with the stationary phase upon using a mobile phase containing 50 mM DEA and 100 mM HFIP (Fig. 2d).

3.1.3. Influence of the concentration of DEA and HFIP

Since nucleotides undergo a change in the number of negative charges with the change in pH, it was decided to maintain a buffered medium of $_{w}^{w}$pH 9–$_{w}^{s}$pH 10 (the medium in which the best results were obtained as regards retention and the shape of the signals) on studying the effect of concentration. For each concentration of HFIP–DEA, we assessed the retention time of the analytes separately by groups (NUs, NMPs, NDPs and NTPs) in the three HILIC columns. Fig. S1 shows the results obtained with the XBridge-Amide column; the behaviour is similar to that observed in the other two HILIC stationary phases.

From the above study, it may be concluded on one hand that the retention time of the NUs was modified slightly with the increase in the salt concentration in the mobile phase. High salt concentrations would produce an increase in the volume or hydrophilicity of the aqueous layer, attracting the solvated analytes and hence causing a slightly greater retention. On the other hand, the retention of NMPs, NDPs and NTPs increased significantly as the concentration of HFIP–DEA in the mobile phase rose. On comparing the behaviour shown by NDPs and NTPs, it may be seen that the retention of the latter was greater than that of the former. It should be noted that in the CoreShell column a higher salt concentration was required (100 mM HFIP–50 mM DEA) for the nucleotides to be eluted in a time similar to or longer than that of the nucleoside of the same family, whereas in the ZIC and XBridge-Amide columns a lower concentration (50 mM HFIP–25 mM DEA) was required for this effect to be achieved.

When working with reversed-phase ion pairing with detection by MS–MS, an increase in the salt concentration in the mobile phase may lead to a decrease in sensitivity due to ion suppression of the signal [36,37]. In our case, an increase in the salt concentration caused a very marked decrease in the sensitivity of the nucleotides only when concentrations higher than 150–75 mM HFIP–DEA were used (Fig. S2b). Similar behaviour was seen for the NUs (Fig. S2a). The failure to observe clear ion suppression for lower HFIP–DEA concentrations could be understood by bearing in mind that DEA and HFIP have low boiling points (54.8–56.4 °C DEA and 58.2 °C HFIP), which reduces the surface tension of the droplets and results in an overall increase in ionization efficiency [38].

3.1.4. Influence of the concentration and nature of different ion-pairing reagents

The separation of nucleotides (mono-di and triphosphates) and different oligonucleotides using reversed-phase ion-pairing liquid chromatography has been studied in depth with a view to finding the most suitable ion-pairing agents [36–39]. However, the formation of ion pairs and later separation by HILIC has received little attention. It should be highlighted the previous work of Zhang and coworkers [33] in 2014 that set the basics of what they called IP-HILIC–MS/MS raising the hypothesis of an ion pair mechanism, although they also confirmed the urge of the hypothesized mechanism to be checked more deeply. To our knowledge, only these authors [33] have proposed a method for the determination of three nucleotides (adenosine mono-, di- and triphosphates) in plasma samples using DEA as the ion-pairing reagent and later HILIC–MS–MS separation–detection. Taking into account the importance of the ion-pairing agent used on the separation process, another three bases susceptible to forming ionic associations with nucleotides (TBA, TEA and NH_3) were studied. Some of the most important properties of the four bases are shown in Table S3. Owing to the low volatility of TBA, and in order to prevent obstructions in the ESI-MS–MS interface, the studies performed with TBA were carried out using only spectrophotometric detection.

Fig. 3 shows the chromatograms obtained for a nucleoside and the nucleotide monophosphate from the same family (A and AMP) in the presence of 100–50 mM HFIP-base with the four ion-pairing reagents. The addition of a cationic base to the mobile phase increased the retention of the nucleotides without significantly affecting the retention of the nucleosides. Moreover, for the

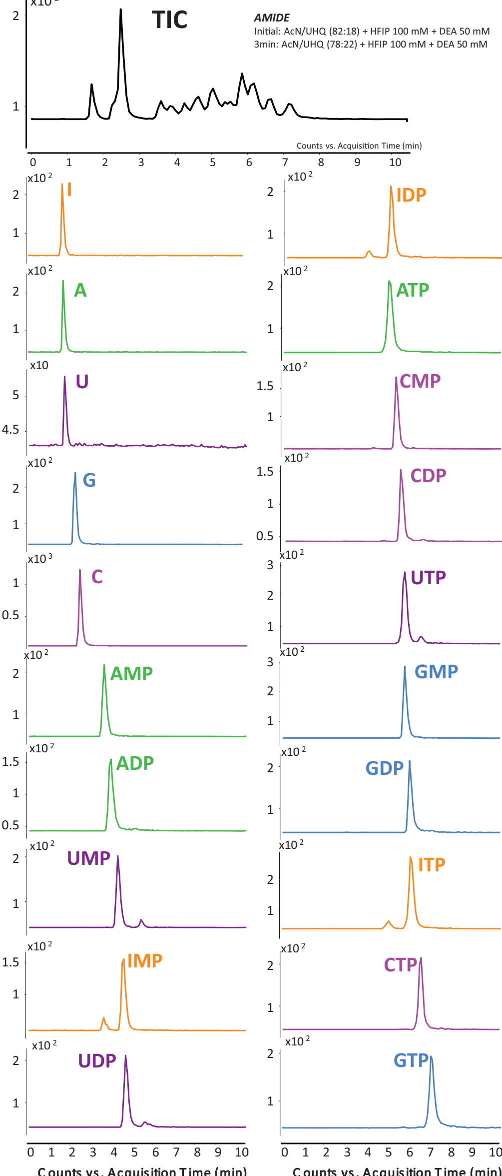

Fig. 5. Total ion chromatogram (TIC) and selected reaction monitoring (SRM) of a standard solution containing all the analytes at a concentration level of 5 μg mL^{-1} using the HILIC XBridge-Amide column. Injection of 5 μL in ACN. LC conditions as described in Section 2.3.

Table 2
Effect of temperature on HILIC XBridge-Amide separation of NUs and NMPs using a mobile phase containing 82% ACN/18% UHQ water and HFIP 100 mM + DEA 50 mM (results showed on the left side) or HFIP 10 mM + DEA 5 mM (results showed on the right side) $_{w}^{w}$pH 9 in the two cases. Thermodynamic parameters calculated from the Van't Hoff equation.[a]

	HFIP 100 mM + DEA 50 mM			HFIP 10 mM + DEA 5 mM		
	b/K	R^2	ΔH/kJ mol^{-1}	b/K	R^2	ΔH/kJ mol^{-1}
G	477.34	0.9797	−4.0	155.06	0.993	−1.3
I	2275.2	0.9888	−18.9	493.02	0.7056	−4.1
A	254.9	0.9446	−2.1	488.67	0.8191	−4.1
U	521.57	0.9922	−4.3	339.8	0.9568	−2.8
C	518.02	0.9947	−4.3	699.01	0.952	−5.8
GMP	1764.9	0.9976	−14.7	463.85	0.774	−3.9
IMP	2432.6	0.9994	−20.2	−2527.6	0.6367	21.0
AMP	2259.4	0.9992	−18.8	−3065.1	0.759	25.5
UMP	2866.4	0.9995	−23.8	−890.62	0.9989	7.4
CMP	2093.8	0.9998	−17.4	515.93	0.6448	−4.3

[a] Temperature variable from 20 to 45 °C. Flow rate: 0.35 mL min^{-1}. Injection: 5 μL in ACN.

same analyte, the more polar the base (NH_3 > DEA > TEA > TBA), the greater the retention of the nucleotides.

The variation in the retention times with respect to the concentration of the four ion-pairing reagents is shown in Fig. 4. The behaviour for the four analytes of the adenosine family is depicted, being similar to that observed for the other analytes of each family. The ionic association compound formed between the nucleotides and the TBA was highly apolar and was therefore not retained in the HILIC stationary phase; instead it was eluted with the solvent front (Fig. 3a). Regarding the other bases studied, on increasing the polarity of the base a greater analyte retention occurred (Fig. 3b–d) and a lower concentration of salts was required to neutralize the negative charges of the stationary phase and for the corresponding association compounds between the nucleotides and the cationic ion-pairing reagent to be formed.

Sensitivity in detection by MS–MS also depended on the base used; it was similar when DEA or NH_3 were used but lower, for all the analytes, in the presence of TEA. This variation in sensitivity was related to the volatility of the ion-pairing agents: $NH_3 \approx$ DEA > TEA > TBA (Table S3). Moreover, when NH_3 was used, slightly broader chromatographic peaks were obtained, both for nucleosides and nucleotides, which affected the resolution between them (Fig. 3d).

In view of the results obtained, and evaluating the effect on chromatographic resolution and sensitivity, the most suitable ion-pairing agent proved to be DEA. Thus, the optimum composition of the mobile phase for the separation of NUs and NMPs was the one formed by ACN/UHQ water mixtures with 100 mM HFIP and 50 mM DEA.

3.2. Stationary-phase selection and optimization of separation

Using the previously optimized mobile phase, we performed a comparative study of analyte separation in the three stationary-phases studied. To accomplish this, we examined, on one hand, separation by groups and, on the other, separation by families.

The results obtained in the analysis of the five NMPs (Fig. S3) revealed that although narrower and better defined peaks were obtained with the HILIC CoreShell column (HETP: 10 μm, 100,000 plates/m, average symmetry factor: 0.57), the resolution between them was poor. In both the XBridge-Amide column (HETP: 25 μm, 40,000 plates/m, average symmetry factor: 0.75) and the ZIC column (HETP: 50 μm, 20,000 plates/m, average symmetry factor: 0.61) good efficiency was achieved in the separation of the five analytes, but in the case of the ZIC column the total time of analysis was longer and the peaks of the analytes most retained in the

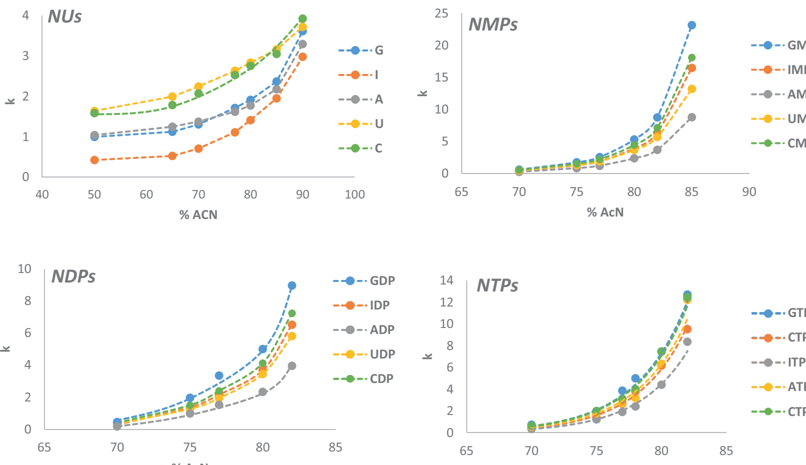

Fig. 6. Retention factors (k) for the target analytes separated by groups (NUs, NMPs, NDPs and NTPs) plotted versus the ACN percentage in the mobile phase, using a HILIC XBridge-Amide column and a mobile phase containing mainly of ACN/UHQ water mixtures with 100 mM HFIP and 50 mM DEA with $_w{}^w$pH 9.

stationary phase tended to broaden. Similar results were obtained for the separation of NDPs and NTPs. In the study of separation by families, bearing in mind both separation sensitivity and efficiency, the best results were also obtained with the XBridge-Amide column.

Thus, with the HILIC-XBridge Amide columns we used different gradients, starting from 82% to 87% of ACN and ending at 78–77% with a fast composition change in the range from 0.01 to 5 min, with a view to achieving the separation of the 20 analytes in the shortest time possible and with good resolution between them. This is important because in each of the four groups of analytes studied (NUs, NMPs, NDPs and NTPs) there were two pairs of analytes that were differentiated only in a single m/z unit; on one hand, the analyte from the adenosine family with that of the inosine family, and on the other the analytes belonging to the cytidine and uridine families (Table S1). Accordingly, it was necessary to optimize the gradient and evaluate the resolution between these pairs of compounds to ensure that no co-elution problems would arise between them.

The optimized mobile phase chosen was the one described in Section 2.3. Fig. 5 shows the total ion chromatogram (TIC) and selected reaction monitoring (SRM) for the injection of 5 μL of a standard solution containing all the analytes at a concentration level of 5 μg mL^{-1} (in ACN). Under these conditions, the separation of the 20 analytes was achieved in a total time of less than 9 min and, with the exception of the pair A–I (NUs group), for the other pairs of compounds resolution values ranging between 1.2 for CTP–UTP and 2.3 for CMP–UMP were obtained. The instrumental limits of detection and quantification (LODs and LOQs) of the method ranged from 5–60 to 15–200 ng mL^{-1}, respectively (Table S4). To show the capabilities of our method to deal with food matrices, Fig. S4 shows the chromatogram of a baby food for babies 8-months-old sample, treated using centrifugal ultrafiltration [40].

3.3. Study of the retention mechanism

In light of the results obtained the HILIC XBridge-Amide column was selected for all the later studies since it seemed to offer the best possibilities for analysis of the target analytes. Thus, our final aim was make a detailed study of the mechanism of retention of NUs, NMPs, NDPs and NTPs under the previously optimized mobile phase and stationary phase conditions.

3.3.1. Effect of temperature

The Van't Hoff equation has been proposed as a means to relate the retention factor and temperature. Likewise, it has been suggested that if the retention of polar compounds in HILIC occurs via partitioning, as proposed by Alpert [41], the Van't Hoff equation should be applicable to HILIC [32,42]. In this study, the effect of temperature on retention was investigated by varying the column temperature from 20 to 45 °C (six calibration points). Table 2 shows the results obtained using the optimized salt concentration and also a lower one in the mobile phase. A good correlation was obtained for NUs and NMPs with 100 mM HFIP and 50 mM DEA, negative enthalpy values being observed for all the analytes. Accordingly, the retention process is exothermic, and its values seem to be compatible with a partition mechanism. This suggests that for this salt concentration hydrophilic ion pairs are formed that interact with the stationary phase through a partition mechanism.

However, with 10 mM HFIP and 5 mM DEA, the NUs afforded the same results but the NMPs did not fit the Van't Hoff equation. This can be related to the results reported in Section 3.1.1, since with low salt concentrations in the mobile phase no ion pairs were formed and neither was the shielding of the negative charges of the stationary-phase observed, thus leading to the appearance of the electrostatic repulsion that prevented retention of the phosphorylated compounds. Thus, a deviation from the ideal behaviour described by the Van't Hoff equation for a partition mechanism is seen.

3.3.2. Correlations of retention with log D

Authors such as Kumar et al. [43] have established a relationship between the values of the correlation coefficient R found on representing log k vs. log D and the mechanism that governs retention in HILIC. R values that depart from unity indicate that as well as partition there are additional influences affecting the retention mechanism. It should be noted that although this criterion may not be conclusive, since the trend is for different algorithms to be used

for the theoretical calculation of log *D*, the *R* value obtained can be used, together with other criteria, to determine the retention mechanism.

Under optimum separation conditions, the correlated coefficient between log *k* vs. log *D* was 0.97 for the 20 analytes studied (Fig. S5). This value indicates that under these conditions the analytes were retained in the stationary-phase by means of a partition mechanism, with no significant influence by any other type of interaction.

3.3.3. Effect of the organic phase content

Next we studied the influence on retention of the percentage of ACN in a mobile phase containing 100 mM HFIP and 50 mM DEA. Fig. 6 shows the results obtained for all the analytes by groups. On comparing the results with those depicted in Fig. 1 (obtained with a mobile phase containing only ACN/UHQ water with no salts) it may be seen that the NUs exhibited the same behaviour in both cases; that is, they were retained in the stationary-phase, even with a low organic component content, and this retention increased with the increase in the percentage of ACN. However, the behaviour of the nucleotides matched a partition mechanism only when the mobile phase contained 100 mM HFIP and 50 mM DEA. Under these conditions, hydrophilic ion pairs were formed and retention was mainly governed by partition.

4. Conclusions

It has been demonstrated that several of the problems that arise when analyzing nucleotide mono-, di- and triphosphates by means of HPLC can be circumvented by the use of HILIC with mobile phases formed of hydrophilic cationic ion-pairing reagents and HFIP. The knowledge about this IP-HILIC separation for polar and charged compounds has been deeply expanded confirming some of the results previously reported. We also observed the mechanism associated with this separation and that the best performance was obtained with a HILIC XBridge-Amide column using a mobile phase containing ACN/UHQ water mixtures with 50 mM DEA and 100 mM HFIP ($_{w}{}^{w}$pH 9–$_{w}{}^{s}$pH 10). In the proposed method, 20 nucleosides and nucleotides were separated by HILIC in less than 9 min.

Acknowledgements

The University of Salamanca, Spain (USAL2015-16) is gratefully acknowledged for financial support of this work. M.M.V. gratefully acknowledges financial assistance from the Ministerio de Educación, Spain (FPU grant).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2015.08.040.

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Electronic Supplementary Material

Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates.

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Supplementary information

2.4. Chromatographic parameters

The chromatographic parameters used along this work were calculated as follow:

Retention time (t_R): elapsed time between the time of injection of an analyte and the time of elution of the peak maximum of that analyte.

Dead-time (t_0): the elution time of an unretained peak.

Retention factor (k'): the period of time that the sample component resides in the stationary phase relative to the time it resides in the mobile phase; $k' = (t_R - t_0)/t_0$.

Resolution (R_s): ability of a column to separate chromatographic peaks; $R_s = (t_{R2} - t_{R1})/[(w_{b1} + w_{b2})/2]$, where w_b is the baseline width of the peaks.

Symmetry factor (tailing factor, As): calculated using the following formula; $As = W_x/2d$, where W_x = peak width at 5% of peak height, measured from the baseline; and d = baseline distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5% of the peak height, measured in the same units as W_x.

Plate number (N): A number indicative of column performance, calculated from the following equation: $N = 16 (t_R/w_b)^2$

Height equivalent to a theoretical plate (HETP): The column length divided by the theoretical plate number.

Table S1. Physical properties and MS/MS transitions of the nucleosides and nucleotides analysed.

Nucleosides and Nucleotides	Mw	log D[a] $_{pH=9}$	Structure	MS/MS transition		
Adenosine (**A**)	267	-0.75	**R1**: OH group	268	→	136
Adenosine monophosphate (**AMP**)	347	-7.68	**R1**: Phosphate group	346	→	79
Adenosine diphosphate (**ADP**)	427	-8.16	**R1**: Diphosphate group	426	→	158.9
Adenosine triphosphate (**ATP**)	507	-10.12	**R1**: Triphosphate group	506	→	158.9
Cytidine (**C**)	243	-1.81	**R2**: OH group	244	→	112
Cytidine monophosphate (**CMP**)	323	-9.63	**R2**: Phosphate group	322	→	79
Cytidine diphosphate (**CDP**)	403	-10.1	**R2**: Diphosphate group	402	→	158.9
Cytidine triphosphate (**CTP**)	483	-12.07	**R2**: Triphosphate group	482	→	158.9
Guanosine (**G**)	283	-1.47	**R3**: OH group	284	→	152
Guanosine monophosphate (**GMP**)	363	-8.64	**R3**: Phosphate group	362	→	79
Guanosine diphosphate (**GDP**)	443	-9.12	**R3**: Diphosphate group	442	→	158.9
Guanosine triphosphate (**GTP**)	523	-11.09	**R3**: Triphosphate group	522	→	158.9
Inosine (**I**)	268	-1.97	**R4**: OH group	269	→	137
Inosine monophosphate (**IMP**)	348	-9.01	**R4**: Phosphate group	347	→	79
Inosine diphosphate (**IDP**)	428	-9.49	**R4**: Diphosphate group	427	→	158.9
Inosine triphosphate (**ITP**)	508	-11.46	**R4**: Triphosphate group	507	→	158.9
Uridine (**U**)	244	-1.76	**R5**: OH group	243	→	110
Uridine monophosphate (**UMP**)	324	-8.1	**R5:** Phosphate group	323	→	79
Uridine diphosphate (**UDP**)	404	-8.55	**R5**: Diphosphate group	403	→	158.9
Uridine triphosphate (**UTP**)	484	-10.51	**R5**: Triphosphate group	483	→	158.9

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2014 ACD/Labs).

Table S2. Characteristics of the chromatographic columns used for the analysis of the nucleosides and nucleotides.

Chromatographic column	Dimensions (mm)	Pore size (Å)	Particle size (µm)	Stationary phase
XBridge Amide	100 x 2.1	144	3.5	SiO_2 — LINKER — $C(=O)NH_2$
HILIC-CoreShell	75 x 4.6	100	2.6	Core / Shell SiO_2
ZIC-HILIC	150 x 4.6	100	3.5	SiO_2 — $CH_2CH_2N^+(CH_3)_2CH_2CH_2CH_2SO_3^-$

Table S3**.** Physical properties of the ion-pair reagents used.

Ion-Pairing reagent[a]	**TBA**	**TEA**	**DEA**	**NH_3**
MW (g/mol)	259.47	101.19	73.14	17.03
Boiling point (°C)	Non volatile	88.6 to 89.8	54.8 to 56.4	-33.35
Chemical structure	N^+	N	N H	NH_3
Solubility (g/L) Unbuffered Water pH 12.05 Temp: 25 °C	Soluble	31	124	31 %
log D (pH 9 Temp: 25 °C)	-	0.03	-1.09	-
Log Pow (Temp: 25 °C)	-	1.647 ± 0.222	0.657 ± 0.199	-2.53
pka	-	10.62 ± 0.25	10.76 ± 0.10	9.25

[a] Experimental properties calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2015 ACD/Labs).

Table S4.LOD and LOQ values obtained for the 20 analytes.

Analyte	*LOD[a] / ng mL^{-1}*	*LOQ[b] / ng mL^{-1}*	*Analyte*	*LOD[a] / ng mL^{-1}*	*LOQ[b] / ng mL^{-1}*
G	25	85	**GDP**	12	40
I	40	135	**IDP**	38	126
A	60	200	**ADP**	12	40
U	24	79	**UDP**	12	41
C	5	15	**CDP**	7	24
GMP	8	28	**GTP**	17	57
IMP	9	31	**ITP**	32	107
AMP	10	34	**ATP**	24	80
UMP	11	37	**UTP**	7	25
CMP	6	19	**CTP**	6	19

[a] LOD: Limits of detection calculated for a signal to noise ratio of 3.

[b] LOQ: Limits of quantification calculated for a signal to noise ratio of 10.

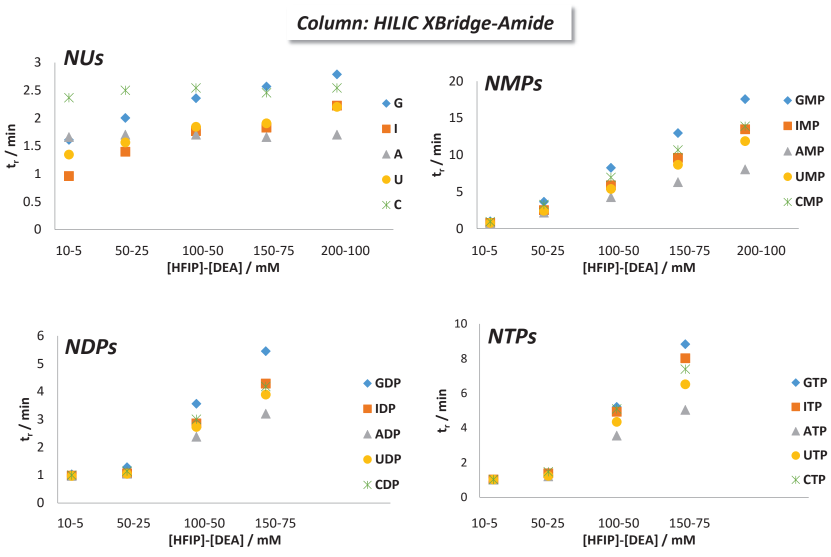

Figure S1. Retention times for the target analytes separated by groups (NUs, NMPs, NDPs and NTPs) plotted versus the HFIP-DEA concentration, using a HILIC XBridge-Amide column and a mobile phase containing 82 % of ACN in the case of NUs and NMPs and 75 % of ACN for NDPs and NTPs.

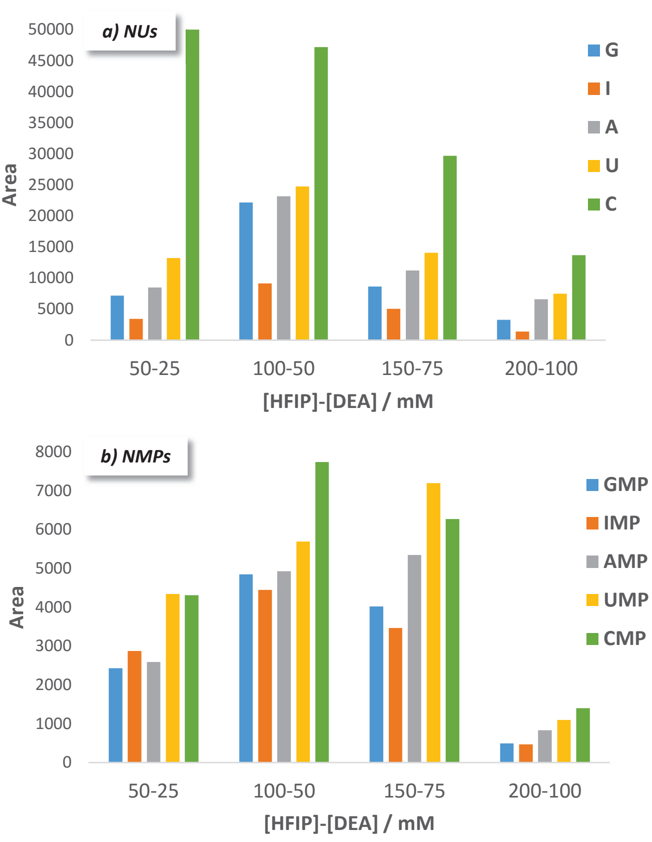

Figure S2. Variation in the peak area of NUs and NMPs, plotted versus the HFIP-DEA concentration, using a HILIC-XBridge Amide column and a mobile phase containing 82 % of ACN.

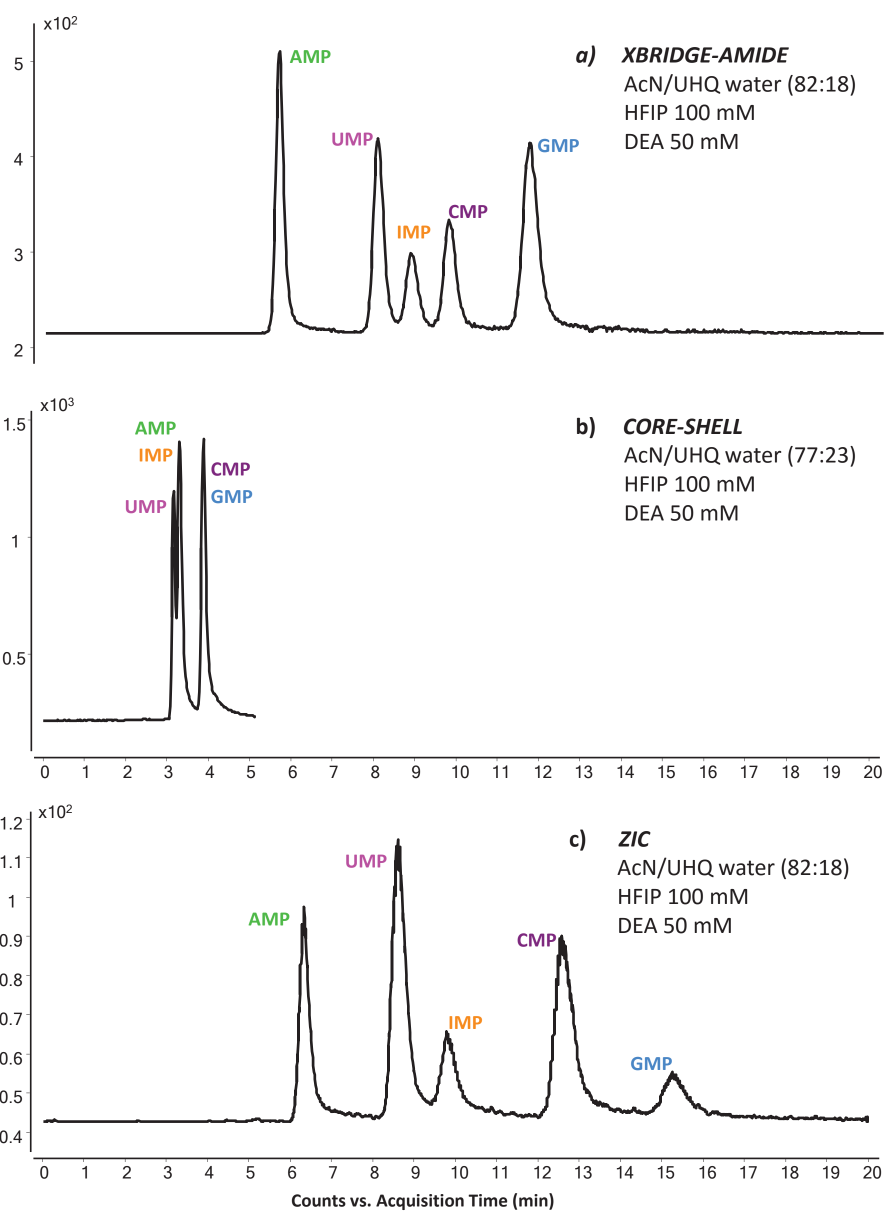

Figure S3. Separation of nucleotide monophosphates (NMPs) on the three stationary phases studied: (a) HILIC-XBridge Amide, (b) HILIC-CoreShell and (c) ZIC-HILIC. Mobile phase containing: (a) and (c) 82 % ACN / 18 % UHQ water with 100 mM HFIP and 50 mM DEA; (b) 77 % ACN / 23 % UHQ water with 100 mM HFIP and 50 mM DEA.

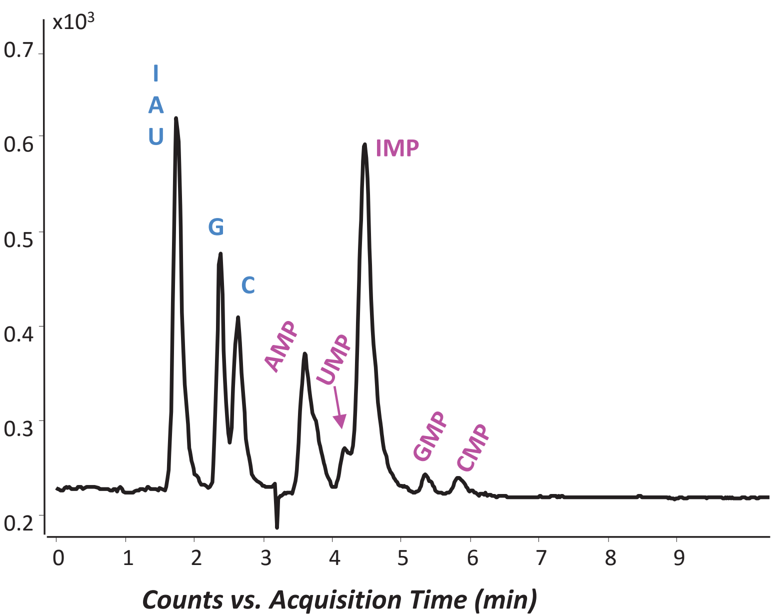

Figure S4. Total Ion Chromatogram (TIC) of a baby food (sole with bechamel sauce) for babies eight months old sample, treated using centrifugal ultrafiltration. Column: HILIC-XBridge Amide and LC conditions as described in Section 2.3.

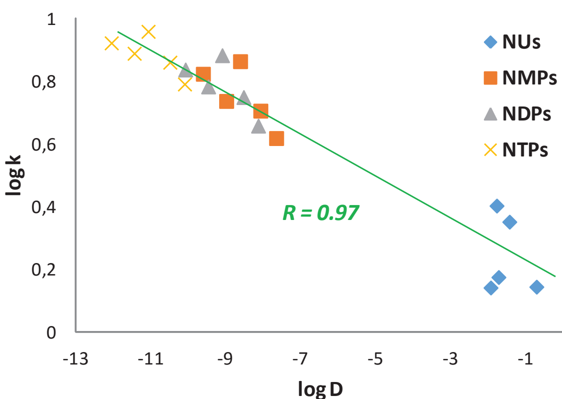

Figure S5. Log k plotted vs. log D in the analysis of the 20 target analytes (NUs, NMPs, NDPs and NTPs) using the HILIC-XBridge Amide column and the LC conditions as described in Section 2.3.

DETERMINACIÓN DE NUCLEÓSIDOS Y NUCLEÓTIDOS EN ALIMENTOS INFANTILES UTILIZANDO CROMATOGRAFÍA DE INTERACCIONES HIDROFÍLICAS ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM, EN PRESENCIA DE REACTIVOS FORMADORES DE PARES IÓNICOS HIDROFÍLICOS

II.3

ARTÍCULO DE INVESTIGACIÓN II.3

RESUMEN

II.3. Determinación de nucleósidos y nucleótidos en alimentos infantiles utilizando cromatografía de interacciones hidrofílicas acoplada a espectrometría de masas en tándem, en presencia de reactivos formadores de pares iónicos hidrofílicos

El primer año de vida de los bebés es crucial para su crecimiento y desarrollo, y por tanto es necesario asegurar una nutrición adecuada para prevenir posibles enfermedades y crear hábitos alimenticios saludables. La Organización Mundial de la Salud recomienda prolongar la lactancia exclusiva hasta los seis meses, momento en el que es necesario introducir una alimentación complementaria que debe tener una composición apropiada en cuanto a energía y nutrientes, ya que en algunos casos es la fuente principal de nutrición para bebés entre 6 y 12 meses.

Aunque existen estudios clínicos sobre la eficacia de las fórmulas infantiles enriquecidas con nucleótidos, no se ha encontrado información sobre los niveles de estos compuestos en otros alimentos infantiles no lácteos. Sin embargo, todos los ingredientes de origen animal o vegetal que contienen material celular son fuentes potenciales de nucleótidos, lo cual aumenta el interés de estudiar este tipo de compuestos en alimentos infantiles teniendo en cuenta que cada vez se añaden más ingredientes a las dietas de los bebés. Ingredientes como el pescado, la proteína animal, las legumbres o los extractos de levadura contienen niveles altos de nucleótidos; mientras que la proteína muscular, la soja, la fruta, los vegetales y los lácteos procesados son fuentes pobres en nucleótidos.

Según nuestro conocimiento, hasta la fecha no se ha descrito ningún método para la determinación conjunta de nucleósidos y nucleótidos en alimentos infantiles no lácteos. Por ello, el objetivo principal de este trabajo es mostrar la aplicabilidad del método basado en cromatografía líquida de interacciones hidrofílicas acoplada a espectrometría de masas en tándem y en presencia de reactivos formadores de pares iónicos hidrofílicos (IP-HILIC-MS/MS), desarrollado por el grupo, al análisis de muestras comerciales de alimentos infantiles, tanto lácteos como no lácteos. Debido a la complejidad de las matrices estudiadas, se requiere el desarrollo de un tratamiento de muestra eficaz, capaz de eliminar los principales interferentes de la matriz y generar así extractos limpios que puedan ser compatibles con la inyección en el sistema cromatográfico. Del mismo modo que en el análisis de fórmulas lácteas de iniciación y de leches maternas, el procedimiento más utilizado para el tratamiento de alimentos infantiles es la precipitación de proteínas en medio ácido. En este trabajo se propone la utilización de la ultrafiltración asistida por centrifugación (CUF), por ser un método simple, eficaz y que evita el uso de disolventes orgánicos.

Para la separación y detección de los analitos se recurre al método HILIC-MS/MS, basado en la formación de pares iónicos hidrofílicos, con una fase móvil que contiene hexafluoroisopropanol (HFIP) 100 mM y dietilamina (DEA) 50 mM ($_{w}^{w}pH$ 9-$_{w}^{s}pH$ 10). En estas condiciones, se consigue la separación de los nucleósidos y nucleótidos mono-, di- y trifosfato en menos de 9 minutos.

La complejidad de las muestras analizadas radica, fundamentalmente, en su elevado número de ingredientes y su alto

contenido en grasa y proteínas. Al igual que en el tratamiento de fórmulas infantiles y leche materna, se planteó aquí la utilización de ultrafiltración asistida por centrifugación (CUF) para el tratamiento de los alimentos infantiles lácteos y no-lácteos. A priori, la principal limitación de este procedimiento es la recogida de los analitos disueltos en medio acuoso, ya que la inyección en un sistema HILIC debe realizarse con un alto contenido de acetonitrilo. Por ello, se hace necesario una dilución 1:5 con acetonitrilo del extracto obtenido después de la etapa CUF. Otros parámetros estudiados fueron la influencia de la temperatura de extracción, agitación con ultrasonidos, la cantidad de muestra usada para el tratamiento, y la cantidad de agua necesaria para su disolución y dilución. Se incluyó, además, una nueva etapa consistente en el lavado del dispositivo CUF con un volumen de 500 μL de agua, seguido de una segunda centrifugación para evitar la pérdida de nucleótidos en la etapa de ultrafiltración. La optimización de estas condiciones permite obtener recuperaciones superiores al 80 % para todos los analitos en el proceso de extracción.

Para la evaluación del posible efecto de matriz se compararon las pendientes obtenidas realizando la calibración con estándares preparados en acetonitrilo con aquellas que se encontraron al aplicar la calibración por adición estándar de una muestra sin dopar. Se comprobó que no existía un efecto de matriz significativo por lo que la cuantificación de los nucleósidos y nucleótidos en alimentos infantiles complementarios con la metodología propuesta se puede llevar a cabo mediante calibración con estándares preparados en acetonitrilo. La validación del método se realizó según la Decisión Europea 2002/657: los valores de repetibilidad y reproducibilidad se mantienen entre 2 y 13 %, con valores de límites de detección entre 0.2 y 2.7 μg g^{-1}. La

precisión obtenida entre diferentes dispositivos CUF es inferior al 10 %. La exactitud del método se evaluó con dos muestras diferentes, comparando en cada una el contenido de nucleósidos y nucleótidos obtenido por calibración con estándares en acetonitrilo y por adición estándar; no encontrándose diferencias significativas entre ambos métodos de cuantificación.

Finalmente, para comprobar la aplicabilidad y robustez del método se analizaron dieciséis muestras de alimentos infantiles, incluyendo la utilizada para la validación, de diferentes marcas comerciales e ingredientes: pescado, carne, fruta, yogur infantil y papilla de cereales. En ninguna de las muestras se encontraron nucleótidos di- y trifosfato, lo que puede deberse a que son alimentos procesados en las que estos compuestos se han degradado a nucleótidos monofosfato y nucleósidos. En las muestras de pescado y carne se encontraron concentraciones variables de todos los nucleósidos y nucleótidos, y en ambos casos la Inosina fue el nucleósido presente en mayor concentración. En las muestras de fruta y en la papilla de cereales no se detectaron nucleótidos y los niveles de nucleósidos son mucho más bajos, siendo Adenosina el nucleósido presente en mayor concentración. En la muestra de yogur infantil se pudieron cuantificar tres nucleótidos monofosfato, CMP, UMP y AMP, que son los tres nucleótidos que también se encontraban en mayor concentración en la leche materna y en las fórmulas infantiles de iniciación.

ARTÍCULO DE INVESTIGACIÓN II.3

Food Chemistry, 211 (2016) 827-835

Food Chemistry 211 (2016) 827–835

Contents lists available at ScienceDirect

Food Chemistry

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

Determination of nucleosides and nucleotides in baby foods by hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents

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ARTICLE INFO

Article history:
Received 21 January 2016
Received in revised form 21 April 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
Hydrophilic interaction chromatography-tandem mass spectrometry
Hydrophilic ion-pairing
Nucleosides
Nucleotides
Centrifugal ultrafiltration
Baby foods

ABSTRACT

In this work we propose a rapid and efficient method for the joint determination of nucleosides and nucleotides in dairy and non-dairy baby foods based on hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of diethylammonium (DEA) as a hydrophilic ion-pairing reagent (IP-HILIC-MS/MS).

Sample treatment of the baby food included dilution with water and centrifugal ultrafiltration (CUF) with an additional washing step that notably improved the global performance of the process. Later dilution of the extract with acetonitrile allowed adequate separation in the HILIC system. With the proposed treatment, we obtained extraction recoveries higher than 80% and, additionally, no matrix effects were observed.

The CUF-IP-HILIC-MS/MS method was validated according to the 2002/657/EC decision and was used for the quantification of nucleotides and nucleosides in sixteen samples of commercial baby foods.

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1. Introduction

The first year of life of babies is crucial for their growth and development and it is therefore crucial to ensure that they will have access to sufficient suitable nutrition to prevent possible diseases and create healthy eating habits. The World Health Organization (WHO) recommends prolonging exclusive lactation up to six months, after which time it becomes necessary to introduce complementary nutrition. Complementary feeding should have an appropriate composition as regards energy and nutrients, since sometimes it is the main source of nutrition for babies between 6 and 12 months (Mir-Marqués, González-Masó, Cervera, & de la Guardia, 2015).

Nucleosides and nucleotides are compounds that are related to the regulation and modulation of many physiological processes (Klawitter, Schmitz, Klawitter, Leibfritz, & Christians, 2007). Their determination is of great interest in food analysis (Studzinska & Buszewski, 2013; Yeung, Ding, & Casley, 2008), among other reasons because their appearance is related to iron absorption, the repair of intestinal damage and an improved immune response (Ranogajec, Beluhan, & Šmit, 2010).

It is known that ingredients such as fish soluble, animal protein soluble, fish meal, legumes and yeast extracts contain high levels of these compounds (Carver & Walker, 1995), whereas muscle protein, soy, fruit, vegetables and processed dairy products seem to be poor sources of nucleotides.

The simultaneous separation of nucleotides and nucleosides is complicated owing to their chemical similarities, their high polarity, which restricts their retention in reverse-phase chromatography, and also owing to the adsorption of the phosphorylated compounds in chromatographic systems, leading to a lack of sensitivity and precision when MS detection is employed (Tuytten et al., 2006; Wakamatsu, Morimoto, Shimizu, & Kudoh, 2005). In order to circumvent these problems, we developed a method based on HILIC coupled to tandem mass spectrometry (MS/MS) in the presence of diethylammonium as a hydrophilic ion-pairing reagent (Mateos-Vivas, Rodríguez-Gonzalo, García-Gómez, & Carabias-Martínez, 2015).

For the determination of nucleotides in baby foods and human milk, different chromatographic methods have been described, based on reversed-phase, (Lin, Li, & Dai, 2007), ion pairing (Viñas et al., 2010), ion exchange (Viñas et al., 2009) and hydrophilic

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http://dx.doi.org/10.1016/j.foodchem.2016.05.091
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interaction chromatography (HILIC) (Inoue, Obara, Akiba, Hino, & Oka, 2010; Sfakianaki, & Stalikas, 2015). Also, several methods employing capillary zone electrophoresis coupled to mass spectrometry (CZE-MS) (Rodríguez-Gonzalo, Domínguez-Álvarez, Mateos-Vivas, García-Gómez, & Carabias-Martínez, 2014; Mateos-Vivas, Rodríguez-Gonzalo, Domínguez-Álvarez, et al., 2015) have been developed.

Regarding the joint separation and detection of nucleotides and nucleosides, few chromatographic applications have been described (Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001; Gill & Indyk, 2007; Ranogajec, Beluhan, & Šmit, 2010; Wu et al., 2015; Yamaoka et al., 2010). A rapid method for the determination of nucleotides and nucleosides in dairy baby foods using LC-MS has been proposed (Gill, Indyk, & Manley-Harris, 2013). However, in some cases, these authors found a loss of sensitivity due to the accumulation of small amounts of phosphate on the cone.

Dairy baby foods and human milk are the matrices most studied in the determination of nucleotide monophosphates. Although there is much less information available about complementary baby foods (non-dairy), what is certain is that the most widely used sample treatment, as in the case of both types of milk, involves the addition acid followed by centrifugation, aimed at protein precipitation (Viñas et al., 2009, 2010). For the determination of different nucleotides and nucleosides in royal jelly, other methods, mainly based on liquid-liquid extraction, have been proposed: with hot water (Zhou et al., 2012), with a hot saline solution (Zhou et al. 2012), with water-ethanol (Xue, Zhou, Wu, Fu, & Zhao, 2009) and with ethanol-acetone-water, also hot (Wu et al., 2015). To our knowledge, until now no method for the joint determination of nucleotides and nucleosides in non-dairy baby foods has been described.

Owing to the complexity of the matrices of baby foods studied here, one of the chief aims was to develop an efficient sample treatment able to remove the main interferents from the matrix and generate clean extracts that would be compatible with injection into the chromatographic system. Here, we propose the use of centrifugal ultrafiltration (CUF), together with additional steps in the sample treatment process.

A further aim was to describe the application of the method based on the HILIC chromatographic mode, a field in which its application is still scant. The complete method proposed (CUF-IP-HILIC-MS/MS) was validated in commercial samples of baby food following EU decision 2002/657, and the contents of NMPs and NUs in different types of sample were evaluated.

2. Materials and methods

2.1. IP-HILIC-MS/MS method

The chemicals, reagents, instrumentation and the method used in separation-detection by IP-HILIC-MS/MS have been described in a previous paper (Mateos-Vivas, Rodríguez-Gonzalo, García-Gómez, et al., 2015). Briefly, chromatographic separation was achieved with a HILIC XBridge-Amide column and the mobile phase consisted of an ACN/UHQ water mixture (3 min of isocratic elution using 82:18%, v/v and then a fast gradient from 18% to 22% of water) with 100 mM hexafluoro-2-propanol and 50 mM diethylammonium. The chromatographic flow rate was set at 0.35 mL min^{-1}. Under these conditions, the separation of the 20 analytes was achieved in less than 9 min. With the exception of the pair adenosine-inosine (nucleosides) that was not resolved, for any other pair of compounds differentiated only in a single *m*/*z* unit, resolution values ranging between 1.2 for cytidine-uridine triphosphates and 2.3 for cytidine-uridine monophosphates were obtained. The CUF devices used in the sample treatment (CUF; Spin-X UF 20, 5 k molecular weight cut-off, polyethersulfone, nonsterile, 20 mL) were purchased from Corning (NY, USA).

2.2. Baby food samples

Sixteen samples of baby foods were analyzed. The samples were purchased at a local supermarket and were of different brands and ingredients, all of them sold for consumption by babies as from 6 to 8 months of age. The sample set was classified in four different blocks: four of the samples with a fish content of 9–16% of the total composition; another six samples with a 10–15% content of meat; a third group comprising four baby foods made of fruit (almost 100% of the composition), and finally a block comprising yoghourt for babies and a cereal purée, both elaborated with continuation milk for babies. The composition, per 100 g, varied between 266 and 368 kJ of energy value, 0.1 and 2.8 g of fat, 6.5 and 17.9 g of carbohydrates, 0.4 and 1.5 of dietary fiber and between 0.5 and 4.1 g of proteins. The lowest values of fat and proteins and the highest values of carbohydrates in the baby foods were found for those made of fruit. The levels of the other parameters were similar, whether they were fish, food, meat, or fruit baby foods, baby yoghourt or cereal purée. All samples were analyzed and variable levels of nucleoside and nucleotide monophosphates were found. One of the fish baby foods, which contained these ten analytes, was used to develop and validate the proposed method. The accuracy of the method was evaluated by analysis of this fish baby food sample and of one of the meat baby food samples. The other samples were used to check the applicability and robustness of the method.

2.3. Sample treatment

To perform the proposed method, 0.5 g of sample was weighed in a 50-mL centrifuge tube and ultrapure water (UHQ) was added to bring volume up to 4.5 g. The sample was shaken manually and then centrifuged for 15 min (2800 g). A 4.0-mL aliquot of the supernatant was collected, attempting to avoid touching the solid residue at the bottom of the tube and was passed through the CUF device (30 min, 2800 g), previously conditioned with 5.0 mL of UHQ water (15 min, 2800 g), to carry out the ultrafiltration. Following this, without removing either the filtrate or the liquid remains that might not have passed through the filter, 0.5 mL of UHQ water was added to the CUF device and this was centrifuged again (30 min, 2800 g) in order to carry out an extra step in washing the filter. The analytes studied that were present in the filtrate were analyzed using IP-HILIC-MS/MS, after dilution at 1:5 with acetonitrile (ACN).

In the cases in which it was necessary to spike the samples, this was done directly on a weighed aliquot of the baby food sample, leaving it to stand for about 12 h before applying the extraction and analysis method to favor the interactions between the analytes and the matrix compounds.

2.4. Quantification

Quantification of the analytes by MS/MS was carried out in the Selected Reaction Monitoring (SRM) mode, using the optimum transition for each compounds, whose precursor ion corresponded to the $[M+H]^+$ ion in the case of adenosine (A), cytidine (C), guanosine (G) and inosine (I) and to the $[M-H]^-$ ion for uridine and nucleotide mono-, di-, and triphosphates. The transitions used in the quantification have been reported in a previous work (Mateos-Vivas, Rodríguez-Gonzalo, García-Gómez, et al., 2015). These transitions, ESI polarity, collision energies and fragmentor voltages, were optimized for each analyte by infusing them

separately. The quantitative variable was the peak area expressed in arbitrary area units.

Sample quantification was conducted using external calibration and the standard additions method. In the latter case (standard additions), 1.0 mL of the filtrate obtained with the CUF devices were collected and 4.0 mL of ACN was added in order to maintain a UHQ/ACN ratio compatible with injection in HILIC. Following this, aliquots of 0.8 mL were collected and spiked with volumes of 0.015–0.060 mL of a standard solution containing the analytes at different concentrations. Since adenosine and inosine are not time-resolved in the proposed method, the contribution of the isotopic adenosine was subtracted from the total peak area found for the 269 → 137 MS/MS transition to isolate the peak area for inosine. This contribution was calculated experimentally from adenosine mass spectra and theoretically by ChemDraw®Ultra software 1985–2003 (CambridgeSoft Corporation), resulting in a value of 12.9%. The unspiked sample and the spiked samples were then analyzed with the IP-HILIC-MS/MS system, obtaining three replicas per sample.

2.5. Method validation

The method was validated with a fish baby food (*sole with béchamel*), evaluating the following parameters: external calibration curves, limits of detection and quantification, repeatability, intralaboratory reproducibility and inter-CUF reproducibility. Accuracy was evaluated with this fish baby food and also with a meat baby food (*garden vegetables with beef*) by comparing the calibration with standards in ACN with the standard additions method, using the latter as quality control.

2.6. Statistical analysis

In order to check for differences from the two calibration procedures (external calibration vs. standard additions) p-values for the comparison between slopes were calculated by means of a two-tailed paired *t*-test (Howell, 2010; Kleimbaum, Kupper, & Muller, 1988). Values of $p < 0.05$ were interpreted as a rejection of the null hypothesis, thus rejecting equal slopes between the two groups.

3. Results and discussion

3.1. IP-HILIC separation – MS/MS detection

As explained in the experimental part, separation and detection were carried out following a method based on the formation of hydrophilic ion pairs in HILIC-MS/MS (Mateos-Vivas, Rodríguez-Gonzalo, García-Gómez, et al., 2015). We used a mobile phase containing hexafluoro-2-propanol (HFIP) and a cationic ion-pairing reagent (DEA) and determined a total of 20 analytes: nucleosides and nucleotide mono-, di-, and triphosphates. Before the method was applied to the analysis of baby food samples, we determined the instrumental characteristics of the system by applying it to standard solutions of the analytes dissolved in ACN. Since no nucleotide di- (NDPs) and triphosphates (NTPs) were found in any of the samples analyzed, Table S1 only shows the analytical characteristics obtained for the nucleosides (NUs) and nucleotide monophosphates (NMPs).

Linear calibration curves were obtained for all the compounds in the 50–3000 ng mL^{-1} range. The instrumental limits of detection (LODs), based on the signal-to-noise ratio of 3 (S/N) varied between 6 and 11 ng mL^{-1} for the NMPs and between 5 and 60 ng mL^{-1} for the NUs. The limits of quantification (LOQs), based on an S/N ratio of 10, varied between 19 and 37 ng mL^{-1} and between 15 and 200 ng mL^{-1}, respectively. It should be noted that LODs and LOQs for the NMPs are more favorable than those obtained in previous works where NMPs were analyzed by means of CE-ESI-MS (Rodríguez-Gonzalo et al., 2014) and by nano-LC-UV (Mateos-Vivas, Fanali, Rodríguez-Gonzalo, Carabias-Martínez, & Aturki, 2016). Other authors also found higher LODs and LOQs in the analysis of NMPs (Viñas et al., 2010; Wu et al., 2015), and similar values for the analysis of NUs (Wu et al., 2015).

The intralaboratory repeatability and reproducibility at three concentration levels (50, 500 and 3000 ng mL^{-1}), expressed as relative standard deviations (RSD) (%), were in all cases lower than 20%. The recoveries at those levels ranged between 80 and 108% (Table S1).

3.2. Baby food pretreatment

The use of a suitable sample pretreatment is crucial when performing a quantification with ESI-MS/MS since co-elution of the matrix interferents may give rise to ion suppression at the electrospray source, affecting the response of the analytes by either increasing or decreasing their ionization (Kohler, Schappler, & Rudaz, 2013; Xiu-Qin, Zong, Qing-He, & Hong-Mei, 2014). There are also other strategies for minimizing matrix effects such as simple dilution or the use of another ionization source.

The baby food samples studied in the present work had a complex matrix, based on their large number of ingredients and their majority content in fat and/or protein. Accordingly, it is necessary to choose an appropriate method for the extraction and clean-up

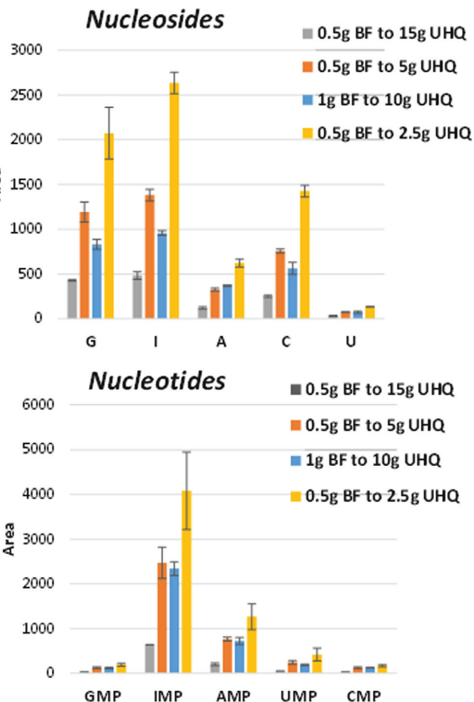
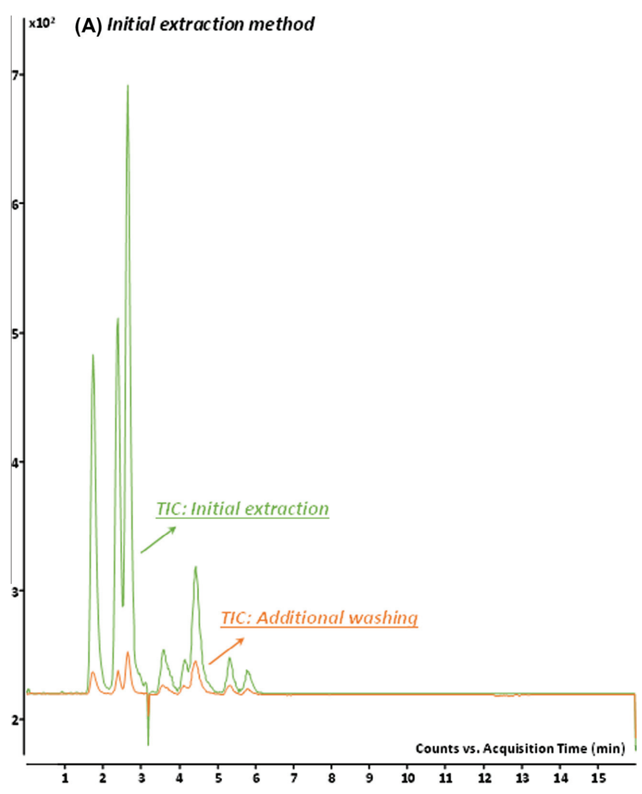


Fig. 1. Influence of the ratio between the amount of baby food and the amount of UHQ water employed for its dilution on the peak areas of the NUs and NMPs.

of the sample. Initially, we evaluated analyte extraction using a modified QuEChERS method; that is, performing salt-assisted extraction with ACN without applying the later clean-up step using PSA (Primary Secondary Amine). This treatment is compatible with chromatographic separation because it is necessary to inject the analytes in organic medium in order to achieve suitable separation in the HILIC chromatographic column. Since nucleosides and nucleotides are highly polar compounds, we introduced a possible modification into the method, consisting of forming an ion pair with the analytes studied in order to facilitate the extraction of the compounds to the organic phase (Kukusamude, Burakham, Chailapakul, & Srijaranai, 2012). To accomplish this, we used the same combination of reagents present in the mobile phase, DEA and HFIP, since in basic medium DEA is in cationic



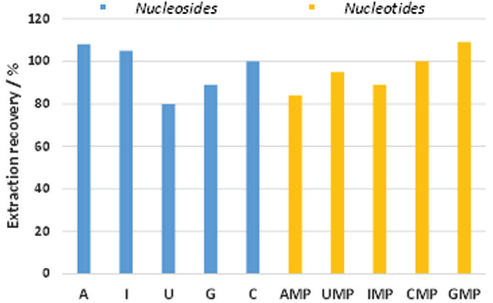


Fig. 2. Comparison of the initial extraction method and the extraction method modified by the inclusion of an additional washing step. (A) Total Ion Chromatograms (TICs) obtained with the unmodified method and after a wash in the CUF device. (B) Extraction recoveries of NUs and NMPs obtained with the modified sample treatment method.

form and, owing to its small size, it is able to form hydrophilic ionic associations (Mateos-Vivas, Rodríguez-Gonzalo, García-Gómez, et al., 2015). We also assayed quite the opposite option, using an apolar cationic reagent (tetrabutylammonium, TBA) to try to create a much less polar reagent. However, in no case was it possible the achieve analyte extraction from the aqueous to the organic phase.

In previous studies addressing the analysis of nucleotides in samples of baby food and human milk (Rodríguez-Gonzalo et al., 2014; Mateos-Vivas, Rodríguez-Gonzalo, Domínguez-Álvarez, et al., 2015) good results were obtained upon performing a sample dilution followed by centrifugal ultrafiltration (CUF) for its treatment. Accordingly, here we were prompted to test that procedure in the case of non-dairy baby foods. *A priori*, the main limitation to this cleaning method using CUF is the collection of the analytes dissolved in an aqueous medium since when working with HILIC injection must be carried out with a high percentage of ACN. Accordingly, it is necessary to introduce an additional step to change to an ACN organic medium in order to achieve separation in the HILIC system.

Two options were tested; evaporation and later reconstitution in an organic medium, and dilution of the extract obtained in ACN. The best results were obtained when working with a 1:5 dilution with ACN of the extract obtained after CUF.

3.2.1. Influence of the extraction temperature

Some authors have reported that the extraction of these analytes from different samples is more effective if hot water is used during the process (Zhou et al., 2012) and also if an extraction step is performed with ultrasound (Xue et al., 2009).

Fig. S1 shows the values obtained for the NUs and NMPs on carrying out the process with ultrapure water at RT and without including the extraction step with ultrasound and also when the extraction step was performed with water at 80 °C and agitation by ultrasound for 15 min. It may be seen that there are no significant differences between either process, such that it was decided to use the simpler procedure, in which neither heat nor ultrasound was used, in order to simplify the sample pretreatment step as much as possible.

3.2.2. Optimization of the extraction method

We then optimized the amount of sample used in its treatment and the amount of water with which the sample was dissolved and diluted. Four different possibilities were studied: 0.50 g of baby food dissolved in 15 g of UHQ water; 1.0 g in 10 g; 0.50 g in 5.0 g and 0.50 g in 2.5 g. The results, expressed in the area of the NUs and NMPs, are shown in Fig. 1. The analytical signal increased proportionally upon decreasing the dilution of the sample. Moreover, the increase in the amount of sample assayed did not affect the shape of the chromatographic peaks negatively or cause any kind of peak broadening. This is probably because after the clean-up step using CUF the extract was diluted 5 times prior to its injection into the chromatographic system. In light of the results obtained, it was decided to perform extraction using 0.50 g of baby food dissolved in 5.0 g of UHQ water.

The extraction recovery of the method was evaluated by analyzing the sample of fish baby food (*sole with béchamel*) spiked at analyte concentrations of 20 μg g^{-1}. The values obtained revealed an appreciable loss of NMPs in some steps of the sample treatment step. In order to determine which step (or steps) of the sample treatment was responsible for this loss, it was determined whether the analytes were present in the solid residue obtained after the first extraction step, for which a re-extraction was carried out with UHQ water and the resulting extract was analyzed. We also analyzed the solutions obtained after performing successive washing steps with UHQ water from the CUF filter used in the sample clean-up step. The results showed that the main loss of NMPs (around 32–44%, depending on the nucleotide) occurred during the clean-up step in the CUF filter. Fig. 2-A shows the total ion chromatogram (TIC) of the initial extraction and also that corresponding to the first wash performed on the CUF device. In light of these results, it was decided to implement a small modification in the extraction procedure by including a CUF washing step using a volume of UHQ water, which was then centrifuged (30 min, 2800*g*). Of the washing volumes assayed, 500 μL of UHQ water

Table 1
Validation parameters for the proposed CUF prior to HILIC-MS/MS method.

	Nucleotide monophosphates					*Nucleosides*				
	AMP	CMP	GMP	IMP	UMP	A	C	G	I	U
Limits										
LOD (μg g^{-1})	0.5	0.3	0.4	0.4	0.5	2.7	0.2	1.1	2.0	1.1
LOQ (μg g^{-1})	1.5	0.9	1.3	1.4	1.7	9.0	0.7	3.8	6.7	3.6
Precision										
Repeatability intraday[a] (%)	5.9	7.8	8.3	3.6	6.8	3.3	5.0	12.4	3.2	6.3
Reproducibility interday[b] (%)	2.4	4.9	3.4	4.2	2.7	11.1	10.6	13.1	10.5	5.7
Inter-CUF reproducibility[c] (%)	5.4	0.6	9.8	2.8	0.6	4.7	4.3	3.3	4.3	3.4
Accuracy // Fish baby food										
St. Add.[d] (μg g^{-1})	19 ± 1	4.5 ± 0.4	3.1 ± 0.4	92 ± 4	10.3 ± 0.4	10.3 ± 0.4	5 ± 1	12 ± 1	65 ± 4	8 ± 1
Aq. Cal.[e] (μg g^{-1})	18 ± 2	4 ± 2	3.6 ± 0.4	104 ± 2	9 ± 1	12 ± 1	4 ± 2	12 ± 1	59 ± 2	6 ± 2
Acc.[f] (%)	95	89	116	113	87	116	80	100	91	75
p-value[g]	0.14	0.23	0.43	0.17	0.06	0.69	0.05	0.11	0.05	0.06
Accuracy // Meat baby food										
St. Add.[d] (μg g^{-1})	49 ± 4	2.2 ± 0.4	5 ± 1	62 ± 3	37 ± 1	12.1 ± 0.4	3.1 ± 0.4	7 ± 1	114 ± 3	7 ± 2
Aq. Cal.[e] (μg g^{-1})	48 ± 3	2 ± 1	4 ± 4	56 ± 3	26 ± 1	10 ± 2	3 ± 1	8 ± 3	88 ± 2	5 ± 2
Acc.[f] (%)	98	91	80	90	70	83	97	114	77	71
p-value[g]	0.93	0.15	0.77	0.08	0.07	0.31	0.11	0.08	0.91	0.05

[a] Repeatability calculated as intraday precision (6 injections) for the analysis of unspiked baby food sample.
[b] Reproducibility calculated as interday precision (3 different days, 6 injections for the analysis of unspiked baby food sample).
[c] Reproducibility calculated as inter-CUF precision (6 injections for the analysis of 4 different unspiked baby food sample).
[d] Concentration in μg mL^{-1} obtained by the standard addition method.
[e] Concentration in μg mL^{-1} obtained by calibration with standards in ACN.
[f] Accuracy calculated using the standard addition method as quality control.
[g] p-values obtaining applying a two-tailed *t* test on the slopes: standard addition method vs external calibration.

was found to be the most suitable for achieving extraction recoveries higher than 80% (Fig. 2-B) for all the analytes.

The matrix effect, defined as ion suppression or enhancement of the ionization of analytes, was determined by comparing the slopes obtained on performing calibration with standard solutions prepared in ACN with those obtained upon applying standard additions calibration on an unspiked sample of fish baby food. Values ranging between −7% for GMP and +20% for I and UMP were obtained, such that it may be concluded that there was no significant matrix effect and that the quantification of these analytes in samples of baby food with the proposed methodology can be accomplished using calibration with standards prepared in ACN.

3.3. Validation of the method on baby food sample: CUF prior to IP-HILIC-MS/MS

Method validation was performed following the directives of the Commission Decision 2002/657/EC, for which one of the fish baby foods (*sole with béchamel*) was used. The results are shown in Table 1. The LODs varied between 0.2 and 2.7 $\mu g\, g^{-1}$ and the LOQs between 0.7 and 9.0 $\mu g\, g^{-1}$. To establish the intraday and interday precision of the method, the intralaboratory repeatability and reproducibility of the method were determined via the RSDs. In the first case, we performed 6 consecutive injections of the baby food sample and in the case of reproducibility the injections were made over 3 consecutive days. Satisfactory results were obtained in all cases, with values between 2 and 13% for interday precision. We also determined the precision between different CUF devices; to accomplish this, 4 different samples were prepared and 6 replicas of each were analyzed, observing values lower than 10% in all cases.

To the best of our knowledge, no Certified Reference Material (CRM) has been described for the joint determination of NUs and NMPs in baby food samples like to ones used in this work. Therefore, the accuracy of the method was assessed by analyzing the content of NUs and NMPs in two different samples of baby food (one of fish: *sole with béchamel*, and the other of meat: *garden vegetables with beef*) using the standard additions method and comparing the results with those obtained by calibration with standards in ACN. No significant differences in the concentrations found with either of the two calibration methods employed were observed. We also compared the slopes of the two methods via a two-tailed *t*-test (Howell, 2010; Kleimbaum et al., 1988). In all cases, p values of >0.05 were found, indicating that there were no significant differences between the two methods (Table 1). The slopes were also compared for one of the fruit baby foods (called *six fruits*) and another of cereals. In both cases, p values in the same range as those found for the fish or meat baby food samples were observed. These results indicate that despite their different compositions the baby foods studied showed similar behaviour.

Fig. 3 shows the TIC and SRM of the fish-based baby food *sole with béchamel*. In these chromatograms it is possible to see the good results obtained regarding sensitivity, resolution and analysis time.

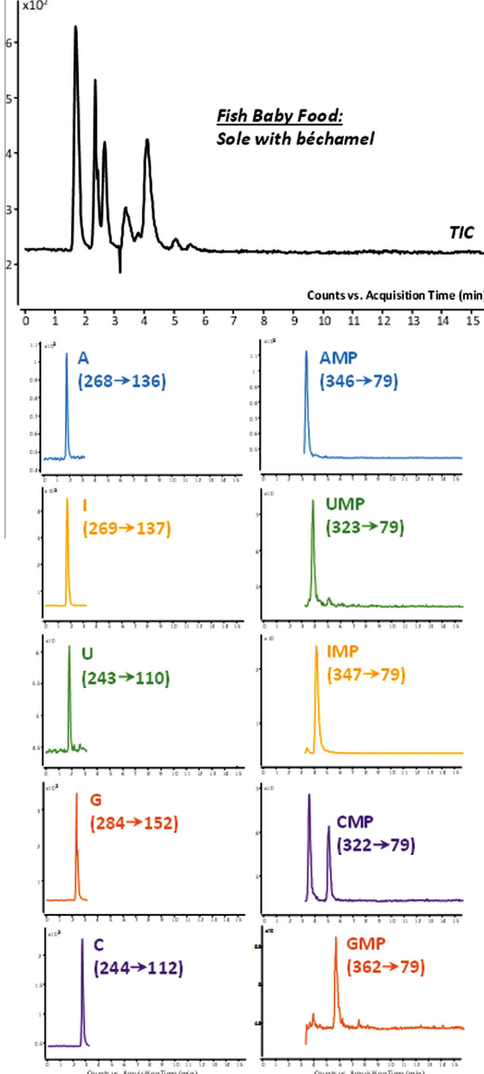


Fig. 3. Total Ion Chromatogram (TIC) and selected reaction monitoring (SRM) of one the fish baby foods (*sole with béchamel*).

3.4. Application to different baby food samples

In order to assess the applicability and robustness of the proposed method, fifteen samples of baby foods were analyzed, as well as the one that had already been used for the development and validation of the method. The parameters related to the composition of these samples are described in Section 2.2. Fig. 4 shows the TIC obtained in the analysis of a standard solution of NUs and NMPs at a concentration of 200 ng mL^{-1}, together with the TICs of some of the baby food samples studied (one of fish, two of meat, one of fruit and the cereal purée).

Table 2 shows the contents of NUs and NMPs obtained upon analyzing each of the samples in triplicate, using external calibration of standard solutions in ACN. Contribution from isotopic adenosine to the inosine MS/MS transition was corrected as indicated in the Experimental (Section 2.4. Quantification). The table also shows the total content of both groups. It should be noted that the presence of NDPs and NTPs was not detected in any of the samples analyzed. The reason for this is that the samples used were not

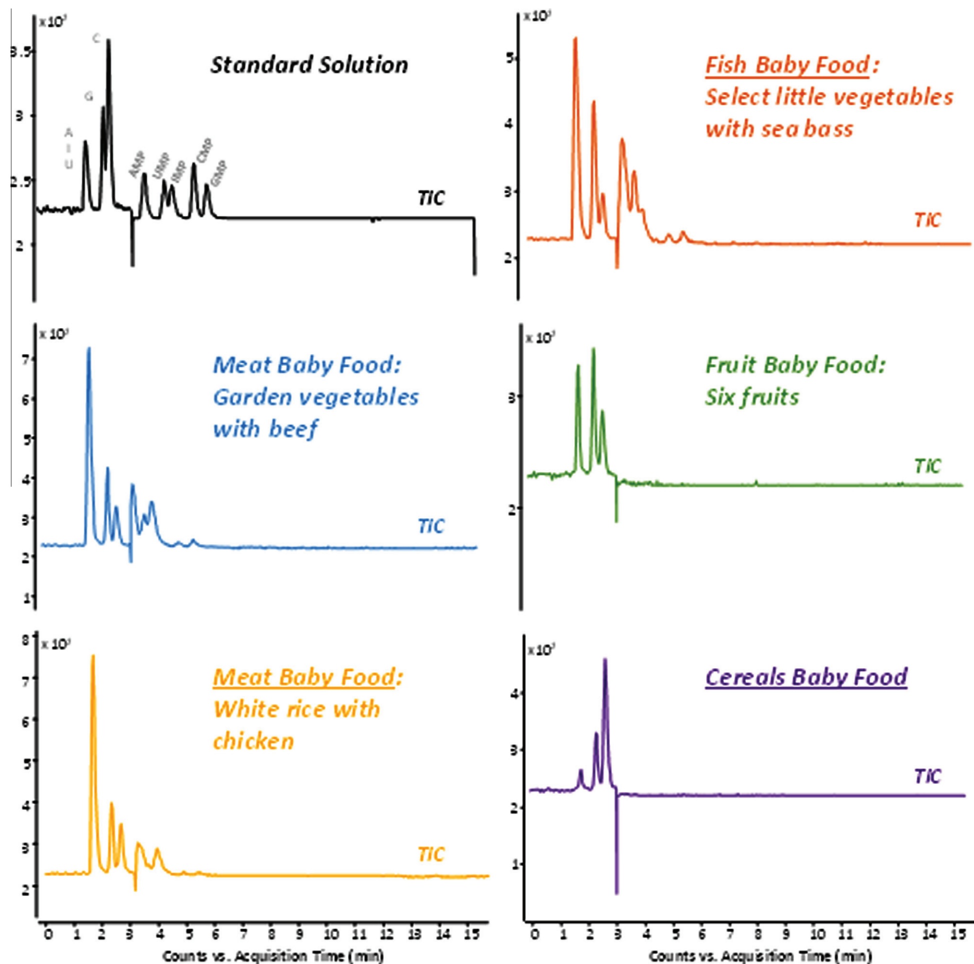


Fig. 4. Total Ion Chromatograms (TICs) for a standard solution of the analytes in ACN at a concentration of 200 ng mL^{-1} and for different samples of baby foods analyzed.

fresh (they were processed) and hence the nucleotide di- and triphosphates had degraded to NMPs and NUs. Different authors have reported that in the muscle of fresh fish a rapid degradation of nucleotide triphosphates occurs. The degradation of ATP generates ADP, AMP, A and, finally, the nucleoside adenosine is converted into inosine (Agustini et al., 2001; Mohan, Ravishankar, Srinivasa Gopal, & Ashok Kumar, 2009; Veracha, Lazzarino, Niven, & Bremer, 2013).

Of the fish baby food samples studied, the one called *sole with béchamel* had the highest level of NMPs. In this type of sample, the NMPs present at the highest concentrations were IMP, AMP and UMP; the nucleoside present at the highest concentration was inosine. In the meat baby food samples, the profiles of the chromatograms and the contents of NUs and NMPs varied, depending on the type of sample (Fig. 4 and Table 2). The *garden vegetables with beef* sample had the highest content of NMPs and in all the meat baby food samples analyzed inosine was also the nucleoside present at the highest concentration. These findings can be interpreted by taking into account that the samples analyzed were not of fresh origin and hence the conversion of the different nucleotides into NUs had already taken place.

The NMP and NU contents shown in Table 2 and the chromatographic profiles (Fig. 4) indicate the absence of NMPs in fruit baby foods and in the cereal purée. In both foods we detected low levels of NUs in comparison with the levels found in fish and above all in meat baby foods, adenosine being the nucleoside present at the highest concentration. In the sample of baby yoghourt CMP, UMP and AMP were found, these being the three NMPs present at the highest concentrations in baby milk formulas and human milk.

Table 2
Nucleotide monophosphate and nucleoside contents (in μg g^{-1}) in different commercial baby foods.

SAMPLE	Nucleotide monophosphates						Nucleosides					
	AMP	CMP	GMP	IMP	UMP	Total	A	C	G	I	U	Total
Fish baby food												
Sole with béchamel	18 ± 2	4 ± 2	3.6 ± 0.4	104 ± 2	9 ± 1	**139 ± 4**	12 ± 1	4 ± 2	12 ± 1	59 ± 2	6 ± 2	**93 ± 4**
Select little vegetables with sea bass	38 ± 2	2.2 ± 0.4	4.5 ± 0.4	17 ± 2	28 ± 1	**90 ± 3**	15 ± 1	1.8 ± 0.4	8 ± 1	47 ± 2	5 ± 2	**77 ± 3**
Select vegetables with sole	11 ± 2	0.9 ± 0.4	1.8 ± 0.4	0.9*	8 ± 1	**23 ± 2**	5.8*	0.4*	6 ± 1	6 ± 2	4 ± 2	**22 ± 4**
Hake with vegetables	41 ± 2	1.3 ± 0.4	2.7 ± 0.4	3 ± 2	16 ± 1	**64 ± 3**	9 ± 1	1.3 ± 0.4	6 ± 1	214 ± 3	3.6 ± 0.4	**234 ± 3**
Meat baby food												
Rice with tender chicken	3.6 ± 0.4	0.4*	ND	ND	1.3*	**5 ± 1**	20 ± 2	1.8 ± 0.4	7 ± 3	118 ± 2	5 ± 2	**152 ± 5**
Baby carrots with beef	4.9 ± 0.4	ND	ND	ND	2 ± 1	**7 ± 1**	26 ± 2	6 ± 1	18 ± 3	90 ± 2	9 ± 2	**149 ± 5**
White rice with chicken	20 ± 3	1.3 ± 0.4	1.8 ± 0.4	34 ± 3	4 ± 1	**61 ± 4**	6.3*	3 ± 1	5 ± 3	80 ± 2	2.2*	**96 ± 5**
Garden vegetables with beef	48 ± 3	2 ± 1	4 ± 4	56 ± 3	26 ± 1	**136 ± 6**	10 ± 2	3 ± 1	8 ± 3	88 ± 2	5 ± 2	**114 ± 5**
Chicken with vegetables	22 ± 3	1.3 ± 0.4	1.3 ± 0.4	ND	9 ± 1	**34 ± 3**	6.7*	3 ± 1	7 ± 3	125 ± 2	4 ± 2	**146 ± 5**
Beef with vegetables	31 ± 3	0.9 ± 0.4	1.3 ± 0.4	ND	10 ± 2	**43 ± 4**	8.1*	4 ± 1	6 ± 3	108 ± 2	8 ± 2	**134 ± 5**
Fruit baby food												
Banana, tangerine and Williams pear	ND	ND	ND	ND	ND	–	10 ± 2	1.3 ± 0.4	2.7*	ND	1.3*	**15 ± 2**
Multifruits	ND	ND	ND	ND	ND	–	3.6*	1.3 ± 0.4	ND	ND	0.9*	**6 ± 1**
Six fruits	ND	ND	ND	ND	ND	–	16 ± 2	1.8 ± 0.4	4 ± 3	ND	1.8*	**24 ± 4**
Varied fruits	ND	ND	ND	ND	ND	–	18 ± 2	1.8 ± 0.4	2.2*	ND	3.1*	**25 ± 2**
Other baby food												
Children yoghourt	0.9*	3 ± 1	ND	ND	1.8 ± 0.4	**6 ± 1**	ND	0.4*	ND	ND	0.9*	**1.3**
Cereals baby food	ND	ND	ND	ND	ND	–	3.6*	7 ± 2	4 ± 3	ND	5 ± 2	**20 ± 5**

Concentration in μg g^{-1} obtained by calibration in ACN.
NDPs and NTPs were not detected (ND) in any sample.
Bold values correspond to the total content in baby food.
* Concentration below LOQ.

4. Conclusions

For the first time, a novel, rapid and highly efficient IP-HILIC-MS/MS method is proposed for the simultaneous detection of nucleosides and nucleotides in baby foods. The sample treatment used for analytes extraction and matrix clean-up included centrifugal ultrafiltration, later washing of the CUF device with water and a further dilution of the extract with acetonitrile. The procedure is simple and compatible with separation in the HILIC system.

The complete method was validated according to the parameters established in the 2002/657/EC decision, providing a reliable and robust method that was applied satisfactorily to the analysis of a large number of dairy and non-dairy baby food samples.

Acknowledgements

The University of Salamanca, Spain (USAL2015-16) is gratefully acknowledged for financial support for this work. M.M.V gratefully acknowledges financial assistance from the Spanish Ministry of Education (FPU Grant).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.05.091.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Determination of nucleosides and nucleotides in baby foods by hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents.

Running title: Nucleosides and nucleotides in baby foods by IP-HILIC-MS/MS

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Table S1. Analytical characteristics of HILIC-MS/MS.

	Nucleotide monophosphates					Nucleosides				
	AMP	**CMP**	**GMP**	**IMP**	**UMP**	**A**	**C**	**G**	**I**	**U**
Calibration Parameters[a]										
Slope / 10^2 AU mL μg^{-1}	13.4 ± 0.3	17.6 ± 0.3	9.8 ± 0.4	10.3 ± 0.3	13.6 ± 0.2	10.4 ± 0.2	48.4 ± 0.8	30.1 ± 0.9	7.4 ± 0.1	4.2 ± 0.1
Intercept / 10^2 AU	0.8 ± 0.4	0.3 ± 0.4	1.1 ± 0.6	0.8 ± 0.5	0.4 ± 0.4	0.2 ± 0.3	0.7 ± 1.1	0.8 ± 0.9	0.2 ± 0.2	0.1 ± 0.2
R	0.9991	0.9995	0.9963	0.9978	0.9992	0.9989	0.9995	0.9983	0.9991	0.9982
LOD[b] **/ ng mL^{-1}**	10	6	8	9	11	60	5	25	45	24
LOQ[c] **/ ng mL^{-1}**	34	19	28	31	37	200	15	85	150	79
CC_α / ng mL^{-1}	33	24	66	51	30	36	25	36	32	45
CC_β / ng mL^{-1}	56	41	112	87	51	61	43	62	54	77
Repeatability[d] ***and (Reproducibility) as RSD / %***										
0.05 µg mL^{-1}	3.0 (8.8)	9.8 (0.2)	7.2 (2.0)	9.3 (3.7)	6.7 (1.7)	13.0 (10.2)	7.6 (4.0)	4.2 (2.4)	13.8 (10.5)	17.0 (10.0)
0.5 µg mL^{-1}	1.4 (8.1)	1.4 (4.8)	2.6 (4.7)	4.7 (6.7)	1.7 (4.8)	4.9 (2.4)	4.4 (4.3)	8.1 (7.5)	8.1 (6.5)	5.1 (2.1)
3 µg mL^{-1}	3.6 (1.0)	2.2 (0.9)	2.3 (5.7)	2.0 (2.7)	1.3 (2.1)	3.5 (2.9)	3.6 (4.3)	4.7 (3.3)	4.6 (5.4)	2.0 (1.3)
Recovery[e] ***/ %***										
0.05 µg mL^{-1}	0.042 ± 0.007 (84 %)	0.05 ± 0.01 (100 %)	0.045 ± 0.007 (90 %)	0.046 ± 0.008 (92 %)	0.05 ± 0.05 (100 %)	0.04 ± 0.01 (80 %)	0.050 ± 0.009 (100 %)	0.047 ± 0.008 (94 %)	0.05 ± 0.04 (100 %)	0.053 ± 0.004 (106 %)
0.5 µg mL^{-1}	0.49 ± 0.05 (98 %)	0.47 ± 0.03 (94 %)	0.49 ± 0.05 (98 %)	0.49 ± 0.08 (98 %)	0.47 ± 0.02 (94 %)	0.53 ± 0.02 (106 %)	0.50 ± 0.04 (100 %)	0.48 ± 0.06 (96 %)	0.50 ± 0.05 (100 %)	0.51 ± 0.07 (102 %)
3 µg mL^{-1}	2.92 ± 0.04 (97 %)	2.96 ± 0.04 (99 %)	3.25 ± 0.06 (108 %)	3.03 ± 0.06 (101 %)	2.98 ± 0.06 (99 %)	3.07 ± 0.06 (102 %)	2.83 ± 0.05 (94 %)	2.9 ± 0.2 (97 %)	2.99 ± 0.06 (100 %)	2.98 ± 0.08 (99%)

a. Calibration range from 50 to 3000 ng mL^{-1} (seven calibration points). AU: Arbitrary Units.
b. LOD: Limits of detection calculated for a signal to noise ratio of 3.
c. LOQ: Limits of quantification calculated for a signal to noise ratio of 10.
d. Repeatability calculated as intraday precisions (6 injections per level). Reproducibility: calculated as interday precisions (3 different days, 6 injections per level each day).
e. Recoveries calculated as the average of 3 injections per concentration level.

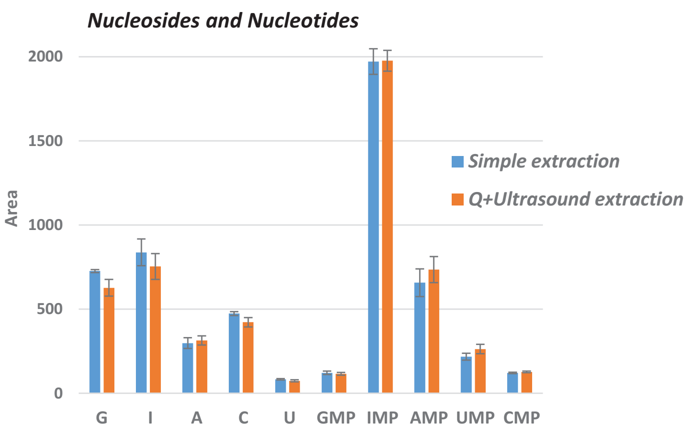

Figure S1. Peak areas obtained for the NUs and NMPs using a process of extraction with UHQ water at RT (*Simple extraction*), compared with those obtained using hot UHQ water and extraction with ultrasound (*Q + Ultrasound extraction*).

CONCLUSIONES

De forma general, puede concluirse que se ha alcanzado el objetivo principal propuesto al inicio de esta Tesis, ya que se han desarrollado de manera satisfactoria nuevas metodologías analíticas basadas en el acoplamiento de la espectrometría de masas a técnicas separativas de alta eficacia. Con las metodologías propuestas se ha conseguido la simplificación de los procedimientos analíticos, tanto en etapas de tratamiento de muestra como en aspectos relacionados con la separación-detección, y se ha demostrado la aplicabilidad de las mismas a través de la resolución de problemas de interés relacionados con el control de calidad de alimentos.

En el primer capítulo de la Tesis, dedicado a las aplicaciones de la electroforesis capilar acoplada a espectrometría de masas (CE-MS), se han puesto de manifiesto las posibilidades que ofrece esta técnica como alternativa a los métodos cromatográficos, teniendo siempre en cuenta las ventajas y condicionantes inherentes a la misma. El tratamiento QuEChERS ha sido propuesto por primera vez como etapa previa al análisis de muestras reales mediante electroforesis capilar, obteniéndose extractos muy limpios en el tratamiento de una matriz alimentaria compleja como es el huevo. Además, la modificación de dicho método, incluyendo una etapa posterior de preconcentración, ha permitido acceder a los niveles de residuos benzimidazólicos en alimentos que establece la actual legislación. Por otra parte, la optimización conjunta de los medios de inyección y separación, tanto en orgánico como en acuoso, ha aportado al método ventajas adicionales a través de su combinación. La inyección de la muestra en medio orgánico ha mejorado notablemente la sensibilidad de la señal analítica, a la vez que ha compatibilizado la técnica de análisis con el tratamiento de

muestra utilizado. La posterior separación en medio acuoso utilizando un modificador orgánico ha originado una buena resolución en la separación electroforética.

Por otra parte, la ultrafiltración asistida por centrifugación (CUF) ha resultado ser una metodología totalmente compatible con la separación electroforética debido al uso exclusivo de medios acuosos. Es un procedimiento simple, que minimiza la manipulación de la muestra y evita el uso de disolventes orgánicos para la extracción. Estas características han permitido su utilización en el análisis de compuestos de elevada polaridad como son los nucleótidos y su aplicación en el tratamiento de muestras complejas como la leche de iniciación y la leche materna. Cabe destacar que ha sido la primera vez que se ha propuesto un método basado en CE-MS para la determinación de nucleótidos en estos dos tipos de alimentos infantiles. En las fórmulas lácteas de iniciación, la aplicabilidad de la metodología propuesta se ha puesto de manifiesto comparando los contenidos de nucleótidos monofosfato obtenidos con aquellos etiquetados por el fabricante. Los resultados han demostrado las posibilidades del método como método de control para la evaluación del contenido de nucleótidos en fórmulas infantiles.

Un procedimiento similar ha sido aplicado al análisis de leches maternas procedentes de un banco de leche. En estas muestras se ha evaluado el contenido de nucleótidos libres después de los tratamientos de pasteurización (HoP) y procesado con altas presiones (HPP) a los que se someten las leches en los bancos para su conservación. Después de un periodo de seis meses de congelación, ninguna de las muestras tratadas sufría variación en sus niveles de nucleótidos. Se ha comprobado que el tratamiento con altas presiones no produce ninguna

variación en el contenido de nucleótidos, por lo que puede ser utilizado como una alternativa adecuada al tratamiento tradicional de pasteurización de las leches maternas, en cuanto a su efecto sobre el contenido de nucleótidos.

En el segundo capítulo se ha abordado el estudio de nuevas tendencias en cromatografía líquida. La nano-cromatografía líquida se ha presentado como una importante alternativa a la cromatografía convencional ofreciendo ventajas únicas propias de las técnicas miniaturizadas: menor consumo de fase móvil y fase estacionaria, menor generación de residuos, pequeños volúmenes de muestra, alta eficiencia en la separación, menor tiempo de análisis y fácil acoplamiento con masas. La nano-LC no había sido muy utilizada en el campo del análisis de alimentos y esta es la primera vez que se ha aplicado a la determinación de nucleótidos en fórmulas infantiles. Cabe destacar el estudio llevado a cabo para la optimización del método, en el que se han utilizado diferentes columnas capilares preparadas en el laboratorio con diferentes fases estacionarias; así como numerosas fases móviles en presencia de reactivos formadores de pares iónicos.

Otro aspecto de este segundo capítulo ha sido constatar el potencial de la cromatografía de interacciones hidrofílicas (HILIC) para la separación de compuestos polares. Se ha comprobado que la utilización de fases móviles ricas en componente orgánico, y en presencia de reactivos formadores de pares iónicos hidrofílicos y hexafluoroisopropanol (HFIP), permitía solucionar los problemas de adsorción que aparecen en el análisis de compuestos fosforilados como los nucleótidos mono-, di- y trifosfato cuando se utiliza cromatografía líquida acoplada a espectrometría de masas. Por otra parte, un estudio

completo del mecanismo de retención nos ha llevado a concluir que, utilizando la concentración adecuada de sales en la fase móvil, los pares iónicos hidrofílicos que se forman interaccionan con la fase estacionaria HILIC a través de un mecanismo de partición, sin que existan influencias significativas de otro tipo de interacción.

El estudio detallado de todas las variables implicadas en la separación con HILIC ha resultado en el desarrollo de un método rápido y eficiente para la separación de un gran número de nucleósidos y nucleótidos, que ha sido aplicado por primera vez al análisis de diferentes alimentos infantiles lácteos y no lácteos. Para el tratamiento previo de estas muestras se ha utilizado la ultrafiltración asistida por centrifugación; una etapa final de dilución del extracto obtenido con acetonitrilo ha hecho compatible este procedimiento con la inyección y posterior separación en el sistema HILIC-MS/MS.

Para finalizar, se puede concluir que los métodos desarrollados han resultado rápidos, sensibles y selectivos, tanto en el análisis de residuos tóxicos como de micronutrientes en alimentos. Todos ellos han mostrado su capacidad para ser utilizados como métodos cuantitativos de confirmación, superando las diferentes etapas de validación exigidas por la Decisión de la Comisión Europea 2002/657. Adicionalmente, se han abordado de forma satisfactoria problemas actuales relacionados con la calidad y seguridad alimentaria a través de la aplicación de estos métodos al análisis de alimentos de elevado consumo.

SUMMARY IN ENGLISH

SUMMARY IN ENGLISH

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INTRODUCTION AND AIMS

Introduction and Justification of the Subject of Study

The presented Doctoral Thesis focuses on the development of new analytical methods based on the coupling of mass spectrometry (MS) and high- efficiency separation techniques, such as capillary electrophoresis (CE) and high performance liquid chromatography (HPLC), that can be used to address issues of common interest related to food quality and safety.

Therefore, two different types of compounds are evaluated in this work: anthelmintic benzimidazoles, wide-range veterinary drugs used in animals destined for food production, and nucleosides and nucleotides, semi-essential dietary nutrients naturally found in food of animal origin. The importance of these compounds in the baby food field is also worth noting, as these compounds are present in human breast milk and are often added as supplements to infant formulas and other complementary baby foods.

In addition to the interest within food analysis, benzimidazoles and nucleotides are compounds with very different chemical properties, and therefore, the steps to extract/preconcentrate these analytes from different food samples should take the particular characteristics of each compound into account. New strategies for sample treatment will be developed to allow processing to be simplified, and sample time preparation and manipulation to be shorten.

The work focuses on the development of new strategies based on the QuEChERS methodology (*Quick, Easy. Cheap, Effective, Rugged and Safe*) and the use of Centrifugal-assisted Ultrafiltration (CUF) devices, which reduce the need for the use of organic solvents. Additionally, the Solid Phase Extraction method (SPE), a well-established technique used in food analysis, will be utilized by employing different sorbents, such as polymers-based or based on polar interactions or on cation and anion exchange. Therefore, by incorporating these strategies, new treatment methods will be designed that are quick, easy, effective and compatible with the following separation and detection techniques.

As a separation technique, capillary electrophoresis is highly efficient and greatly compatible with aqueous media, a condition that favours the separation of compounds that are highly polar, ionic or ionisable. Moreover, the coupling of CE with mass spectrometry (CE-MS) is especially important as this combines the power of identification of CE together with the detection capacity of MS, and is nowadays regarded as a complementary technique or alternative to LC-MS.

One important aspect of the Thesis will be dedicated to the development of methodological innovations in liquid chromatographic systems. Hence, *Hydrophilic Interaction Liquid Chromatography* (HILIC)

will be utilized for separating highly polar compounds. In addition, the coupling of HILIC with tandem mass spectrometry (MS/MS) will allow not only the detection of analytes but also the study of fragmentation processes and the transitions that could result in unequivocal identification of the compounds. A second aspect of this part will be to address the use of nano-liquid chromatography (nano-LC) that allows us to exploit the intrinsic advantages of miniaturized separation techniques: high separation efficiency, shorter analysis time, lower consumption of eluents and samples, and a good coupling with MS owing to a reduced chromatographic flow.

In all cases, the innovative methodologies incorporated throughout this Doctoral Thesis are focused on obtaining analytical methods that are quick, sensitive and selective, and that comply with the established legislation validating their use as methods of quantification and confirmation in the analysis of compounds of interest.

Working Hypothesis and Main Objectives

This Doctoral Thesis includes two well-defined objectives. On one hand, new methodologies will be developed that respond to the demands posed by Analytical Chemistry nowadays, such as the simplification of analytical processes; including the laborious sample treatment steps and the aspects related to separation and detection. On the other hand, the second objective is the application of these new methodologies to resolve problems of interest related to food quality control.

In the first instance, a method for the detection of veterinary drug residues will be designed, in particular for anthelmintic benzimidazoles in egg samples, as eggs are a commonly consumed food. To achieve this, a highly efficient separation technique, such as CE-MS, will be employed to obtain fast separation with a high power of resolution. Furthermore, different strategies for sample treatment based on SPE and the QuEChERS method will be evaluated. The QuEChERS methodology consist of a salts-assisted extraction with acetonitrile to efficiently extract benzimidazoles and make a suitable clean-up of a complex matrix like the case of eggs.

Following on, work will be carried out to analyse micronutrients such as nucleosides and nucleotides. Initially CE-MS will be used, as this technique is highly suitable for the detection of hydrophilic compounds. Nucleotides are very relevant compounds in the developmental stages of infants owing to their regulatory function in the immune response, among other purposes. Hence, samples related to baby food products, such as infant formulas and human breast milk will be analysed.

In addition, the work will also include the evaluation of different sample treatment procedures based on SPE and CUF. And lastly, the developed method will be used to analyse human milk samples originating from a human milk bank. In addition, others parameters will be analysed in these samples, including the influence of the pre-treatments that are frequently applied to human milk for its storage and conservation in milk banks. These pre-treatments involve pasteurization and high-pressure processing.

Within the field of liquid chromatography, a theoretic study will be conducted to elucidate the mechanisms of retention that are

generated between the different HILIC stationary phases and highly polar compounds like nucleotides. These analytes are phosphorylated and very polar compounds that are difficult to resolve using other chromatographic mechanisms and their determination could be a complex issue mainly due to the interactions with different parts of the chromatographic system. Different options to avoid these problems will be evaluated in order to develop an analytical method that is fast and efficient in the analysis of a high number of nucleosides and nucleotides in dairy and non-dairy baby food products.

Complementary to the methods developed to detect nucleosides and nucleotides via the electrophoretic and chromatographic techniques available at the Department of Analytical Chemistry at the University of Salamanca, the work carried out during a short stay at the well-known and internationally prestigious research centre, *Istituto di Metodologie Chimiche, Consiglio Nazionale delle Ricerche* (CNR, Roma), will also be included within the Thesis. The research group "*Electromigration and Chromatographic Methods"*, led by Dr. Fanali, was one of the first groups in the development and application of miniaturized separation techniques, specifically nano-liquid chromatography (nano-LC). Nano-LC is a miniaturized analytical technique that has recently emerged as a complementary separation method or alternative to conventional LC, due to the number of advantages that it offers in relation to efficiency, time of analysis, and the use of less amounts of stationary and mobile phases. As a result of the work carried out during the stay at the CNR, a new method for the detection of nucleotides in infant formulas was designed, where samples are previously treated using CUF and subsequently analysed using nano-LC.

CONCLUSIONS

Overall, it can be concluded that the main objective proposed at the start of the Thesis has been achieved, as new analytical methods based on the coupling of mass spectrometry to highly effective separation techniques were satisfactorily developed. The proposed methodologies have permitted the simplification of analytical processes, not only regarding sample treatment but also the aspects related to separation and detection. Additionally, the application of these processes has been clearly demonstrated through solving issues of common interest related to the quality control of food.

In the first chapter of the Thesis, dedicated to the application of capillary electrophoresis coupled with mass spectrometry, the use of this technique as an alternative to chromatographic methods was manifested, although always taking into account its advantages and inherent constraints. For the first time, the QuEChERS treatment has been proposed as an initial step previous to sample analysis using capillary electrophoresis, which has led to the attainment of very clean extracts from a complex food matrix, such as that found in eggs. In

addition, the modification of the described method, including the subsequent step of preconcentration of the sample, has allowed to obtain the levels of benzimidazolic residues established by the legislation. Furthermore, the combined optimization of the injection and separation media, either organic or aqueous, has provided additional advantages to the method. The injection of the sample in an organic medium has greatly improved the sensitivity of the analytical signal, and at the same time made this technique more compatible with the sample treatment utilized. Subsequent separation in an aqueous medium using an organic modifier has also produced good resolution of electrophoretic separation.

Moreover, centrifugal-assisted ultrafiltration (CUF) has proven to be a methodology totally compatible with electrophoretic separation due to the exclusive use of aqueous media. CUF is a simple procedure that minimizes sample manipulation and avoids the use of organic solvents in the extraction process. These characteristics have allowed CUF to be used in the analysis of highly polar compounds, such as nucleotides, and in its application in the treatment of complex samples, such as first-stage infant formulas and human breast milk. It is worth noting that this is the first time that the use of a method based on CE-MS has been proposed for the detection of nucleotides in these types of baby foods. The applicability of the proposed method in the analysis of infant formulas has been demonstrated by comparing the contents of nucleotide monophosphates obtained with those outlined within the manufacturing labels. The results obtained confirmed the possibility of this method to be used as a control method for the evaluation of the nucleotide content in infant formulas.

A similar procedure has been applied to the analysis of human milks coming from a human milk bank. In these samples the content of free nucleotides after the pasteurization (HoP) and high-pressure processing (HPP) treatments administered to milk for its subsequent storage, was evaluated. After being frozen for a six-month period, none of the treated samples exhibited variation within their nucleotides levels. It has been confirmed that HPP technique did not reveal any modification in the nucleotide content, thus it could be a suitable alternative to traditional HoP in the treatment of human milk, in relation to its effect on nucleotide content.

In the second chapter of the Thesis new trends in liquid chromatography were assessed. Nano-liquid chromatography has been suggested as a significant alternative to conventional chromatography, offering its own advantages as a miniaturized technique, such as the lower use of mobile and stationary phases, generating fewer residues, the need for smaller amount of sample, high separation efficiency, shorter analysis time and easy coupling with mass spectrometry. Nano-LC had not been very frequently used in food analysis and this was the first time that it has been applied to the determination of nucleotides in infant formulas. It is worth highlighting the study carried out to optimize the method, in that different capillary columns have been prepared in the laboratory with different stationary phases, as well as numerous mobile phases in the presence of ion-pairing reagents.

Another aspect of the second chapter was to confirm the potential of Hydrophilic Interaction Liquid Chromatography (HILIC) for the separation of polar compounds. The use of mobile phases rich in organic components and in the presence of hydrophilic ion-pairing reagents and

hexafluoroisopropanol (HFIP) provided a solution to the adsorption problems that appear in the analysis of phosphorylated compounds, such as nucleotide mono-, di- and triphosphates, when the analysis is performed with liquid chromatography coupled to mass spectrometry. Furthermore, a complete study of the retention mechanisms permitted us to conclude that, by using the adequate concentration of salts in the mobile phase, the formed hydrophilic ion-pairs interact with the HILIC stationary phase via a partition mechanism, without significant influences incited by another type of interaction.

The detailed study of all of the variables implicated in HILIC separation has resulted in the development of a quick and efficient method for the separation of a great number of nucleosides and nucleotides, which has been applied for the first time to the analysis of different types of dairy and non-dairy baby food. The previous treatment of these samples has included the use of centrifugal-assisted ultrafiltration, where a final dilution step with acetonitrile of the extract obtained has made the procedure compatible with the injection and subsequent separation using the HILIC-MS/MS system.

Finally, it can be concluded that the methods developed have proven to be quick, sensitive and selective, not only in the analysis of toxic residues but also in the detection of micronutrients in food. All of them have shown the capacity to be used as methods of quantification and confirmation, surpassing the different validation steps required by the European Commission Decision 2002/657. In addition, current issues related to food quality and securities have been successfully addressed by the application of these methods to the analysis of commonly consumed food.

APÉNDICE: MENCIÓN “DOCTORADO INTERNACIONAL”

Con la elaboración y defensa de esta memoria de tesis se pretende optar a la obtención de la mención de "Doctor Internacional" en el título de Doctor por la Universidad de Salamanca, al considerar que la doctoranda reúne los requisitos para optar a tal mención:

- La doctoranda ha realizado una estancia de tres meses en el *Istituto di Metodologie Chimiche, Consiglio Nazionale delle Ricerche* (CNR), en Roma (Italia), relacionada con la presente tesis.
- Esta memoria incluye un resumen en inglés, incluyendo objetivos y conclusiones.
- La tesis cuenta con los informes favorables de dos doctores expertos pertenecientes a instituciones de educación superior no españolas.
- Uno de los miembros que ha de formar parte del tribunal evaluador de la tesis es un experto perteneciente a un centro de investigación de prestigio no español.

Se incluyen, en este apéndice, la certificación de la estancia breve así como los pertinentes informes favorables.

PROGRAMA ESTATAL DE PROMOCIÓN DEL TALENTO Y SU EMPLEABILIDAD

CERTIFICADO DEL CENTRO RECEPTOR TRAS LA ESTANCIA BREVE O TRASLADO TEMPORAL

CERTIFICATE OF STAY IN A FOREING INSTITUTION

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Dirección/ *Adress:* Area della Ricerca di Roma I, Via Salaria km 29,300-00015
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3. Investigador responsable en el centro de la estancia/ *Responsable person in the Host*
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Nombre/ *Name:* Dr. Giovanna Mancini
Cargo/ *Post:* Director of IMC
CERTIFICO: que el becario arriba mencionado ha realizado una estancia en este centro en las siguientes fechas: desde 01 / 09 / 2014 hasta 30 / 11 / 2014 ***THIS IS TO CERTIFY:*** *that the above mentioned person has performed a stay in this Institution in the following dates:* *From: 01 / 09 / 2014 To: 30 / 11 / 2014*
Firma y Sello/ *Signature & Stamp*

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TÍTULO DE LA TESIS OBJETO DE INFORME / TITLE OF THE TESIS

Mass spectrometry coupled to capillary electrophoresis and liquid chromatography for the determination of contaminants and micronutrients in foodstuff.

NOMBRE Y APELLIDOS DEL DOCTORANDO / NAME OF THE CANDIDATE

María Mateos Vivas

NOMBRE Y APELLIDOS DEL DIRECTOR/A(S) DE LA TESIS / NAME OF THE THESIS DIRECTOR(S)

Encarnación Rodríguez Gonzalo
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INFORME RAZONADO/REASONED REPORT

SOBRE LA CALIDAD CIENTÍFICA DE LA TESIS DOCTORAL (puede añadir las hojas que crea necesarias adjuntando a este informe)

ABOUT THE SCIENTIFIC QUALITY OF PhD (you can use all the pages you might need including this form)

The summary of the PhD Thesis entitled "*Mass spectrometry coupled to capillary electrophoresis and liquid chromatography for the determination of contaminants and micronutrients in foodstuff*" is organized within two main chapters, plus aims and conclusions. The first chapter is dedicated to different applications of capillary electrophoresis coupled to mass spectrometry (CE-MS), and the second one is focused on new trends in liquid chromatography such as hydrophilic interaction chromatography (HILIC) and nano-liquid chromatography (nano-LC).

Aims of this work are very well-defined: on the one hand, the development of modern analtycal methods based on different techniques and on the other hand, the application of these methods to the analysis of contaminants and micronutrients in food samples. It should be noted that several techniques have been employed in this thesis: CE-MS, HILIC-MS/MS and nano-LC-UV, fact that increases the interest and the scientific quality of the work. In addition, investigations about the chromatographic mechanisms involved in the separation process of very highly polar compounds by HILIC-MS/MS were carried out. This study adds an additional value to the work due to its important theoretical contribution and its application possibilities.

Moreover, new strategies for the sample treatment have been assayed considering the different nature of the analytes studied in this work, i.e., benzimidazoles and nucleotides that present very different chemical properties; and also taking into account the complexity of the food samples analyzed.

Furthermore, the objetives described at the beginning of the work have been achieved by proposing analytical methodologies that could be applied to the resolution of problems related to food safety and quality.

At last but not least, results obtained have been successfully published in high ranking journals like Journal of Chromatography A, Electrophoresis or Food Chemistry, placed at the first quartile of their respective areas. Therefore, my conclusion, based on the whole work, is going into favour to the confirmation of an exceptional contribution to analytical sciences. I can confirm the scientific quality of the work and its presentation for getting the PhD degree is highly recommended.

Informo que la calidad científica de esta Tesis es merecedora de obtener la Mención Doctor Internacional (marcar con una cruz una opción)

I inform that the scientific quality of this thesis is worthy of obtaining the International Doctor Mention (mark with across one option)

Favorable [X] No favorable/Not favorable [ ]

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Consiglio Nazionale delle Ricerche

TÍTULO DE LA TESIS OBJETO DE INFORME / TITLE OF THE TESIS

Mass spectrometry coupled to capillary electrophoresis and liquid chromatography for the determination of contaminants and micronutrients in foodstuff.

NOMBRE Y APELLIDOS DEL DOCTORANDO / NAME OF THE CANDIDATE

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ABOUT THE SCIENTIFIC QUALITY OF PhD (you can use all the pages you might need including this form)

In the present doctoral thesis, the development of different analytical methods for the determination of two different classes of compounds including

1) anthelmintic benzimidazoles, used as veterinary drugs in animals for food production

2) nucleosides and nucleotides in human milk and infant food

have been proposed. In the thesis, novel, selective, rapid analytical methods for the determination of the mentioned compounds have been developed. The use of CE hyphenated to mass spectrometry allowed to develop alternative fast, efficient and sensitive methods to HPLC. Novelties were presented also in chromatography by developing a nano-LC and HILIC-LC methods for the determination of nucleotides in human milk and infant food.

Part of the research was dedicated to sample pre-treatment, by evaluating fast and effective extraction methods.

In conclusion the main objectives of the thesis were achieved by the student Maria Mateos Vivas with very good results. In addition, the present research allows to solve problems related to quality control in food.

The results obtained during the doctoral thesis allowed to obtain six publications in impacted valuable journals with good IF.

In my opinion, this is an interesting scientific research with several novelties both in the evaluation of methodologies and applications.

My decision is certainly favorable.

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