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DEPARTAMENTO DE QUÍMICA ANALÍTICA

"Profesor Fermín Capitán García"

TESIS DOCTORAL

"DESARROLLO Y VALIDACIÓN DE METODOLOGÍAS ANALÍTICAS APLICADAS A LA INVESTIGACIÓN ESTRATÉGICA NUTRICIONAL EN CIENCIAS DE LA SALUD"

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DESARROLLO Y VALIDACIÓN DE METODOLOGÍAS ANALÍTICAS APLICADAS A LA INVESTIGACIÓN ESTRATÉGICA NUTRICIONAL EN CIENCIAS DE LA SALUD

por

Ángela Santos Fandila

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A mis padres y hermana.

A Javi y a nuestro "miniyo".

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RESUMEN

La presente tesis doctoral se ha centrado en el desarrollo y validación de nuevas metodologías analíticas para la determinación de diversas moléculas, relacionadas con procesos bioquímicos específicos, en distintas matrices de carácter biológico. Los métodos se han sido validados siguiendo las recomendaciones de la *Guía Internacional para la Industria, Validación de Métodos Bioanalíticos* de la FDA, EE.UU.

El desarrollo y la validación analítica, el diseño experimental en modelos animales y productos nutricionales, la manipulación de los animales o los procesos de toma de muestra, son aspectos fundamentales en el desarrollo de cualquier método de buenas características analíticas. En la presente Memoria de Tesis Doctoral se exponen y discuten cada uno de estos aspectos para los casos particulares de los métodos propuestos.

Las biomoléculas evaluadas están relacionadas con estudios dirigidos al descubrimiento de nuevos ingredientes y/o tecnologías que podrían ser aplicables a nuevos productos de nutrición (para niños o adultos), o a los productos que ya existen en el mercado para mejorar y reforzar la salud general de la sociedad. Este trabajo se divide en varios capítulos, estando cada uno de ellos dirigido a las biomoléculas o analitos de interés que en cada momento han sido estudiados en función del proyecto científico en el que están involucrados. Estas biomoléculas son neurotransmisores, oligosacáridos de la leche humana (HMOs), leucina y su metabolito β-hidroximetilbutirato (HMB), y otros compuestos biológicos relacionados con procesos de oxidación. Se trata de compuestos de gran importancia en procesos esenciales de la bioquímica humana, relacionados con las fisiológicas.

La técnica analítica utilizada para la determinación de los compuestos de interés ha sido principalmente la cromatografía de líquidos acoplada a espectrometría de masas en tándem triple cuadrupolo y de tiempo de vuelo.

La presente Memoria de Tesis Doctoral ha sido realizada por compilación de publicaciones. Está conformada por cuatro artículos publicados en revistas pertenecientes a la Editorial Elsevier (Talanta), Springer (Analytical Bioanalytical

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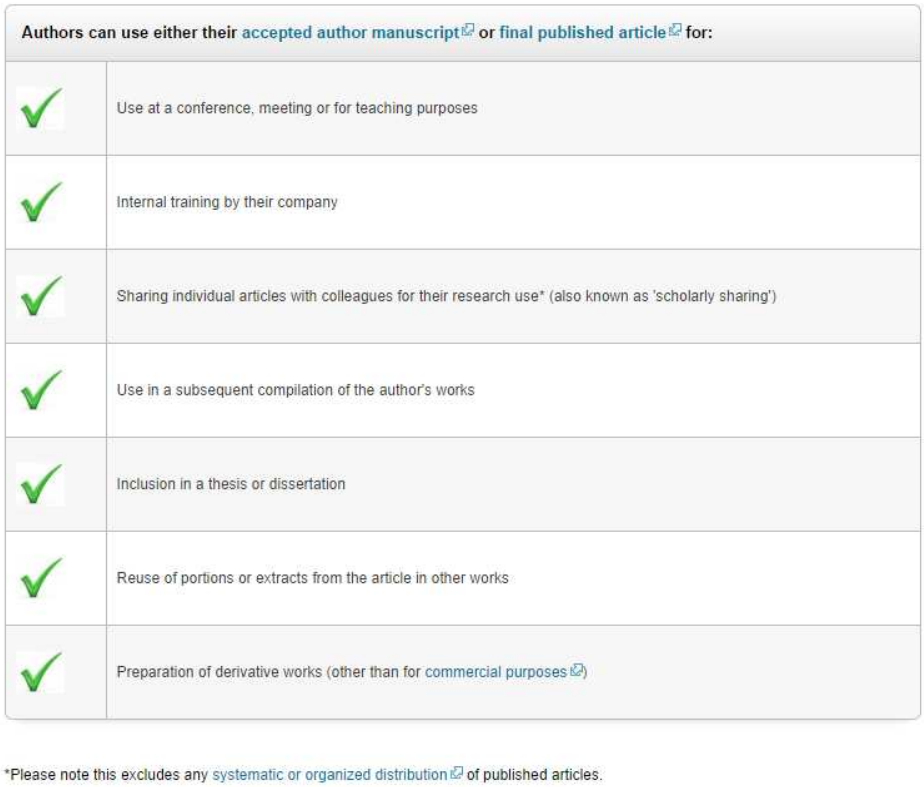

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ACRÓNIMOS Y ABREVIATURAS

$\%P_{lof}$	Valor P del test de fallo de ajuste (Lack-of-fit)
2'-FL	2'-Fucosilactosa, 2'-Fucosyllactose
3-FL	3-Fucosilactosa
3'-SL	3'-Sialilactosa, 3'-Sialyllactose
4-HHE	4-hidroxihexanal
4-HNE	4-hidroxinonenal
5-HIAA	Ácido 5-hidroxiindolacético
5-HT	5-Hidroxitriptamina, Serotonina
5-HTP	5-Hidroxi-L-triptófano
6'-SL	6'-Sialilactosa, 6'-Sialyllactose
8-OHdG	8-oxo-deoxiguanosina, 8-oxo-deoxyguanosine
a	intercept
AA	Absorción atómica
ABC	Analytical Bioanalytical Chemistry
Abs	Absorbancia
AcCh	Acetilcolina, Acetylcholine
Ac-CoA	Acetil-Coenzima A, Acetyl-Coenzyme A
AChE	Acetilcolinesterasa
aCSF	Artificial cerebrospinal fluid

ACTH	Hormona adrenocorticotropa
AD	Alzheymer disease
ADC	Arginina descarboxilasa
ADN	Ácido desoxirribonucleico
ADP	Adenosina difosfato
ADP	Adenosina difosfato
AG	Ácidos grasos
AGE	Ácidos grasos esenciales
AGI	Ácidos grasos insaturados
Agm	Agmatina
AGPI	Ácidos grasos poliinsaturados
ALT	Alanina aminotransferasa
APCI	Ionización química a presión atmosférica
ATP	Adenosina trifosfato; Adenosin triphosphate
ATV	Área tegmental ventral
b	Slope
b.w.	Body weight
BAChE	Acetilcolinesterasa sérica
BBB	Blood brain barrier

BCKAD	Branched chain keto acid dehydrogenase
Bet	Betaina, Betaine
BHMT	Betaína-homocisteína metiltransferasa
BM	Biological matrix
CDP	Citidina difosfocolina
CDP-Cho	5'-Citidinfosfocolina, 5'-Cytidinephosphocholine
CE	Collision energy
CF-FAB	Flujo continuo y bombardeo con átomos rápidos
$CHCl_3$	Cloroformo
Cho	Colina, Choline
CK	Colina quinasa
CML	Carboximeltilisina
CMP	Citidina monofosfato
COP	Productos de oxidación del colesterol
CPT	CDP-colina
CTP	Citidina trifosfato
DA	Dopamina, Dopamine
Da	Dalton
DAD, PDA	Detector de diodos Array

DAG	Diacilglicerol
DHA	Docosahexaenoic acid
DMG	Dimetilglicina
DNPH	2,4-Dinitrophenylhydrazine
dpf	Days post fecundation
DSC	Differential Scanning Calorimetry
E	Epinefrina, Epinephrine
ECD	Detector electroquímico
ELISA	Ensayo por inmunoabsorción ligado a enzimas
ELSD	Detector evaporativo de dispersión de la luz
ERNs	Especies reactivas de nitrógeno
EROs	Especies reactivas de oxígeno
ESCI	Combined electrospray and atmospheric pressure ionization source
ESI	Ionización por electrospray
EtOH	Etanol
eV	Electronvoltio
FA	Ácido fórmico
FD	Detector de fluorescencia
FDA	US Food and Drugs Administration

FI	Fórmula infantil
FID	Detector de ionización de llama, Flame ionization detector
FPD	Detector fotométrico de llama
FT-ICR	Transformada de Fourier-Resonancia ciclotrónica
FTIR	Detector de infrarrojos con Transformada de Fourier
Fuc	Fucosa, Fucose
GABA	Ácido gamma-aminobutírico, Gamma aminobutiric acid
Gal	Galactosa
GC	Cromatografía de Gases
GC-oaTOF	Cromatografía de gases ortogonal con detección de espectrometría de masas tiempo de vuelo
GD	Gestational day
GDA	Glutamato descarboxilasa
GlcNAc	N-acetylglucosamine
Gln	Glutamina, Glutamine
Glu	Glutamato, Glutamate
Glu	Glucosa, Glucose
Gluc	Glucosa, Glucose
GnRH	Hormona liberadora de la gonadotropina
GPCho	Glicerofosfocolina, Glycerophosphocholine

h	hour
HAL	Histidinoalanina
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFBA	Heptafluorobutyric acid
HMB	β-Hidroximetilbutirato, β-Hydroxymetylbutirate
HMG-CoA	β-hidroximetilglutaril-coenzima A
HMOs	Human milk oligosaccharides
HPAEC–PAD	High performance anion–exchange chromatography with pulsed amperometric detection
hpf	Hours post fecundation
HPLC–Chip/TOF–MS	Microfluidic chips and mass spectrometry technology
HPTLC	Cromatografía de líquidos de alta resolución en capa fina
ICP	Plasma acoplado inductivamente
ICUMSA	International Commision on Uniform Methods of Sugar Analysis
IF	Infant Formula
IPA	Isopropanol
IR	Infrarrojo
IT	Trampa de iones
KDa	Kilodaltons
KIC	α-cetoisocaproato

Lact	Lactosa, Lactose
LC-MS/MS	Cromatografía de líquidos acoplada a espectrometría de masas en tándem
LDR	Rango dinámico linear, Linear dynamic range
Leu	Leucina, Leucine
LNFP III	Lacto-N-fucopentosa III
LNnT	Lacto-neotetraosa, Lacto-neotetraose
LOD	Límite de detección, Limit of detection
LOQ	Límite de cuantificación, Limit of quantification
m/z	Relación masa/carga
MALDI	Desorción/ionización asistida por una matriz
MAT	Metionina adenosiltransferasa
MDA	Malondialdehído, Malondialdehyde
MeCN	Acetonitrilo
MeOH	Metanol
Met	Methionine
MHPG	3-Metoxi-4-hidroxifenilglicol
min	Minutos
mpf	Months post fecundation
MR	Maillard Reaction

MRM	Seguimiento de múltiples iones, Multiple Reation Monitoring
MS	Espectrometría de Masas, Mass Spectrometry
MS	Metionina sintasa
MSD	Detector selectivo de masas
MW	Molecular weight
MΩ	Megaohmios
n	Number of determinations
N	Normal
NE	Norepinefrina, Norephinephrin
NMDA	N-metil-D-aspartato
NPD	Nitrogen-phosphorus detector;
OSI	Oil Stability Index
OSLs	Oligosacáridos presentes en la leche
o-Tyr	orto-Tirosina, orto-Tyrosine
OVX	Ovariectomized
p–AnV	p -Anisidine value
PAG	Glutaminasa activada por fosfato
PC	Fosfatidilcolina
PCA	Principal component analysis

PCho	Fosfocolina, Phosphocholine
PCR	Polymerase chain reaction
Pcyt1: CTP	Fosfocolina citidil transferasa1
PE	Fosfatidietanolamina
Pe	Endothelial permeability coefficient
PEMT	Fosfatidiletanolamina-N-metiltransferasa
PID	Detector de fotoionización
PL	Fosfolípidos
PND	Postnatal day
PNMT	Feniletanolamina N-metiltransferasa
POP	Productos de oxidación de los fitoesteroles
PPi	Pirofosfato
ppm	Partes por millón
PPOs	Polifenol oxidasas
PSe	PS value for the endothelial monolayer
PSf	Slope of the clearance curve for the filter only covered with collagen
PSt	Slope of the clearance curves for the co-culture
PUFAs	Polyunsaturated fatty acids
PV	Índice de peróxidos, Peroxide value

Q	Cuadrupolo
QqQ	Triple cuadrupolo
QTOF	Cuadrupolo-tiempo de vuelo
R	Recuperación
R&D	Research and Development
R^2	Coeficiente de determinación
RM	Reacción de Maillard
RMN, NMR	Resonancia Magnética Nuclear, Nuclear Magnetic Resonance
RNS	Reactive nitrosative species
ROS	Reactive oxidative species
rpm	Revoluciones por minuto
RSD	Relative standard deviation
SA	Ácido siálico (ácido 5-neuramínico), Neuraminic Acid
SAH	S-adenosilhomocisteína
SAM	S-adenosilmetionina
SD	Standard deviation
SD rats	Sprague Dawley rats
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Serotonina, Serotonin

SHAM	Simulated surgery without remove anything
SIM	Modo de iones seleccionados, Selected Ion Monitoring
SM	Esfingomielina
SMGs	Soluble milk glycans
SNC	Sistema nervioso central
SOP	Productos de oxidación de los esteroles
SPE	Solid phase extraction
SPME	Solid-phase microextraction
SRM	Selective reaction monitoring
T3	Triyodotironina
T4	Tiroxina
TBA	Thiobarbituric acid
TCD	Detector de conductividad térmica
TDAH	Trastorno por deficit de atención con hiperactividad
TG	Triglicéridos
TMG	Trimetilglicina, Trymethylglycine
TOF	Tiempo de vuelo, Time of flight
TQD	Detector de triple cuadrupolo, Triple quadrupole detector
Trp	Triptófano, Tryptophan

TS	Ionización por termonebulización
TV	Totox value
Tyr	Tirosina, Tyrosine
UHPLC-MS/MS	Cromatografía líquida de ultraresolución acoplada a espectrometría de masas; Ultra high performance Liquid Chromatography-Mass Spectrometry
UHPLC-MS/MS (QqQ)	Cromatografía líquida de ultraresolución acoplada espectrometría de masas (Triple cuadrupolo), Ultra high performance Liquid Chromatography-Mass Spectrometry (Triple quadrupole)
UPLC®	Cromatografía de líquidos de ultraresolución, Ultra performance Liquid Chromatography
UV	Ultravioleta
V	Voltio
vis	Visible
VA	Volume of the abluminal chamber
WS	Work standard
ZLR	Zucker Lean rats

OBJETIVOS DE LA TESIS

La presente Tesis Doctoral tiene como objetivo principal el **desarrollo de métodos de buenas características analíticas** para su aplicación en el campo de las ciencias de la salud y más específicamente dirigidas a la ciencia e investigación estratégica de la industria nutricional.

Se pretende demostrar la necesidad y la importancia de buscar soluciones analíticas para la determinación de diferentes **biomoléculas**, que despiertan un gran interés por su posible actividad biológica *in vivo*. Es importante disponer de nuevas metodologías analíticas dirigidas a la protección de la salud humana para poder atender a las exigencias establecidas en el marco actual tanto legislativo como social.

En este trabajo se van a presentar moléculas de alto interés por su implicación en la mejora de la salud de la población en general, ya que están relacionadas con mecanismos de acción básicos en el organismo que pueden mejorar o paliar ciertas dolencias y/o enfermedades, ralentizar y/o evitar enfermedades degenerativas que la población sufre cada día. El interés inicial que despiertan estas biomoléculas en nuestra investigación, se debe fundamentalmente a sus **propiedades cognitivas**, aunque no son únicas, ya que están interrelacionadas con otras muchas funciones y rutas bioquímicas. Se trata por tanto de biomoléculas de alto interés para la población por su capacidad de mejorar la salud en general.

Además de este enfoque, con el estudio de estas moléculas, y gracias a las técnicas analíticas usadas, podremos conocer rutas metabólicas y dar explicación científica con resultados cuantificables a ciertos procesos que hasta ahora no han sido explicados. El conocimiento de dichos procesos podrá servir para establecer sistemas de **mejora cognitiva en niños y adultos, desarrollo del sistema músculo-esquelético y del sistema inmune**, tanto en individuos sanos como enfermos.

En esta Tesis Doctoral se han desarrollado, optimizado y **validado** diferentes métodos analíticos, siguiendo las guías internacionales de aplicación en cada caso particular. Los diseños experimentales de los **modelos animales**

utilizados, la manipulación animal o la obtención de las muestras son aspectos muy importantes en este trabajo que han sido desarrollados y expuestos de igual manera.

Las biomoléculas estudiadas están involucradas en distintos proyectos de investigación dirigidos al descubrimiento de nuevos ingredientes o tecnologías que podrían ser aplicados a nuevos productos nutricionales o a productos ya existentes en el mercado, para mejorar y reforzar la salud de la población en general. Este trabajo se divide en distintos capítulos, cada uno dirigido a las biomoléculas o analitos de interés estudiados en cada momento según el proyecto de investigación donde han estado involucradas.

Por un lado, se presenta el estudio de una serie de compuestos pertenecientes a distintas familias, directamente relacionados con la función cognitiva, los **neurotransmisores**, incluyendo algunos metabolitos y precursores: betaina, glutamina, ácido glutámico, ácido γ-aminobutírico, fosfocolina, glicerofosfocolina, citidina 5′-difosfocolina, colina, acetilcolina, dopamina, norepinefrina, serotonina, tirosina, epinefrina, triptófano, ácido 5-hidroxiindolacético y agmatina. Por otro lado, una serie de hidratos de carbono de muy elevado interés para la biología humana. Son los llamados **oligosacáridos solubles presentes en leche (human milk oligosaccharides)**: 2'-fucosilactosa, 3'-sialilactosa, 6'-sialilactosa, Lacto-N-neo-tetraosa, y los compuestos básicos relacionados con estos carbohidratos, como el ácido siálico, la fucosa, la lactosa y la glucosa. En otra línea de investigación se presentan dos moléculas de gran interés biológico, la **leucina y su metabolito β-hidroximetilbutirato**, relacionadas clásicamente con el sistema músculo-esquelético, pero que en los últimos años también han sido relacionadas con la función cognitiva. El siguiente capítulo se centra en el desarrollo de métodos analíticos que permitan tener una medida cuantificable del **envejecimiento de un sistema**. Por un lado será estudiado un producto nutricional y por otro un organismo o sistema biológico. Estos métodos nos permiten determinar marcadores de degradación tanto nuevos como clásicos en productos nutricionales, y biomarcadores relacionados con el deterioro de un ser vivo, ya sea por envejecimiento natural o por enfermedad. Estos biomarcadores son las

llamadas especies reactivas del oxígeno (ROS) o del nitrógeno (RNS). Malondialdehido, o-tirosina y 8-oxo-deoxiguanosina son biomoléculas que permiten cuantificar el deterioro de los animales observando el detrimento orgánico en tres líneas o familias distintas: oxidación lipídica, de proteínas y de ADN, respectivamente.

Los objetivos específicos de la Tesis Doctoral son los que se enumeran a continuación:

1. Realizar una **revisión bibliográfica exhaustiva** hasta la fecha en relación a las **biomoléculas** estudiadas para entender en qué punto se encuentra el conocimiento sobre los cambios bioquímicos generados en el organismo por la manipulación nutricional u otros procedimientos.

2. Llevar a cabo una revisión de la **tecnología** clásica utilizada para dar apoyo analítico en los estudios de investigación de las moléculas de interés.

3. Aportar soluciones analíticas, y mejoras sobre las ya existentes, para determinar los **neurotransmisores** mencionados en matrices biológicas. Por un lado, el método incluye la optimización de un método de muestreo que permita medir los analitos seleccionados en el cerebro de un animal *in vivo*, mediante la técnica de **microdiálisis cerebral**. Gracias a estas metodologías *in vivo* se puede aportar más información para entender los mecanismos de acción de muchos neurotransmisores, metabolitos y precursores en procesos biológicamente más cercanos a la realidad. Por otro lado, el método ha sido optimizado, validado y aplicado a una matriz biológica adicional, **pez cebra** *ex vivo*.

4. Poner a punto un método para la determinación de un grupo de carbohidratos denominados genéricamente oligosacáridos solubles presentes en leche humana (**human milk oligosaccharides)** en matrices biológicas.

5. Poner a punto un método para la determinación de dos compuestos de gran interés biológico como son la **leucina y su metabolito el β-hidroximetilbutirato**, e investigar su biodisponibilidad y mecanismos de acción, para experimentos *in vitro*, *in vivo* y *ex vivo*. Estos estudios requieren un método analítico sensible y preciso para medir niveles bajos de HMB en diferentes fluidos biológicos.

6. Poner a punto un método analítico para determinar **ROS en muestras biológicas como suero, hígado y cerebro**, que permita explorar cómo se puede manipular el deterioro o envejecimiento orgánico, llamado también estrés oxidativo, a través de la nutrición. Estudio de los procesos de degradación de un **producto nutricional** a través de una oxidación acelerada.

7. **Validar** los métodos según las guías internacionales aplicables en cada caso.

8. **Aplicar** los métodos propuestos en procedimientos ***in vivo, ex vivo* e *in vitro***.

Las técnicas que se han empleado para la consecución de los objetivos de la presente Tesis Doctoral han sido las siguientes:

- Cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas en tándem de triple cuadrupolo.

- Cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas de alta resolución de tiempo de vuelo.

- Técnicas de **microdialisis** para la obtención de microdializados de cerebro a partir de animales vivos.

- Cámara de **oxidación acelerada**.

- Software específico en el procedimiento de datos adquiridos con screening total mediante TOF: **UNIFI**.

- **Herramientas estadísticas** para el estudio de la acumulación, distribución y procedencia de los compuestos de interés. Uso de softwars estadísticos como GraphPad Prism, Statgraphics y Minitab.

INTRODUCCIÓN GENERAL

Son varias las etapas a seguir para la creación de un proyecto científico en general. El proceso comienza con una búsqueda bibliográfica y la generación de ideas por parte de los distintos grupos de científicos que se ven implicados en el mismo. En esta primera etapa, deben quedar claramente definidos los objetivos. A continuación, se define el apoyo financiero al proyecto y se comienzan a diseñar los distintos experimentos a realizar, específicamente con el uso de animales de experimentación en el caso de los trabajos que se han desarrollado en la presente Tesis Doctoral. A partir de aquí, se procede al desarrollo de una serie de metodologías experimentales, incluyéndose en este caso las metodologías analíticas para el análisis de compuestos de interés en las muestras generadas en el modelo experimental. Finalmente, una vez obtenidos los resultados, el proceso concluye con la generación de diferentes informes, publicaciones científicas y/o patentes.

La presente Tesis Doctoral se centra fundamentalmente en la etapa analítica del proceso. Se han desarrollado, según las necesidades específicas de cada proyecto, diferentes métodos analíticos, que han sido validados siguiendo las guías internacionales adecuadas en cada caso particular, y se han aplicado a casos reales.

Las biomoléculas a estudiar están relacionadas con estudios dirigidos al descubrimiento de nuevos ingredientes o tecnologías que podrían ser aplicados a nuevos productos nutricionales o a productos ya existentes en el mercado, mejorando y reforzando así la salud en general. Este trabajo está dividido en distintos capítulos, cada uno dirigido a las biomoléculas o analitos de interés estudiados en cada momento según el proyecto de investigación donde han estado involucradas.

1. COMPUESTOS OBJETO DE ESTUDIO

1.1 Neurotransmisores y compuestos relacionados

Se trata de compuestos pertenecientes a distintas familias que actúan en diversos mecanismos que trascurren a nivel cerebral y su función principal es trasmitir información de una neurona a otra consecutiva, unidas mediante una sinapsis.

La transmisión sináptica entre neuronas es el mecanismo por el cual la información se transmite en el sistema nervioso central[1,2]. La mayor parte de esta transmisión se produce a través de la liberación y la actividad neurotransmisora. Los neurotransmisores pueden clasificarse en función de su estructura química y la actividad que presenten en:

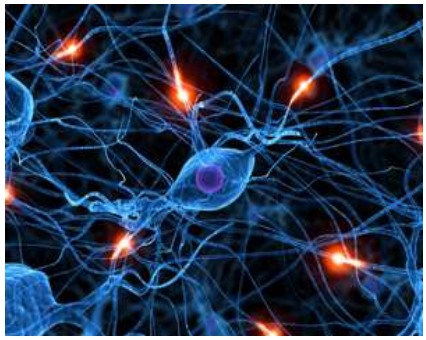

Figura 1. *Dibujo del impulso eléctrico entre neuronas*

- **Aminas:** acetilcolina (AcCh), serotonina (SE), histamina y catecolaminas (dopamina (DA), epinefrina (E) o adrenalina y norepinefrina (NE) o noradrenalina) como monoaminas; agmatina (Agm) o aminoguanidina como triamina.

- **Aminoácidos:** glutamato (Glu), ácido γ-aminobutírico (GABA) y glicina.

- **Péptidos y hormonas.**

- **Otros compuestos** que también pueden trabajar en la comunicación neuronal como óxido nítrico, adenosina trifostafo (ATP) o adenosina que no pertenecen a ninguno de los anteriores grupos químicos.

[1] *Samper L. Neuroquímica cerebral: "Las moléculas y la conducta". Biosalud, Revista de Ciencias básicas.*

[2] *Perea G., Araque A. "Sinapsis tripartita". Mente y cerebro 27 (2007) 50-55.*

Por otro lado existen importantes moléculas relacionadas con los neurotransmisores por ser precursores y/o metabolitos de los mismos: colina (Cho), fosfocolina (PCho), glicerofosfocolina (GPCho), 5-citidinfosfocolina (CDP-Cho) y betaína (Bet), que son precursores y derivados de la AcCh; triptófano (Trp) y ácido 5-hidroxiindolacéctico (5-HIAA), como precursor y metabolito de SE, respectivamente; glutamina (Gln) vinculada al ciclo de Glu; y Tirosina (Tyr) precursor de la DA.

Estas biomoléculas han sido y seguirán siendo estudiadas por las funciones tan importantes que tienen en los organismos vivos, estando además relacionadas con la incidencia de enfermedades mentales como la depresión o la hiperactividad; enfermedades neurodegenerativas como el Alzheimer o el Parkinson; y con otros problemas de salud como la desregulación del sueño, la concentración, el aprendizaje o la memoria[3,4].

A continuación se muestra la estructura química y propiedades generales de los 17 neurotransmisores y compuestos relacionados estudiados en la presente Tesis Doctoral:

1.1.1 Monoaminas

Son neurotransmisores y neuromoduladores que contienen un grupo amino conectado a un anillo aromático a través de una cadena de dos carbonos ($-CH_2-CH_2-$). Todas las monoaminas derivan de aminoácidos aromáticos como la fenilalanina, tirosina, triptófano, y las hormonas tiroideas a través de la acción de las enzimas L-aminoácido aromático descarboxilasa.

En este grupo se incluyen la SE, AcCh y las catecolaminas: DA, NE y E. Sus estructuras químicas pueden observarse en la Figura 2.

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[3] *Franco J., Ballesteros P., et al. Principales neurotransmisores involucrados en la regulación del ciclo sueño-vigilia. Rev. Invest. Clin. 64 (2012) 182-191.*

[4] *Romanides A.J., Duffy P., et al. Glutamatergic and dopaminergic afferents to the prefrontal cortex regulate spatial working memory in rats. Neurosc. 92 (1999) 97-106.*

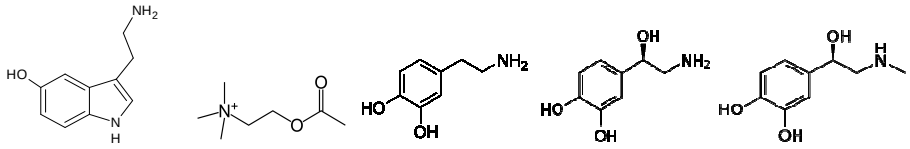

Figura 2. *Estructura química de las monoaminas seleccionadas*

En la clasificación inicial expuesta se puede observar que hay subfamilias dentro de las monoaminas, así por ejemplo la SE pertenece al grupo de las indolaminas, también llamadas triptaminas.

A. Serotonina (SE)

La SE se sintetiza a partir del Trp, por acción de las enzimas triptófano hidroxilasa y la descarboxilasa de aminoácidos aromáticos, en las neuronas serotoninérgicas del sistema nervioso central (SNC) y en las células enterocromafines (células de Kulchitsky) del tracto gastrointestinal de los animales y del ser humano (Figura 3). Hay que destacar que el paso de 5-hidroxi-L-triptófano (5-HTP) a SE es dependiente de la acción de la vitamina B6. Conocida también como 5-hidroxitriptamina, es un mediador periférico de la señal y se encuentra en altas concentraciones en el tracto gastrointestinal (cerca del 90%). El principal almacén son las plaquetas en la circulación sanguínea. Estudios recientes sugieren que la SE juega un papel importante en la regeneración hepática y actúa como mitógeno (induce la división celular)[5].

[5] *Lesurtel M. Platelet-derived serotonin mediates liver regeneration. Science 312 (2006) 104–107.*

La función serotoninérgica es fundamentalmente inhibitoria. Ejerce influencia sobre el sueño y se relaciona también con las emociones y los estados de ánimo depresivos. Afecta al funcionamiento vascular, así como a la frecuencia del latido cardiaco. Regula la secreción de hormonas como la del crecimiento. La desregulación de este neurotransmisor se asocia con desequilibrios mentales como la esquizofrenia o el autismo infantil. También desempeña una función importante en el trastorno obsesivo compulsivo (un desorden de ansiedad). Entre las funciones fisiológicas de la SE destaca la inhibición de la secreción gástrica, la estimulación de la musculatura lisa y la secreción de hormonas por parte de la hipófisis. Los bajos niveles de SE en personas con fibromialgia explican, en parte, los dolores que sufren y los problemas que tienen para dormir. Esos niveles bajos se han relacionado también con estados agresivos, depresión y ansiedad e incluso a las migrañas, debido a que cuando los niveles de SE bajan, los vasos sanguíneos se dilatan. Por último, desempeña también una función importante en la proliferación linfocitaria dependiendo del tipo de receptor estimulado[6].

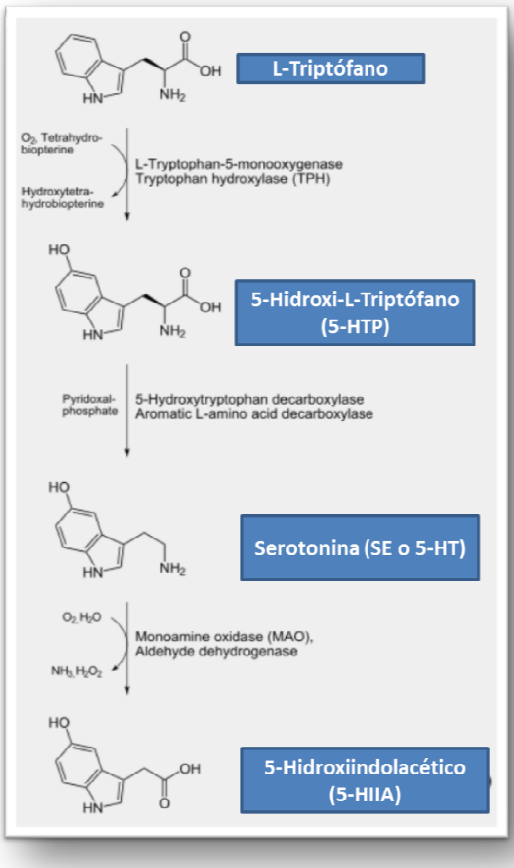

Figura 3. *Síntesis de la SE (versión traducida a partir del trabajo "Serotonin biosíntesis" by NEUROtiker)*

[6] *Medina Martel M., Fazzino F., et al. Efecto de la restricción física sobre el papel de los receptores 5-HT1A en la proliferación linfocitariaArchivos Venezolanos de Farmacología y Terapéutica. Disponible en: <http://www.redalyc.org/articulo.oa?id=55925532004> ISSN 0798-0264.*

En resumen, los efectos de la SE en el humor, en el estado mental de los humanos y el papel que tienen en la conciencia son muy difíciles de determinar, pero pueden destacarse sus principales funciones como la de regular el apetito mediante la saciedad, equilibrar el deseo sexual, controlar la temperatura corporal, la actividad motora y las funciones perceptivas y cognitivas. La SE interviene en otras funciones con conocidos neurotransmisores como la DA y la NE, que están relacionados con la angustia, ansiedad, miedo, agresividad, así como los problemas alimenticios. Interviene en los parámetros de densidad ósea[7,8]. Está demostrado que las personas que toman antidepresivos del tipo inhibidores de la recaptación de la SE pueden generar osteoporosis, reduciendo su densidad ósea.

B. Acetilcolina (AcCh)

La AcCh fue el primer neurotransmisor identificado como tal. Fue aislado y caracterizado farmacológicamente en 1914 por Henry Hallett Dale, y después confirmado como un neurotransmisor por Otto Loewi; estos dos científicos recibieron en 1936 el premio Nobel en Fisiología y Medicina por este descubrimiento. Antes de su trabajo la sinapsis se explicaba como una región en donde las corrientes eléctricas simplemente saltaban de un nervio a una célula efectora. Dale, junto con Loewi, demostraron que esa comunicación sináptica era a través de un intermediario químico como la AcCh[9].

Se trata de un éster de ácido acético y Cho con fórmula química $CH_3COOCH_2CH_2N^+(CH_3)_3$. Se sintetiza en las neuronas mediante la enzima colina acetiltransferasa también llamada colinoacetilasa, a partir de Cho y acetil-coenzima A (Ac-CoA), derivados del metabolismo de la glucosa, en la hendidura sináptica. Normalmente se elimina rápidamente una vez realizada su función; esto lo realiza la enzima acetilcolinesterasa (AChE) que transforma la AcCh en

[7] *Frost M., Andersen T.E., et al. Patients with high-bone-mass phenotype owing to Lrp5-T253I mutation have low plasma levels of serotonin.* J. Bone Miner. Res. *25 (2010) 673–5.*

[8] *Rosen C.J. Breaking into bone biology: serotonin's secrets.* Nat. Med. *15 (2009) 145–6.*

[9] *Fishman M. C. Sir Henry Hallett Dale and the Acetylcholine Story. Yale Journal of biology and medicine 45 (1972) 104-118.*

Cho y acetato (Figura 4). La enzima posee dos isoformas, una ubicada a nivel de la hendidura sináptica (AChE) y otra a nivel sérico, sintetizado principalmente a nivel hepático, denominada acetilcolinesterasa sérica (BAChE). Ésta última es la responsable de impedir el uso terapéutico de la AcCh, por degradarla rápidamente cuando se administra en forma intravenosa.

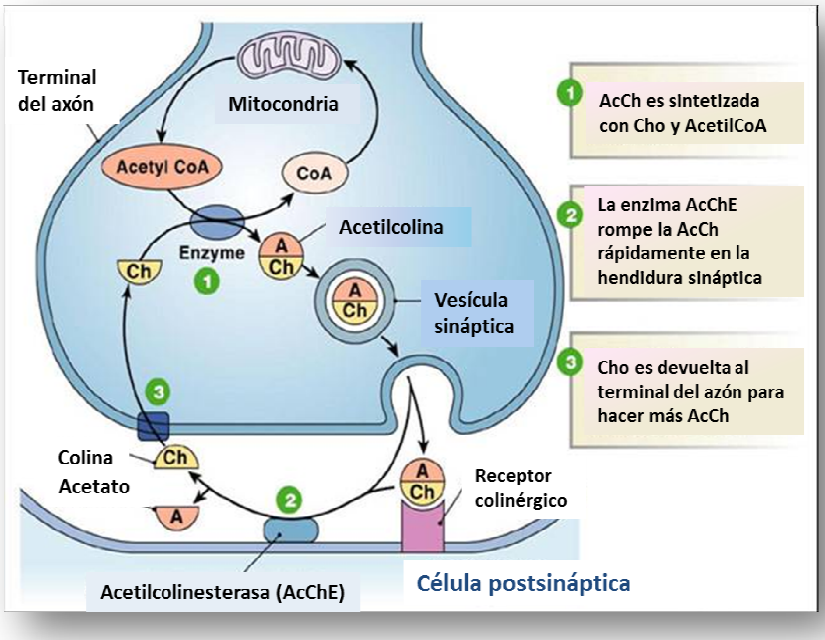

Figura 4. *Sinapsis de la AcCh en la neurona. Versión traducida de la página web Neuroquímica Clínica, Dr. Ernesto Bonilla*

La AcCh está ampliamente distribuida en el sistema nervioso central, particularmente implicada en los circuitos de la memoria, la recompensa ("reward") y los circuitos extrapiramidales, y en el sistema nervioso periférico, a nivel del sistema nervioso autónomo (a nivel de la sinapsis en lo ganglios autónomos, las células cromafines de la médula suprarrenal, todas las terminaciones parasimpáticas y también en la inervación simpática de las glándulas sudoríparas).

En cuanto a su metabolismo, es conocido que cuando la AcCh se une a los muchos receptores nicotínicos de la placa motora de las fibras musculares, causa los llamados *Potenciales Excitatorios Postsinápticos*, que derivan en la generación de un potencial de acción en la fibra muscular con su correspondiente contracción. La AcCh además tiene una alta importancia en el cerebro, donde tiende a causar acciones excitatorias. Las glándulas que reciben impulsos de la parte parasimpática del sistema nervioso autónomo se estimulan de la misma forma. Por eso un incremento de AcCh causa una reducción de la frecuencia cardíaca y un incremento de la producción de saliva. Además posee efectos importantes que median la función sexual eréctil, la micción, así como efectos broncoconstrictores a nivel pulmonar.

La inhibición de la enzima AcChE provoca efectos devastadores en los agentes nerviosos, con el resultado de una estimulación continua de los músculos, glándulas y el SNC. Desde que se asoció una reducción de AcCh con la enfermedad de Alzheimer, se están usando algunos fármacos que inhiben esta enzima para el tratamiento de esta enfermedad. La interrupción en la señalización normal de la AcCh no sólo está relacionada con la enfermedad de Alzheimer, también tiene relación directa con la enfermedad de Huntington, la esquizofrenia y también la enfermedad de Parkinson.

Se sabe que nutricionalmente, al igual que otros neurotransmisores, la concentración de AcCh a nivel cerebral puede ser manipulada[10]. De la misma manera la realización de tareas de aprendizaje y memorización, entre otras, también influyen en los niveles de estos neurotransmisores en el cerebro[11].

C. Dopamina (DA)

La DA, 4-(2-aminoetil)benceno-1,2-diol, $C_6H_3(OH)_2$-CH_2-CH_2-NH_2, es un neurotransmisor producido en una amplia variedad de animales vertebrados e invertebrados. Según su estructura química, la DA es una feniletilamina, una catecolamina que cumple funciones de neurotransmisor en el sistema nervioso

[10] *Messier C. Glucose improvement of memory: a review. Eur. J. Pharm. 490 (2004) 33-57.*
[11] *Das UN. Cognitive performance and glucose. Am. J. Clin. Nutr. 74 (2001) 409-11.*

central, activando los cinco tipos de receptores celulares D1 (relacionado con un efecto activador), D2 (relacionado con un efecto inhibidor), D3, D4 y D5, y sus variantes. Se produce en muchas partes del sistema nervioso, especialmente en la sustancia negra. También es considerada como una neurohormona liberada por el hipotálamo. Su función principal en éste es inhibir la liberación de prolactina del lóbulo anterior de la hipófisis.

Como miembro de la familia de las catecolaminas, la DA es un precursor de la NE y E en las vías de biosíntesis de estos neurotransmisores.

Fue sintetizada artificialmente por primera vez en 1910 por George Barger y James Ewens en los Laboratorios Wellcome en Londres (Inglaterra)[12]. Es una monoamina, y su precursor sintético es la 3,4-dihidroxifenilalanina (L-DOPA). En 1952, Arvid Carlsson y Nils-Åke Hillarp, del Laboratorio de Farmacología Química del Instituto Nacional del Corazón en Suecia, demostraron su papel tan importante como neurotransmisor. Éste y otros logros en la transducción de señales en el SNC hicieron que Carlsson ganara el Premio Nobel en Fisiología o Medicina en 2000[13].

La DA se biosintetiza en el organismo principalmente en el tejido nervioso de la médula de las glándulas suprarrenales. Primero ocurre la hidroxilación de los aminoácidos L-tirosina a L-DOPA mediante la enzima tirosina 3-monooxigenasa, también conocida como tirosina hidroxilasa, y después se da la descarboxilación de la L-DOPA mediante la enzima DOPA-descarboxilasa[14]. En algunas neuronas, la DA es transformada a NE por la dopamina β-hidroxilasa.

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[12] *Fahn S. The history of Dopamine and Levodopa in the treatment of Parkinson's disease. Movement disorders 23 (2008) S497-508.*

[13] *Benes F.M. Carlsson and the discovery of dopamine. Trends Pharmacol. Sci. 22 (2001) 46-47.*

[14] *Qi Z., Miller G.W., et al. Computational Systems Analysis of Dopamine Metabolism. PLoS ONE 3 (2008): e2444.*

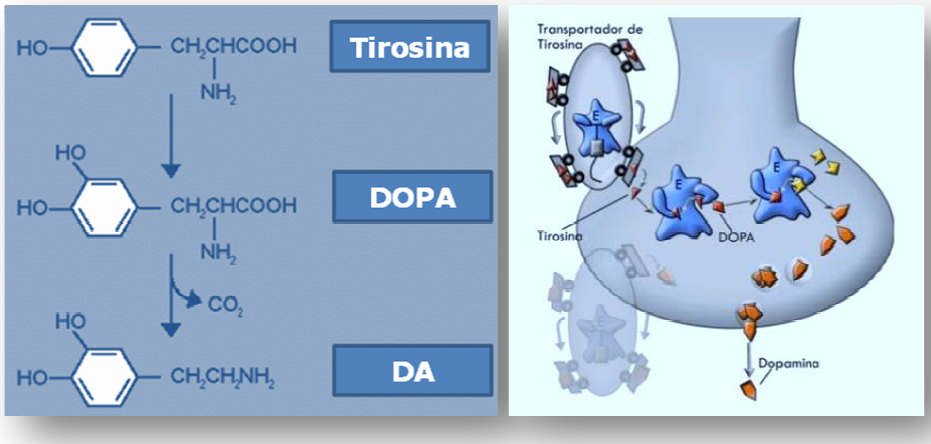

Figura 5. *Biosíntesis de DA (programa del curso Neurobioquímica, Jairo Alfonso Tovar Franco); y liberación de DA en forma de vesículas*

En la Figura 5 se aprecia la biosíntesis de la DA. Una vez sintetizada, se empaqueta en forma de vesículas dentro de las neuronas, y se liberan en la sinapsis en respuesta a un impulso eléctrico presináptico.

La DA juega un rol importante en el cerebro, está relacionada con el comportamiento y la función cognitiva, la actividad motora, la motivación y la recompensa, la regulación de la producción de leche, el sueño, el humor, la atención y el aprendizaje.

En relación a la función cognitiva, la DA es quien controla el flujo de información. Las alteraciones en los lóbulos frontales pueden causar una disminución en las funciones neurocognitivas, especialmente en la memoria, atención y resolución de problemas. Se cree que las concentraciones reducidas de DA en la corteza prefrontal contribuyen al trastorno por déficit de atención con hiperactividad (TDAH)[15]. Por el contrario, la medicación anti-psicótica actúa como antagonista de la DA y se usa en el tratamiento de los síntomas positivos

[15] *Mulas F., Díaz Heijtz R., et al. Alteraciones de los patrones de los marcadores de la dopamina en el trastorno por déficit de atención e hiperactividad. Revista de neurología 42 (2006) 19-23.*

en esquizofrenia, ya que en estos casos la DA se encuentra en concentraciones anormalmente altas[16].

La biosíntesis insuficiente de DA en las neuronas dopaminérgicas pueden causar la enfermedad de Parkinson[17], en la cual una persona pierde la habilidad para ejecutar movimientos finos y controlados.

La sociabilidad se encuentra también muy ligada a la DA. En personas con ansiedad social se encuentra una baja captabilidad de ésta. Las características comunes a los síntomas negativos de la esquizofrenia (apatía, anhedonia) son importantes en relación al estado hipodopaminérgico en ciertas áreas del cerebro.

Como ya se ha mencionado la DA se asocia con el sistema del placer del cerebro, suministrando los sentimientos de gozo y refuerzo para motivar a la persona proactivamente en la realización de ciertas actividades. Se libera desde las neuronas situadas en el área tegmental ventral (ATV) hasta las estructuras como el núcleo accumbens, la amígdala, el área septal lateral, el núcleo olfatorio anterior, el tubérculo olfatorio y el neocórtex mediante las proyecciones que tiene el ATV sobre estas estructuras. Participa en experiencias naturalmente recompensantes tales como la alimentación, el sexo[18,19], algunas drogas y los estímulos neurales que se pueden asociar con éstos.

La DA aumenta la presión arterial. A dosis bajas aumenta el filtrado glomerular y la excreción de sodio. Inhibe la producción de prolactina en la lactancia.

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[16] *Tajima K., Fernández H., et al. Tratamientos para la esquizofrenia. Revisión crítica sobre la farmacología y mecanismos de acción de los antipsicóticos. Actas Esp. Psiquiatr. 37 (2009) 330-342.*

[17] *Micheli F.E. Enfermedad de Parkinson y trastornos relacionados. Ed. Médica Panamericana, 2006.*

[18] *Giuliano F., Allard J. Dopamine and male sexual function. Eur. Urol. 40 (2001) 601-608.*

[19] *Giuliano F., Allard J. Dopamine and sexual function. Int. J. Impot. Res. 13 (2001) S18-S28.*

Por último, se conocen las polifenol oxidasas (PPOs) como una familia de enzimas responsables de la oxidación de frutas frescas y vegetales al ser cortados o golpeados. Estas enzimas usan oxígeno molecular (O_2) para oxidar varios difenoles a su correspondiente quinonas. El sustrato natural para los PPOs en la banana es la DA. El producto de su oxidación, la quinona dopamina se oxida espontáneamente en presencia de otras quinonas. Las quinonas entonces se polimerizan y condensan con amino ácidos para formar pigmentos marrones denominados melaninas. Se cree que estas quinonas y melaninas derivadas de la DA podrían ayudar a proteger a las frutas y vegetales dañados de bacterias y hongos[20].

D. Noradrenalina o norepinefrina (NE)

Es una catecolamina y una feniletilamina. El estereoisómero natural es L-(−)-(R)-norepinefrina. Tiene diversas funciones fisiológicas y homeostáticas, actuando como hormona y como neurotransmisor. Las áreas del cuerpo donde se producen o se ven afectadas por la NE son descritas como noradrenérgicas.

Los términos noradrenalina (del latín) y norepinefrina (del griego) son intercambiables, siendo el primero más común en la mayor parte del mundo. Sin embargo, para evitar confusión y obtener consistencia, las autoridades médicas promovieron la norepinefrina (NE) como la nomenclatura favorecida, y éste es el término usado a lo largo de este capítulo.

Una de las funciones más importantes de la NE es su rol como neurotransmisor. Es liberada de las neuronas simpáticas afectando al corazón. Un incremento en los niveles de NE del sistema nervioso simpático incrementa el ritmo de las contracciones[21].

[20] *Mayer A.M. Polyphenol oxidases in plants and fungi: Going places? A review. Phytochemistry 67 (2006) 2318-2331.*

[21] *Guyton A., Hall J. Chapter 10: Rhythmical Excitation of the Heart. En Gruliow, Rebecca (Book). Textbook of Medical Physiology (11th edición). Elsevier Inc. (2006) 122.*

Como hormona del estrés, la NE afecta partes del cerebro como la amígdala cerebral, donde se controlan la atención y respuestas neuronales[22]. Junto con la E, la NE también está involucrada en la reacción de lucha o huida, incrementando directamente la frecuencia cardiaca, desencadenando la liberación de glucosa de las reservas de energía, e incrementando el flujo sanguíneo hacia el músculo esquelético. Incrementa además el suministro de oxígeno del cerebro. Y también puede suprimir la neuroinflamación cuando es liberada difusamente en el cerebro por el locus coeruleus[23].

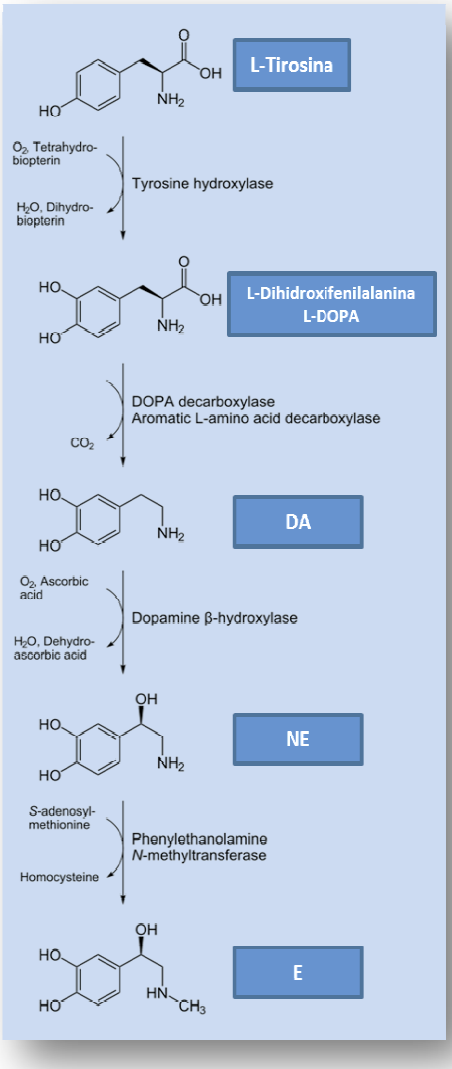

Figura 6. *Biosíntesis de NE y E (versión traducida a partir de "Catecholamines biosíntesis" by NEUROtiker)*

La NE se biosintetiza a partir de la DA en las vesículas o depósitos de almacenamiento. En la Figura 6 se muestra la cadena de reacciones que se da en esta ruta. En un primer paso, la Tyr se convierte en DOPA por la acción de la tirosina hidroxilasa. La DOPA se convierte en DA en las vesículas de almacenamiento. Finalmente, y por la acción intravesicular de la dopamina β-hidroxilasa, se transforma en NE. Es liberada por la médula suprarrenal en el torrente

[22] *Tanaka M., Yoshida M., et al. Noradrenaline systems in the hypothalamus, amygdala and locus coeruleus are involved in the provocation of anxiety: basic studies. Eur. J. of Pharmacol. 405 (2000) 397-406.*

[23] *Heneka M.T., Nadrigny F., et al. Locus ceruleus controls Alzheimer's disease pathology by modulating microglial functions through norepinephrine. Proc. Natl. Acad. Sci. 107 (2010) 6058-6063.*

sanguíneo como una hormona, aunque también es un neurotransmisor en el sistema nervioso central y sistema nervioso simpático donde es liberada por neuronas noradrenérgicas en el locus coeruleus. Las acciones de la NE se llevan a cabo a través de la unión a los receptores adrenérgicos.

Las neuronas noradrenérgicas en el cerebro forman un sistema de neurotransmisores que cuando es activado ejerce efectos en grandes áreas del cerebro. Interviene en los procesos de atención, en el despertar e influye en el sistema de recompensas.

Anatómicamente, las neuronas noradrenérgicas se originan tanto en el locus coeruleus como en el campo tegmental lateral. Los axones de las neuronas en el locus coeruleus actúan sobre los receptores adrenérgicos en el hipocampo, hipotálamo, giro cingulado, amígdala cerebral, circunvolución del cíngulo, tálamo, neocórtex, médula espinal y cuerpo estriado. Por otra parte, los axones de las neuronas del campo tegmental lateral actúan sobre los receptores adrenérgicos del hipotálamo, por ejemplo. Esta estructura explica algunos de los usos clínicos de la NE, ya que una modificación del sistema afecta a grandes áreas del cerebro.

En mamíferos, la NE es rápidamente degradada en varios metabolitos, siendo los principales: normetanefrina, ácido 3,4-dihidroximandélico, ácido vanililmandélico (ácido 3-metoxi-4-hidroximandélico o VMA), 3-metoxi-4-hidroxifenilglicol (MHPG) y E. En la periferia, el VMA es el metabolito principal de las catecolaminas, y se excreta no conjugado en la orina. Como metabolito minoritario, aunque es el metabolito principal en el sistema nervioso central, se presenta el MHPG, que se conjuga parcialmente con sulfato o derivados de glucurónidos y se excreta en la orina[24].

La NE puede ser usada para el tratamiento de trastornos de déficit de atención/hiperactividad, depresión e hipotensión. Está relacionada, junto a la DA, en el trastorno TDAH. Al igual que otras catecolaminas, la NE no puede

[24] *Flower R., Rang H.P., et al. Chapter 11 in: Rang & Dale's pharmacology (2007).*

atravesar sola la barrera hematoencefálica, así drogas como las anfetaminas son necesarias para incrementar los niveles en el cerebro.

E. Epinefrina (E)

También llamada adrenalina, es una hormona y un neurotransmisor[25]. El término epinefrina se deriva de las raíces griegas epi- y nephros-, y significa literalmente "sobre el riñón," en referencia a la localización anatómica de la glándula adrenal, donde se sintetiza.

Incrementa la frecuencia cardíaca, contrae los vasos sanguíneos, dilata los conductos de aire, y participa en la reacción de lucha o huida del sistema nervioso simpático[26]. Químicamente, la adrenalina es una catecolamina, una monoamina producida mayoritariamente por las glándulas suprarrenales a partir de los aminoácidos fenilalanina y Tyr.

El fisiólogo polaco Napoleon CybulskiLos[27] y los científicos Oliver and Schaefer[28] consiguieron en 1895 obtener extractos suprarrenales que contenían E. Estos extractos contenían, además de E, otras catecolaminas. El químico japonés Jokichi Takamine y su asistente Keizo Uenaka descubrieron independientemente la E en 1900. En 1901, Takamine aisló y purificó con éxito la hormona de las glándulas suprarrenales de ovejas y bueyes. En 1902 Abel pudo cristalizarla[28]. Pudo ser sintetizada en 1904 en un laboratorio por Friedrich Stolz y Henry Drysdale Dakin[29].

Como hormona, la E actúa en casi todos los tejidos del cuerpo. Sus acciones varían según el tipo de tejido y la expresión de los distintos receptores

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[25] *Berecek B.M., Brody M.J. Evidence for a neurotransmitter role for epinephrine derived from the adrenal medulla. Am. J. Physiol. 242 (1982) H593–H601.*

[26] *Cannon W.B., Newton H.F., et al. Some aspects of the physiology of animals surviving complete exclusion of sympathetic nerve impulses. Amer. J. Physiol. 89 (1929) 84–107.*

[27] *Grzybowski A., Pietrzak K. Napoleon Cybulski (1854–1919). J. Neurol. 260 (2013) 2942–2943.*

[28] *Mefford I.M. Epinephrine in mammalian brain. Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 12 (1988) 365-388.*

[29] *Bennett M. One hundred years of adrenaline: the discovery of autoreceptors. Clin. Auton. Res. 9 (1999) 145–159.*

adrenérgicos en cada uno de ellos. Por ejemplo, causa la relajación del músculo liso en las vías respiratorias pero también causa contracciones en el músculo liso de las arteriolas.

Actúa uniéndose a una gran variedad de receptores adrenérgicos. Es un agonista no selectivo de todos los receptores adrenérgicos. La unión a estos receptores origina una serie de cambios metabólicos: inhibe la secreción de insulina en el páncreas, estimula la glucogenolisis en el hígado y el músculo, estimula la glucólisis en el músculo[30], provoca la secreción de glucagón en el páncreas, acrecienta la secreción de la hormona adrenocorticotropa en la glándula pituitaria e incrementa la lipólisis en el tejido adiposo. Juntos, estos efectos llevan a un incremento de la glucemia y de la concentración de ácidos grasos en la sangre, proporcionando sustratos para la producción de energía dentro de las células de todo el cuerpo. La E es el activador más potente de los receptores α, es de 2 a 10 veces más activa que la NE.

La E se sintetiza mayoritariamente en la médula de la glándula suprarrenal en una ruta enzimática que convierte el aminoácido Tyr en una serie de intermediarios hasta llegar a ella. La Tyr es primero oxidada para obtener levodopa, que posteriormente se descarboxila para dar DA. La oxidación de esta molécula proporciona NE que luego se metila para dar E (Figura 6). También se sintetiza al metilarse la amina distal primaria de la NE por la acción de la enzima feniletanolamina N-metiltransferasa (PNMT) en el citosol de las neuronas adrenérgicas y células de la médula adrenal (llamadas células cromafínicas). La PNMT sólo se encuentra en el citosol de las células de la médula suprarrenal. La PNMT usa la S-adenosilmetionina como cofactor para donar el grupo metilo a la NE, creando la E. La síntesis comienza cuando el hipotálamo indica al lóbulo anterior de la glándula pituitaria en el cerebro que libere una hormona proteíca llamada adrenocorticotropa (ACTH) en el torrente sanguíneo, estimulando la corteza suprarrenal.

Por otro lado, en los últimos tiempos la E se ha encontrado en el cerebro de diferentes especies de animales como mamíferos, aves o reptiles. También

[30] *Sabyasachi S. Medical Physiology. Thieme Publis. Group. 536 (2007).*

está presente en peces pero en niveles inferiores. Se encuentra en diversas áreas del cerebro, mayoritariamente en hipotálamo, estructuras de la línea media y tronco cerebral con diversas concentraciones en función de la especie y clase estudiada. En rata, por ejemplo, la E se encuentra principalmente co-almacenada en las terminaciones del nervio noradrenérgico, mientras que se sintetiza en un compartimento extraneuronal, quizás glia o una neurona disociada del sitio de almacenamiento. Además en el tronco cerebral, las neuronas que hay presentes contienen el complemento perfecto de enzimas biosintéticas. Por lo tanto, estas neuronas pueden formar E como productos finales de catecolaminas[28].

Se libera principalmente en situaciones de alta tensión y momentos de estrés, como por ejemplo, las amenazas físicas, las emociones intensas, los ruidos, las luces brillantes y la alta temperatura ambiental. Todos estos estímulos se procesan en el sistema nervioso central[31].

1.1.2 Triamina: Agmatina (Agm)

La Agm posee un grupo guanidino básico (ver Figura 7), y en condiciones fisiológicas se encuentra protonada, por tanto tiene propiedades que la diferencian de las monoaminas. Aunque puede encontrarse en concentraciones bajas en muchos órganos, su concentración en determinadas regiones cerebrales es elevada. La enzima encargada de sintetizarla es la arginina descarboxilasa (ADC). La Agm se almacena en vesículas sinápticas en el cerebro y la medula espinal. Posteriormente a la despolarización de la membrana, es liberada y actúa sobre receptores transmembrana. En los últimos años se ha propuesto que es un neurotransmisor con efectos anticonvulsivante, antineurotóxico y antidepresivo.

[31] *Nelson L., Cox M. Lehninger Principles of Biochemstry (4th edición). New York: Freeman. (2004) p. 908.*

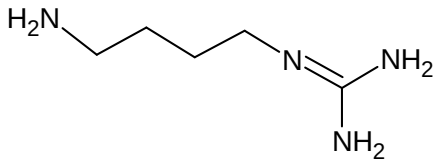

Figura 7. *Estructura química de la Agm*

Fue descubierta en el tejido cerebral de mamíferos en 1994 y surgió como neurotransmisor candidato con acción directa sobre los receptores imidazolínicos. Su importancia fue cuestionada rápidamente, apareciendo interpretaciones y metodologías nuevas que derivaron en el descubrimiento de otras de sus funciones y características. En la actualidad es sabido que la Agm actúa sobre otros receptores además de los imidazolínicos, como los α2 adrenérgicos y los glutamatérgicos N-metil-D-aspartato (NMDA), e inhibe de manera irreversible a la enzima óxido nítrico sintetasa neuronal. Su concentración aumenta en presencia de estresores o traumatismos cerebrales, que inducen su síntesis por parte de las neuronas y los astrocitos. De ahí surge la importancia del uso de la Agm en el campo psiquiátrico[32].

En plantas y bacterias, la Agm es un intermediario metabólico en la biosíntesis de poliaminas. El descubrimiento y la caracterización de la forma humana de la ADC permitieron su detección en tejido cerebral de ratas[33]. En la actualidad es sabido que existe un transportador específico de la Agm que permite su ingreso a las células de los mamíferos. La acción de la agmatinasa sobre la Agm genera putrescina, una poliamina (ver Figura 8). Adicionalmente, en algunos tejidos como el hígado, la Agm puede ser metabolizada por la diaminooxidasa y convertida en ácido guanido-butanoico, que es excretado rápidamente. Las enzimas cerebrales ADC y agmatinasa son las principales involucradas en la vía biosintética de la Agm.

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[32] *Halaris A., Plietz J. Agmatine: Metabolic Pathway and Spectrum of Activity in Brain. CNS Drugs 21 (2007) 885-900.*

[33] *Feng Y., Halarisa A.E., et al. Determination of agmatine in brain and plasma using high-performance liquid chromatography with fluorescence detection. J. Chromatogr. B 691 (1997) 277-286.*

Los receptores y las enzimas que interactúan con la Agm son numerosos y se encuentran distribuidos en varias regiones cerebrales; en consecuencia, la vía de la Agm afectaría a numerosos sistemas. Existen datos que indican que tiene un papel neuroprotector, específicamente antiinflamatorio. Se ha demostrado que la administración extracelular de Agm reduce la liberación de glutamato durante crisis convulsivas en ratas[32].

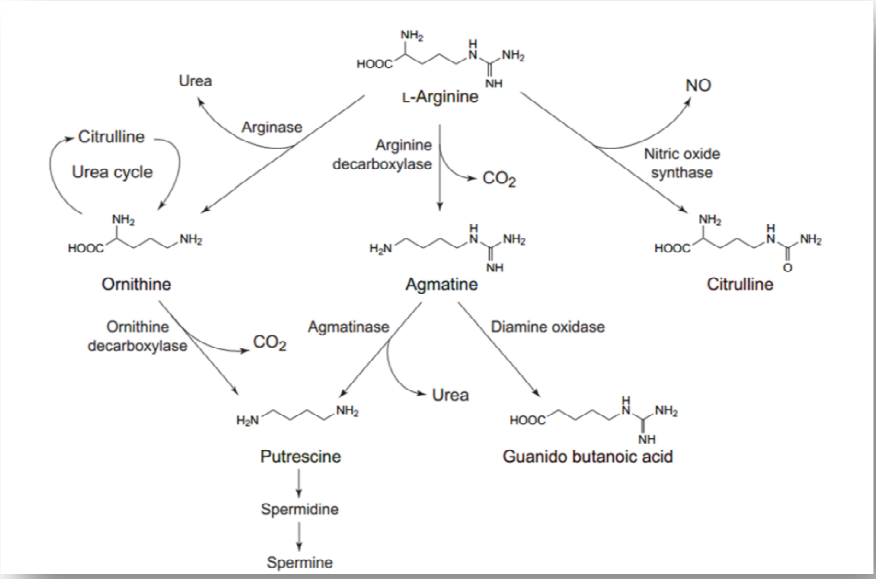

Figura 8. *Vías metabólicas de Agm y compuestos relacionados. Agm se produce a partir de L-arginina por la acción de la arginina descarboxilasa. A continuación puede ser metabolizada para producir espermina o ácido guanido-butanoico (adaptación de A. Halaris et al.[32])*

La Agm puede considerarse como neurotransmisor ya que es sintetizada y almacenada en vesículas, liberada por la despolarización dependiente del calcio, recaptada, inactivada por una enzima específica llamada agmatinasa y se une a receptores de alta afinidad ubicados en la superficie celular. Además, funciona como neuromodulador de receptores en nervios periféricos y centrales. Se desconoce si posee receptores propios y si actúa a nivel presináptico o postsináptico[34].

[34] *Gümrü S., Şahin C., et al. A review on agmatine as a novel neurotransmitter / neuromodulator. J. Marmara Univ. Inst. Health Sci. (2013) doi:10.5455/musbed.20130412103633.*

1.1.3 Aminoácidos

A este grupo pertenecen los neurotransmisores: glutamato o ácido glutámico (Glu) y ácido γ-aminobutírico (GABA). Y los neurocompuestos relacionados por ser metabolitos o precursores: glutamina (Gln), tirosina (Tyr), ácido 5-hidroxiindolacético (5-HIAA) y triptófano (Trp), cuyas estructuras químicas son similares por contener al menos un grupo amino (ver Figura 9).

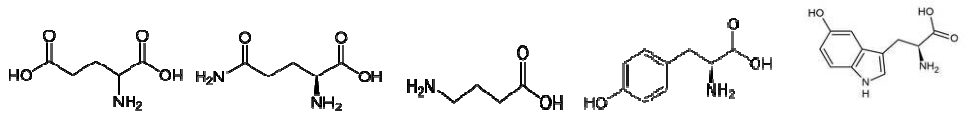

Figura 9. *Estructura química de los aminoácidos seleccionados*

A. Ácido glutámico/glutamato (Glu)

Aunque no es un aminoácido esencial, ya que puede sintetizarse a partir de otros compuestos, es crítico para la función celular. Pertenece al grupo de los llamados aminoácidos ácidos, por tener carga negativa a pH fisiológico, debido a que presenta un segundo grupo carboxilo en su cadena secundaria. Es un neurotransmisor excitatorio primario en el SNC. Su papel como está mediado por la estimulación de receptores específicos, los receptores de glutamato (ionotrópicos y metabotrópicos).

Todas las neuronas contienen Glu, pero sólo unas pocas lo usan como neurotransmisor. Es potencialmente excitotóxico, por lo que existe una compleja maquinaria para que los niveles de esta sustancia estén siempre regulados. Desempeña un papel principal en los procesos de transaminación y en la síntesis de distintos aminoácidos que necesitan la formación previa de este ácido, como es el caso de la prolina, hidroxiprolina, ornitina y arginina. Se acumula en altas concentraciones en el cerebro.

El Glu es uno de los aminoácidos más abundantes del organismo y un comodín para el intercambio de energía entre los tejidos. Es un sustrato para la síntesis de proteínas y un precursor del metabolismo anabólico en el músculo, además de regular el equilibrio ácido/básico en el riñón y la producción de urea en el hígado. También interviene en el transporte de nitrógeno entre los diferentes órganos. Las células de la mucosa intestinal lo consumen en altas concentraciones, al igual que las células del sistema inmunitario que lo necesitan como fuente de energía. Además, el Glu es precursor del glutatión, compuesto con alta capacidad antioxidante.

Varios estudios han demostrado que el estómago, intestino, páncreas y bazo consumen un 95% del Glu ingerido en la dieta, razón más que suficiente para tomar una dieta rica en proteínas y no alterar el equilibrio de estos aminoácidos.

El Glu parece que también interviene en la liberación de la hormona liberadora de la gonadotropina (GnRH), fundamental para el dimorfismo cerebral y corporal. Un estudio realizado en la Universidad Estatal de Michigan encontró que una inflamación en el cerebro puede producir altos niveles de Glu, y esto podría producir pensamientos suicidas en la persona afectada[35].

B. Glutamina (Gln)

Es uno de los 20 aminoácidos que intervienen en la composición de las proteínas y que tienen codones referentes en el código genético; es la amida del ácido glutámico, formada mediante el reemplazo del hidroxilo del ácido glutámico con un grupo funcional amina. Es un aminoácido no esencial, es decir que el organismo puede sintetizarlo a partir otros aminoácidos presentes en las proteínas o en otros alimentos. Es uno de los aminoácidos más abundantes en los músculos humanos (llegando a casi el 60%), así como en la sangre, y está muy relacionado con el metabolismo que se realiza en el cerebro.

[35] *Countering Brain Chemical Could Prevent Suicides. http://msutoday.msu.edu (13 de diciembre de 2012).*

La Gln tiene dos átomos de N que pueden ser proporcionados en las actividades metabólicas del cuerpo. En la Figura 10 se representa su biosíntesis en el organismo, y se puede apreciar la gran ayuda que ejerce "limpiando" en algunos tejidos el exceso de NH_3, reduciendo la toxicidad por altas concentraciones, en especial en el cerebro haciendo que se transporte a otras regiones del organismo. Se emplea en la síntesis de proteínas (frecuentemente se usa como suplemento culturista debido a los efectos ergogénicos de reparación de las fibras musculares) y en la biosíntesis del glutation. Los niveles de Gln en sangre son a veces indicadores de un trastorno en el organismo de carácter catabólico, como puede ser una necrosis intestinal.

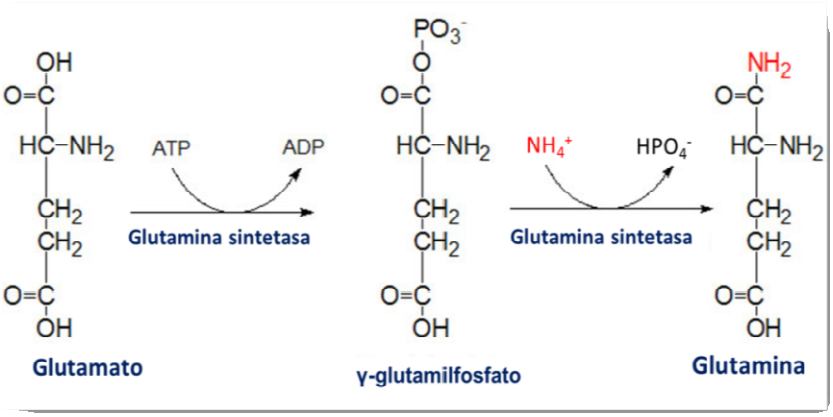

Figura 10. *Fijación de amonio en la ruta glutamato-glutamina (adaptación de themedicalbiochemistrypage.org)*

C. Ácido γ-aminobutírico (GABA)

El GABA era un aminoácido inicialmente asociado a las plantas, hasta que en 1950 fue encontrado en el tejido cerebral[36]. En el cerebro actúa como neurotransmisor inhibitorio en varios de sus circuitos. Se encuentra muy concentrado en el cerebelo y menos concentrado en el tálamo e hipocampo. Las alteraciones en estos circuitos GABAérgicos están asociadas con la

[36] *Schousboe A., Waagepetersen H.S. Handbook of Neurochemistry and Molecular Neurobiology Neurotransmitter Systems. Springer US. (2008) 214-221.*

enfermedad de Huntington, el Parkinson, la demencia senil, la enfermedad de Alzheimer y la esquizofrenia.

Existen tres tipos de receptores de GABA. Unos de acción rápida, receptores ionotrópicos $GABA_A$ y $GABA_C$; y otros de acción lenta, los receptores metabotrópicos $GABA_B$.

Entre otras funciones del GABA, está la inhibición de GnRH (hormona liberadora de gonadotropinas). Cuando hay descenso de GABA y un aumento de Glu suele coincidir con una alta concentración de GnRH durante la pubertad. Ayuda a la recuperación muscular en deportistas y mejora el sueño junto con la ornitina.

Hay una relación directa entre Glu y GABA, a través de la glutamato descarboxilasa (GDA), que cataliza la descarboxilación del Glu generando GABA. Ver Figura 11.

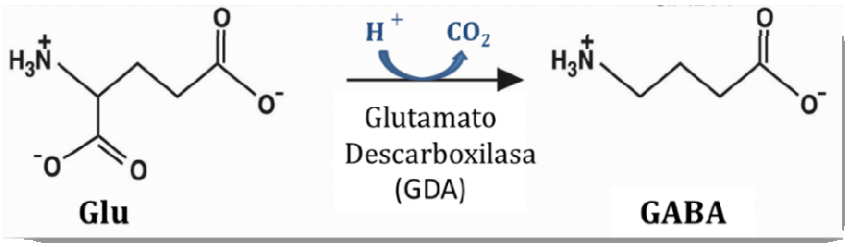

Figura 11. *Descarboxilación del glutamato*

Glu, Gln y GABA están correlacionadas entre sí a través del ciclo mostrado en la siguiente figura.

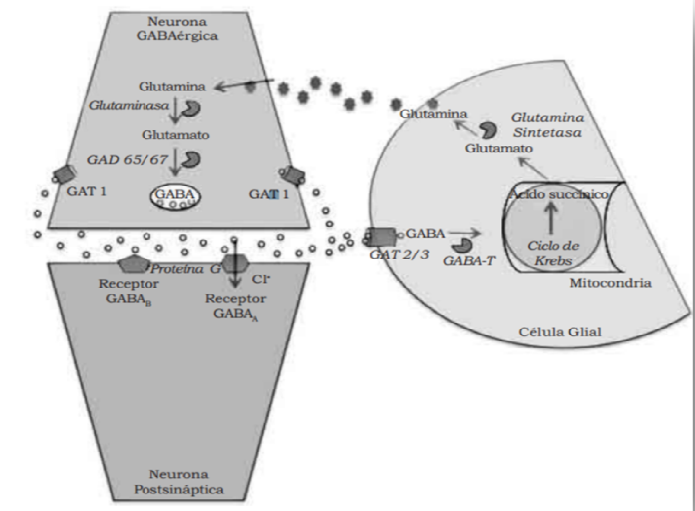

Figura 12. *Sinapsis GABAérgica. La síntesis de GABA se inicia por la descarboxilación de Glu por la glutamato descarboxilasa (GAD); se empaqueta en vesículas y después se libera el contenido en la hendidura sináptica para luego unirse a receptores ionotrópicos (GABAA) y receptores metabotrópicos (GABAB). Después de su unión a receptores, el GABA puede recapturarse por la glía o interneuronas y se convierte de nuevo a glutamato*[37]

El GABA sintetizado es almacenado en vesículas y liberado a la hendidura sináptica mediante exocitosis. Después de su liberación, es retomado por la terminal presináptica mediante un transportador y empacado otra vez en vesículas para su uso posterior[38]. Otra parte del GABA puede ser captado por células de la glia para convertirlo en Glu por la GABA-T y éste a su vez puede ser convertido en Gln que es transportada a la terminal nerviosa donde será convertida en Glu y posteriormente en GABA. El GABA es transferido de las neuronas GABAérgicas a los astrocitos adyacentes en las cuales es transaminado, utilizando 2-oxoglutarato, para producir succinato-semialdehído y Glu. Este último es convertido en Gln mediante la glutamina sintetasa y la Gln generada es llevada de vuelta a la neurona GABAérgica donde es hidrolizada por la glutaminasa activada por fosfato (PAG) para producir el Glu que es decarboxilado para formar GABA y cerrar el ciclo.

[37] *Sandoval-Salazar C., Ramírez-Emiliano J., et al. El sistema de inhibición GABAérgico implicado en la regulación de la ingesta alimentaria y obesidad. Rev. Mex. Neuroci. 14 (2013) 262-271.*

[38] *Siegle A. Essential Neuroscience. Lippincott Williams & Wilkins, Baltimore USA (2005).*

D. Tirosina (Tyr)

Es un aminoácido no esencial cuya síntesis se produce a partir de la hidroxilación del aminoácido esencial fenilalanina. La palabra tirosina proviene del griego tyros, que significa queso. Se llama así porque este aminoácido fue descubierto por el químico alemán Justus Von Liebig[39] a partir de la caseína, proteína que se encuentra en el queso. Se conocen tres isómeros distintos de la Tyr: para-Tyr, meta-Tyr y orto-Tyr. Aunque la forma más conocida y estudiada es la primera, también llamada L-Tyr.

Se considera un aminoácido polar y protonable. Aunque normalmente se clasifica como aminoácido hidrofóbico a causa de su anillo aromático, aunque se debe tener en cuenta que también contiene un grupo hidroxilo, con lo que su carácter anfipático es claro. Normalmente se encuentra en estado neutro sin carga, aunque a pH muy básico presenta carga negativa.

Su biosíntesis se puede dar de dos formas distintas. En los mamíferos se obtiene a partir de la hidroxilación de la fenilalanina, mientras que en algunos microorganismos se consigue directamente a partir del profenato.

En el metabolismo de la Tyr se producen dos moléculas: fumarato y acetoacetato. El primero puede ser utilizado para producir energía en el ciclo de Krebs (o ciclo del ácido tricarboxílico) o bien para la gluconeogénesis. El segundo puede ser utilizado para la síntesis lipídica o para la producción de energía en forma de Ac-CoA. La producción de Tyr está regulada por la demanda de varias moléculas de las cuales es precursor como con la melanina, las catecolaminas (DA, NE y E, ver Figura 6) y las hormonas del tiroides (tiroxina (T4) y triyodotironina (T3)).

El déficit o mala regulación de la Tyr puede conllevar a distintas enfermedades como tirosinemias (acumulación y/o excreción de la Tyr y de sus metabolitos por la ausencia o deficiencia de la enzima tirosina

[39] *Just the facts 101, textbook key facts. Biology, Preliminary Volume 1: The Dynamic Science, Units 1 & 2, by Brooks/Cole, 2nd edition.*

aminotransferasa, conlleva disfunción de túbulos renales, raquitismo, polineuropatía, generación de tumores, lesiones en los ojos y piel, etc.); alcaptonuria (se excreta casi toda la Tyr que ingieren en su forma de ácido homogentísico mediante la orina, se produce ocronosis, enfermedad autosómica recesiva, etc.); albinismo (falta de la enzima β-zetasa, la cual actúa en el proceso de formación de melanina a partir de Tyr, se produce sensibilidad a las radiaciones solares, quemaduras en la piel y/o carcinomas, fotofobia); y Parkinson (temblores causados por la degeneración de unas células que se encuentran en la sustancia negra y en el locus coeruleus del cerebro que producen DA a partir de la Tyr).

F. Triptófano (Trp)

Es un aminoácido esencial incorporado a través de la alimentación, siendo uno de los 20 aminoácidos incluidos en el código genético. Se clasifica entre los aminoácidos apolares, también llamados hidrófobos. Es esencial para promover la liberación de la SE, involucrado en la regulación del sueño y el placer.

Es el precursor de la SE, melatonina, quinurenina, quinurénico, ácidos xanturénico y niacina, moléculas que cumplen todas ellas funciones biológicas importantes en el organismo. Posee actividades biológicas importantes gracias a su metabolismo dentro de los tejidos. Entre otras propiedades se encuentran las siguientes: ayuda a que el organismo elabore sus propias proteínas; interviene en la elaboración de la melatonina (hormona cerebral) a través de la SE favoreciendo la regulación del sueño; tiene efecto antidepresivo y tranquilizante debido a la SE que puede actuar como ansiolítico (se ha demostrado que su deficiencia produce ansiedad y depresión en ratas[40] e irritabilidad en humanos[41]); controla el apetito y a la formación de vitamina B3 o niacina a través de la SE.

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[40] *Blokland A., Lieben C., et al. Anxiogenic and depressive-like effects, no cognitive deficits, after repeated moderate tryptophan depletion in the rat. J. Psychopharmacol. 16 (2002) 39-49.*

[41] *Russo S., Kema I., et al. Tryptophan as a link between psychopathology and somatic states. Psychosom. Med. 65 (2003) 665-671.*

1.1.4 Otros metabolitos y precursores

Los seleccionados en la presente Tesis Doctoral son los que se muestran en la Figura 13:

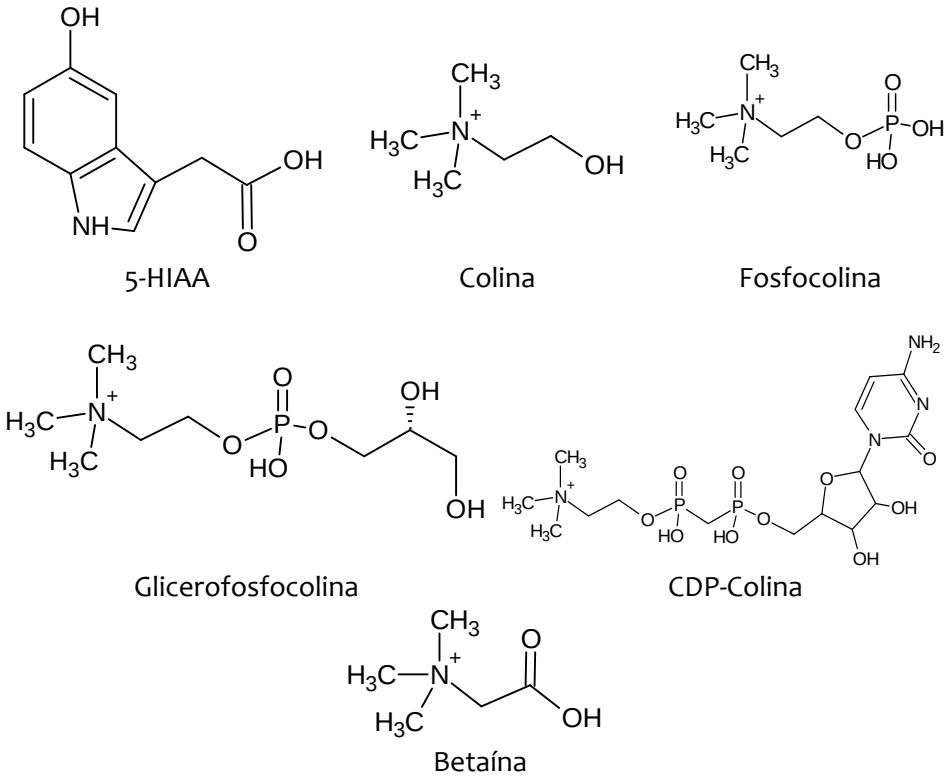

Figura 13. *Estructuras químicas de metabolitos y precursores de los neurotransmisores seleccionados*

A. Ácido 5-hidroxindolacético (5-HIAA)

Es el metabolito principal de la SE en el cuerpo humano. Se incluye en el estudio con el objeto de controlar la posible degradación de la SE, ya que tiene una importante relevancia desde el punto de vista clínico y nutricional.

Debido a que 5-HIAA es un metabolito de SE, su análisis sirve para el diagnóstico de tumores carcinoides de células enterocromafines del intestino delgado, caracterizadas por una elevada síntesis de SE. Valores altos de 5-HIAA

en orina podrían indicar tumores carcinoides, aunque también esos niveles basales pueden ser aumentados con la ingesta de ciruelas, aguacates, glicerol, paracetamol, fenociaticacinas, metanfetamina, etc. Niveles bajos de 5-HIAA en el líquido cefalorraquídeo se asocia con una conducta agresiva y tendencias suicidas con violencia, estando correlacionado con niveles bajos de SE cerebral. Está demostrado que la SE inhibe comportamientos agresivos[42].

La SE compite con las catecolaminas, especialmente con la DA durante la recaptura en el cerebro, por lo tanto, el desequilibrio entre SE-DA puede interferir con la función neuronal. Las razones que provocan estos desequilibrios son diversos, pero entre las que más nos interesan en este caso, desde el punto de vista nutricional, podrían ser debidas a un alto contenido de Trp dietario (precursor de la SE) y el bajo de Tyr (precursor de la DA). Es clara la importancia del análisis de estos aminoácidos y de sus metabolitos, ya que un tratamiento mediante la dieta, como la suplementación con Tyr, podría mejorar los síntomas de la depresión.

En un estudio publicado en la literatura científica se comparan animales alimentados ad libitum y animales privados de alimento durante 24 y 48 h. Se evalúa la relación entre SE, Trp y 5-HIAA y se determinan los niveles tisulares en cerebro, estómago e intestino[43]. El estudio alcanza conclusiones interesantes cuando la dieta es eliminada durante un largo periodo de tiempo:

- La concentración de Try en cerebro no cambia. En estómago e intestino aumenta.

- La concentración de SE en cerebro disminuye. En estómago e intestino aumenta.

- La concentración de 5-HIAA en cerebro disminuye. En estómago e intestino aumenta.

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[42] *Winberg S., Overli O., et al. Suppression of aggression in rainbow trout (Oncorhynchus mykiss) by dietary L-tryptophan. J. Exp. Biol. 204 (2001) 3867-3876.*

[43] *Bubenik G.A., Ball R.O., et al. The effect of food deprivation on brain and gastrointestinal levels of tryptophan, serotonin, 5-hydroxyindoleacetic acid, and melatonin. J. Pineal Res. (1992) 7-16.*

Si se tiene en cuenta que el hecho de encontrar bajas concentraciones de SE en cerebro se puede relacionar con comportamientos de cierta agresividad o malestar, y aquí se explicaría este comportamiento en algunos individuos cuando sufren periodos de inanición[44]. Se ha demostrado que la ingestión de dietas enriquecidas en Trp aumenta la concentración de SE y 5-HIAA en el duodeno. Además, en situaciones de estrés y cambios inmunológicos se presentan concentraciones de SE y Trp elevadas en el cerebro.

Por otro lado, la SE plasmática elevada (hiperserotoninemia) es uno de los síntomas más comunes del autismo[45], y el 5-HIAA puede estar elevado en pacientes con trastornos autisticos. La actividad alterada de las células enterocromafines y desórdenes gastrointestinales también se han relacionado con la etiología del autismo.

B. Colina (Cho)

Es un nutriente esencial soluble en agua. Se suele agrupar con las vitaminas del grupo B. Su nombre hace referencia a una serie de sales cuaternarias de amonio que contienen el catión N,N,N-trimetiletanolamina. El catión aparece en la cabeza de compuestos como la fosfatidilcolina (PC) y esfingomielina (SM), dos clases de fosfolípidos que son abundantes en las membranas celulares. Su fórmula química es $(CH_3)_3N^+CH_2CH_2OHX$-, donde X– es un contraión como el cloruro, hidroxilo o tartrato.

Como ya se ha comentado, la Cho es el precursor de la AcCh, el neurotransmisor que está involucrado en muchas funciones biológicas, entre las más importantes la memoria y el control del músculo. Fue descubierta por Adolph Strecker[46] en 1862. Sin embargo, fue el científico francés Charles

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[44] *Gibbonsa J.L., Barra G.A., et al. Manipulations of dietary tryptophan: Effects on mouse killing and brain serotonin in the rat. Brain Res. 169 (1979) 139–153.*

[45] *Burgess N.K., Sweeten T.L., et al. Hyperserotoninemia and altered immunity in autism. J. Autism Dev. Disord. 36 (2006) 697-704.*

[46] *Strecker A. Ueber einige neue bestandtheile der schweinegalle. Annal der Chemie u Pharm. 123 (1862) 353-360.*

Adolph Wurtz[47] quien la sintetizó químicamente por primera vez en 1866. En 1868, se demostró que la Cho formaba parte de la lecitina de la yema de huevo[48] y posteriormente se observó que era un componente de la SM[49,50]. Tras diversos experimentos y estudios para demostrar la importancia de la Cho en el metabolismo animal, en 1998 fue clasificada como un nutriente esencial por el "*Food and nutrition Board*" del "*Institute of Medicine*" (U.S.A.)[51].

Su importancia como nutriente empezó con una investigación sobre funciones de la insulina. En algunos experimentos se vio que individuos alimentados con dietas deficientes en Cho desarrollaron hígado graso y daño hepático, presentando una concentración elevada de alanina aminotransferasa (ALT), un marcador bioquímico de daño hepático[52]. En 1975 los científicos descubrieron que la administración de Cho aumentaba la síntesis y liberación de AcCh por parte de las neuronas. Estos descubrimientos hicieron que aumentara el interés de la dieta basada en Cho y de su función cognitiva. Hoy en día está más que demostrada la importancia que tiene la Cho como nutriente dietético. Es básica en la dieta para poder llevar a cabo una vida normal, pues es indispensable para la síntesis de componentes esenciales de membranas y es una fuente importante de grupos metilos.

La Cho y sus metabolitos son necesarios para tres fines fisiológicos importantes: integridad estructural y funciones de señalización para las membranas celulares; vías colinérgicas (síntesis de AcCh); y fuente muy importante de grupos metilos gracias a su metabolito betaína que participa en la síntesis de las secuencias de S-adenosilmetionina (Figura 14). Las consecuencias más graves de la deficiencia de Cho en humanos son la aparición de disfunción orgánica, principalmente hepática (hígado graso o esteatosis)

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[47] *Wurtz A. [Título no disponible]. Annal der Chem u Pharm. 148 (1868) 116-197.*
[48] *Strecker A. Ueber das lecithin. Annal der Chemie u Pharm. 148 (1868) 77-90.*
[49] *Levene P.A. On sphingomyelin II. J. Biol. Chem. 18 (1914) 453-463.*
[50] *Levene P.A. Spingomyelin III. J. Biol. Chem. 24 (1916) 68-69.*
[51] *Institute of Medicine Food Nutrition Board. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin b6, folate, vitamin b12, pantothenic acid, biotin, and choline. Washington DC: The National Academies Press; 1998. 592.*
[52] *Zeisel S.H., Da Costa K.A., et al. Choline, an essential nutrient for humans. FASEB J. 5 (1991) 2093-2098.*

pero también renal o muscular[53,54]. Además aparecen diversos problemas como infertilidad, crecimiento deteriorado, anormalidades en los huesos, hipertensión y posiblemente desórdenes neurológicos[55,56]. También se cree que el aporte de Cho en la dieta puede influir positivamente en el tratamiento de cáncer[57]. Nutricionalmente con dietas o complementos nutricionales ricos en Cho se pueden solucionar o mejorar algunos de esos problemas.

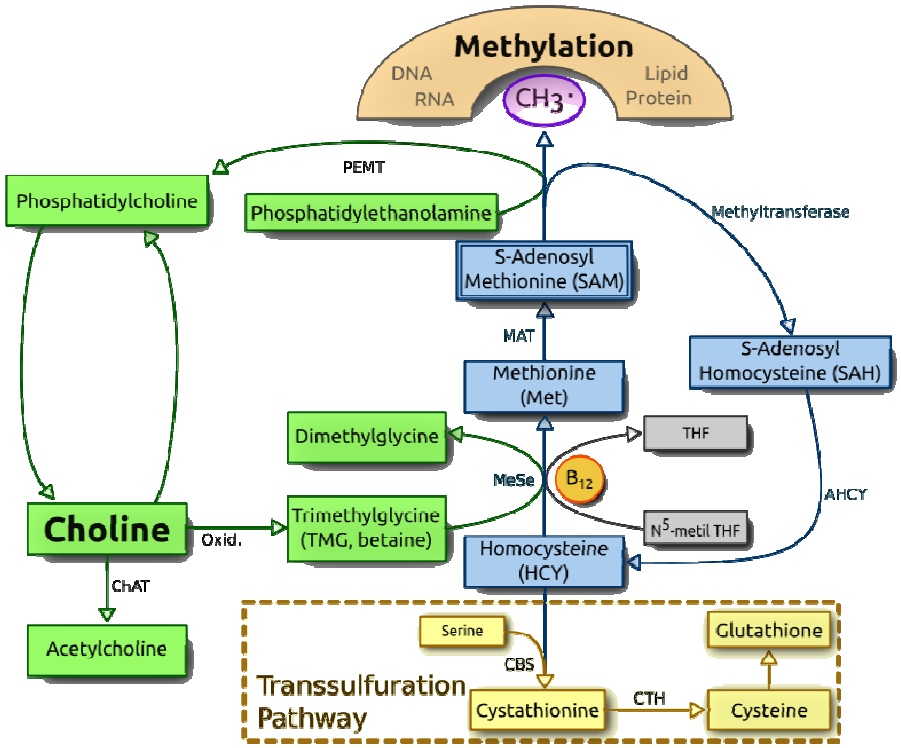

Figura 14. *Metabolismo de la Cho*
(obtenido de asturnatura.com, La colina. Aditivos)

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[53] *da Costa K.A., Niculescu M.D., et al. Choline deficiency increases lymphocyte apoptosis and DNA damage in humans. Am. J. Clin. Nutr. 84 (2006) 88-94.*

[54] *Vance D.E., Li Z., et al. Hepatic phosphatidylethanolamine N-methyltransferase, unexpected roles in animal biochemistry and physiology. J. Biol. Chem. 282 (2007) 33237-33241.*

[55] *Zeisel S.H., Da Costa K.A. Choline: an essential nutrient for public health. Nutrition Reviews 67 (2009) 615–623.*

[56] *Li Z., Vance D. Phosphatidylcholine and choline homeostasis. J. Lipid Res. 49 (2008) 1187–1194.*

[57] *Xu X., Gammon M.D., et al. High intakes of choline and betaine reduce breast cancer mortality in a population-based study. FASEB J. 23 (2009) 4022-4028.*

La Cho es un precursor de la trimetilamina, la cual muchas personas no pueden descomponer debido a un desorden genético llamado trimetilaminuria. Las personas que sufren este desorden suelen manifestar un olor desagradable a pescado, debido a que su cuerpo libera olores de trimetilamina. El olor puede darse incluso teniendo una dieta normal, con niveles de Cho no muy altos.

Hay grupos en la población que presentan riesgo de deficiencia de Cho como son los vegetarianos, atletas de alta resistencia y los alcohólicos. En general, la gente que no consume muchos huevos enteros tendría que prestar atención en incluir suficiente Cho en su dieta[58]. Se ha descubierto en estudios realizados en varias poblaciones que el consumo de Cho está por debajo del recomendado[59]. El Dr. Steven Zeisel afirmó que: *"Un análisis de NHANES en 2003-2004 reveló que los niños más mayores, hombres, mujeres y embarazadas, tenían un consumo de Cho muy inferior al recomendado, y sólo un 10 % o menos consumía habitualmente Cho en un nivel igual o superior al recomendado"*[55].

Se encuentra en los alimentos de forma libre o esterificada. Una de las formas más utilizadas por el cuerpo humano son las formas liposolubles, como es el caso de la PC.

Hay dos vías por las que la Cho aparece en el organismo, a partir de la metilación de fosfatidiletanolamina para formar PC en el hígado; o a través de la dieta. Ambas son necesarias para un correcto funcionamiento en hígado y en músculo. Las concentraciones plasmáticas de Cho pueden variar según los alimentos que se ingieran. Además, dado que atraviesa fácilmente la barrera hematoencefálica por difusión facilitada, estos cambios en plasma pueden producir cambios paralelos en los niveles del cerebro. Cabe destacar que la vía de actuación de la Cho es muy compleja, donde intervienen otros elementos como el folato, la metionina o la vitamina B12.

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[58] *Hasler C.M. The changing face of functional foods. J. Am. Coll. Nutr. 19 (2000) 499S-506S.*

[59] *Bidulescu A., Chambless L.E., et al. Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) study. Nutr. J. 8 (2009) 14.*

Durante el embarazo y la lactancia, la demanda de Cho aumenta drásticamente. Su síntesis se puede aumentar incrementado los niveles de estrógenos que señalizan la producción de Cho a partir de la metilación de PC. En el embarazo la Cho se acumula en la placenta, mayoritariamente en forma de AcCh. Así el feto tiene fácil disponibilidad a la Cho, siendo la concentración en el líquido amniótico diez veces superior al nivel que se encuentra en la sangre materna[60]. Es un compuesto muy importante en estas primeras etapas de la vida por sus diversas funciones:

- Es el substrato para construir membranas celulares y para incrementar la producción de lipoproteínas[61,62].

- Es muy importante en la formación y desarrollo del cerebro. El crecimiento del cerebro es muy rápido durante el tercer trimestre del embarazo y continúa desarrollándose hasta los 5 años de edad[63]. Durante este periodo hay una demanda muy alta de SM, que se forma a partir de PC, siendo su precursor la Cho. Además, como ya se ha dicho anteriormente la Cho también es precursor de AcCh, el cual puede influir en la estructura y organización de determinadas regiones del cerebro, en la neurogénesis, mielinización y formación de la sinapsis.

- Interviene en la metilación de los dinucleótidos en el DNA del cerebro, metilación que puede cambiar la expresión genómica[64,65].

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[60] *Zeisel S.H., Niculescu M. Perinatal choline influences brain structure and function. Nutr Rev. 64 (2006) 197–203.*

[61] *Caudill M.A. Pre- and Postnatal Health: Evidence of Increased Choline Needs. J. Am. Diet. Assoc. 1 (2010) 1198-1206.*

[62] *King J.C. Physiology of pregnancy and nutrient metabolism. AM. J. Clin. Nutr. 71 (2000) 1218S-1225S.*

[63] *Morgane P.J., Mokler D.J., et al. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci. Biobehav. Rev. 26 (2002) 471-483.*

[64] *Waterland R.A., Jirtle R.L. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhance susceptibility to adult chronic diseases. Nutrition 20 (2004) 63-68.*

[65] *Davison J.M., Mellott T.J., et al. Gestational histone methyltransferases G9a (Kmt1C) and Suv39h1 (Kmt1a) and DNA methylation of their genes in rat fetal liver and brain. J. Biol. Chem. 284 (2009) 1982-1989.*

- Dependiendo de la concentración de la Cho tendrá distintas funciones. A concentraciones bajas se utiliza para la formación de PL. Cuando la concentración aumenta, la Cho libre se convierte en Bet en las mitocondrias del hígado (se utiliza como fuente de grupos metilo para metilaciones del ADN). La concentración también es muy importante para que pueda llegar Cho al cerebro y así asegurar una correcta formación de este órgano. El transporte de Cho hacia el cerebro se hace gracias a un transportador específico. Por otro lado, se almacena unida a la membrana en forma de PC, y podrá ser utilizada por ejemplo, para la síntesis del neurotransmisor AcCh.

La Cho de la dieta, incluida la leche materna, se puede encontrar de forma libre, en forma de PC, GPCho o SM[66]. La concentración de Cho en la leche materna está relacionada con la concentración de ésta en la sangre de la madre[67,68]. La Cho consumida a través de la leche materna aumenta los niveles en la sangre del lactante.

C. Fosfocolina (PCho), glicerofosfocolina (GPCho), citidin 5'-difosfocolina (CDP-Cho) y betaína (Bet)

Todas estas biomoléculas tienen una relación importante con la Cho y AcCh. La Cho se utiliza principalmente para la síntesis de PC en un 95% aproximadamente del total que pueda acumular el organismo (Figura 15). El 5% restante se encuentra en forma de Cho libre, PCho, GPCho, CDP-Cho y AcCh[69].

La PCho es una biomolécula intermedia en la síntesis de PC en los tejidos, catalizada por la colina quinasa. Se encuentra en la lecitina. Tiene una importante función en la placenta humana ya que suprime la respuesta inmune de sus huéspedes.

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[66] *Zeisel S.H. The fetal origins of memory: the role of dietary choline in optimal brain development. J. Pediatr. 149 (2006) S131–S136.*

[67] *Holmes-McNarry M.Q., Cheng W.L., et al. Choline and choline esters in human and rat milk in infant formulas. Am. J. Clin. Nutr. 64 (1996) 572-576.*

[68] *Ilcolemail Y.O., Ozbek R., et al. Choline status in newborns, infants, children, breast-feeding women, breast-fed infants, and human breast milk. J. Nutr. Biochem. 16 (2005) 489-499.*

[69] *Li Z., Vance D.E. Phosphatidylcholine and choline homeostasis. J. Lipid Res. 49 (2008) 1187-1194.*

La GPCho es un compuesto de Cho natural que se encuentra en el cerebro. También es un precursor de la AcCh y puede ser un fármaco potencial para el tratamiento de la enfermedad de Alzheimer[70] y la demencia[71]. Es capaz de generar Cho en el cerebro, pasando a través de la barrera hematoencefálica, y a su vez es precursor biosintético del neurotransmisor AcCh.

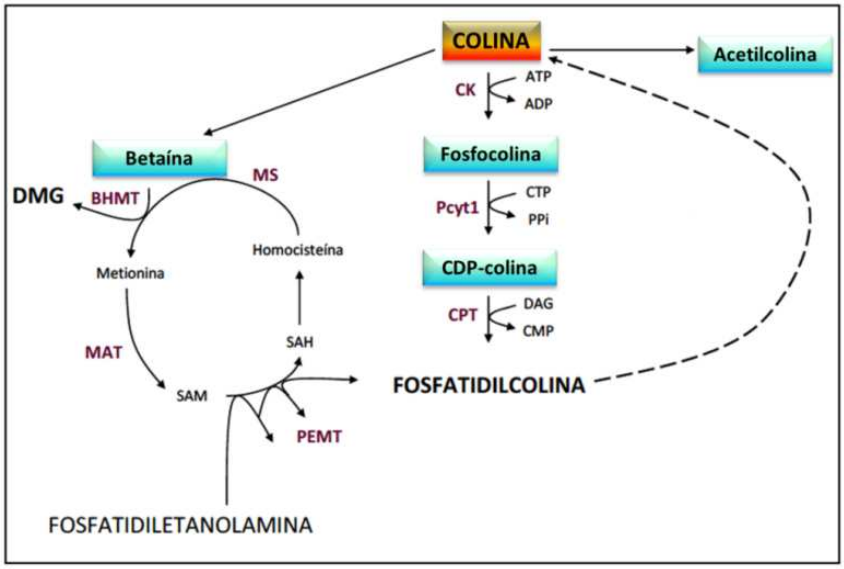

Figura 15. *Metabolismo de la Cho[72]. Intervienen PCho, CDP-Cho y Bet. CK: colina quinasa; ATP: adenosina trifosfato; ADP: adenosina difosfato; Pcyt1: CTP: fosfocolina citidil transferasa1; CTP: citidina trifosfato; PPi: pirofosfato; CDP: citidina difosfocolina; CPT: CDP-colina:DAG colinafosfotransferasa; DAG: diacilglicerol; CMP: citidina monofosfato; BHMT: betaína-homocisteína metiltransferasa; MS: metionina sintasa; MAT: metionina adenosiltransferasa; SAM: S-adenosilmetionina; SAH: S-adenosilhomocisteína; PEMT: fosfatidiletanolamina-N-metiltransferasa; DMG: dimetilglicina*

La CDP-Cho es un intermedio en la generación de PC a partir de la Cho. Hay estudios que sugieren que como suplemento aumenta la densidad del receptor de DA y ayuda a prevenir las pérdidas de memoria debidas a malas condiciones ambientales. Ha sido asociada a la mejora de concentración, siendo útil en el

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[70] *Parnetti L., Mignini F., et al. Cholinergic precursors in the treatment of cognitive impairment of vascular origin: Ineffective approaches or need for re-evaluation? J. Neur.Sci. 257 (2007) 264–269.*

[71] *Doggrell S.A., Evans S. Treatment of dementia with neurotransmission modulation. Expert Opin. Investig. Drugs 12 (2003) 1633–1654.*

[72] *Tesis: Fernández S. Contribución de la colina y betaína al metabolismo de la homocisteína durante la gestación. Universitat Rovira i Virgili. Dipòsit Legal: T. 1431-2012.*

tratamiento del trastorno de déficit de atención[73]. Además también mejora la función visual en diversos problemas como el glaucoma.

La Bet, también conocida como glicina-betaína (N,N,N-trimetilglicina), se descubrió en el siglo XIX en la savia de la remolacha azucarera. Es un aminoácido trimetilado (Figura 13) que a pH neutro se encuentra en su forma zwiteriónica. Su fórmula es $(CH_3)_3N^+CH_2COO^-$. Los humanos obtienen la Bet mediante la dieta y también endógenamente mediante la oxidación de la Cho (Figura 15). Se elimina principalmente a través de su catabolismo, ya que su excreción urinaria es ínfima[74]. Tiene tres funciones principales en los mamíferos: 1) Es un osmolito orgánico, ya que la célula regula su volumen ajustando la concentración de osmolitos intracelulares. Los osmolitos pueden ser inorgánicos, como el sodio, el potasio y los iones clorados, o bien orgánicos como la Bet, la GPCho, el myo-inositol, el sorbitol y los aminoácidos[75]. 2) Actúa como sustancia estabilizadora de las estructuras de las proteínas bajo condiciones de desnaturalización. 3) Por último, sirve como dador de grupos metilo en la reacción que convierte la homocisteína a metionina. Por su participación en esta reacción, Bet y Cho están directamente relacionadas con el metabolismo monocarbonado.

1.2 Oligosacáridos presentes en la leche humana

Los denominados oligosacáridos solubles presentes en leche (OSL), son compuestos de gran importancia en las primeras fases de la vida de un ser humano que clásicamente han sido relacionadas con el correcto funcionamiento del sistema inmune. Son habitualmente conocidos por sus siglas en inglés *"Human Milk Oligosaccharides"* (HMOs), aunque cuando nos referimos a oligosacáridos solubles presentes en leche de animales no

[73] *Silveri M.M., Dikan J., et al. Citicoline enhances frontal lobe bioenergetics as measured by phosphorus magnetic resonance spectroscopy". NMR in Biomedicine 21 (2008) 1066–1075.*

[74] *Schwahn B.C., Hafner D., et al. Pharmacokinetics of oral betaine in healthy subjects and patients with homocystinuria. Br. J. Clin. Pharmacol. 55 (2003) 6-13.*

[75] *Wehner F., Olsen H., et al. Cell volume regulation: Osmolytes, osmolyte transport, and signal transduction. Rev. Physiol. Biochem. Pharmacol. 148 (2003) 1-80.*

humanos se conocen como OSL o por el término en inglés *"Soluble Glycans in Milk"* (SGMs).

Hoy en día están siendo estudiados ampliamente por estar relacionados con funciones esenciales en el organismo, atribuyéndoles importantes propiedades[76], incluyendo la función cognitiva[77].

La leche humana es un fluido biológico complejo compuesto básicamente por lípidos, proteínas e hidratos de carbono (lactosa (Lact)). Los oligosacáridos son el tercer componente más abundante en la leche humana. Están presentes en cantidades que oscilan entre 5 y 23 g L^{-1} y son biomoléculas de naturaleza muy diversa y compleja. Los monosacáridos usados para la biosíntesis de glicanos de leche humana son glucosa, galactosa, N-acetilglucosamina, fucosa y ácido N-acetilneuramínico (más conocido como ácido siálico (SA)). A pesar de estas estructuras básicas, las posibles combinaciones de monosacáridos y sus uniones contribuyen a la diversidad global y a la complejidad de las estructuras de los OSL, hasta el punto de que hoy día han sido identificados más de 150 OSL estructuralmente distintos[78].

Figura 16. *Momento de la Lactancia*

En general, se acepta que los OSL ejercen diversos efectos biológicos destacables sobre la salud. Por ejemplo, poseen un efecto prebiótico que sirve selectivamente como fuente de energía y de nutrientes para las bacterias, ayudando a colonizar el intestino infantil. También se ha descrito que pueden beneficiar al bebé alimentado con leche materna a través de otros múltiples mecanismos relacionados con el sistema inmune: pueden proteger contra las

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[76] *Isolauri E., Salminen S., et al. Microbial-gut interactions in health and disease. Probiotics. Best Pract. Res. Clin. Gastroenterol. 18 (2004) 299-313.*

[77] *Brand Miller J.C., McVeagh P. Human milk oligosaccharides: 130 reasons to breast-feed. Brit. J. Nutr. 82 (1999) 333–335.*

[78] *Ninonuevo M.R., Lebrilla C.B. Mass spectrometric methods for analysis of oligosaccharides in human milk. Nutr. Rev. 67 (2009) S216-226.*

infecciones imitando los sitios de unión para ciertos patógenos bloqueando su adherencia[79]; influir en diversas etapas de la maduración intestinal *in vitro*[80]; y prevenir la enterocolitis necrotizante en ratas neonatales[81].

Inicialmente los OSL fueron considerados no digeribles en el organismo. Sin embargo, algunos autores han hablado recientemente de sus cambios de estructura antes de alcanzar el colon y la posibilidad de ser absorbidos, ya que algunos de ellos han sido detectados en el plasma de ratas neonatales[82] y en la orina de lactantes[83].

Los OSL también pueden ejercer efectos sistémicos. La perspectiva de que los OSL puedan afectar la funcionalidad del cerebro, directa o indirectamente, ha sido menos explorada y ofrece una nueva e interesante aplicación. El mecanismo de acción de los OSL en los tejidos neuronales podría ser como una fuente de oligosacáridos que pueden funcionar como ligandos para otras moléculas. Los oligosacáridos se encuentran a menudo como un componente de los glicolípidos o glicoproteínas y hay evidencias del papel ejercido por estos en funciones de adaptación de los componentes de la membrana neuronal que determinan la eficacia de las conexiones interneuronales[84].

1.2.1 Oligosacáridos seleccionados

Los OSL son estructuras de hidratos de carbono lineales o ramificados constituidos por cinco tipos de monosacáridos: glucosa, galactosa, N-acetylglucosamine, L-fucosa y SA.

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[79] *Bode L. Human milk oligosaccharides: prebiotics and beyond. Nutr. Rev. 67 (2009) S183-191.*

[80] *Kuntz S., Rudloff S., et al. Oligosaccharides from human milk influence growth-related characteristics of intestinally transformed and non-transformed intestinal cells. Br. J. Nutr. 99 (2008) 462-471.*

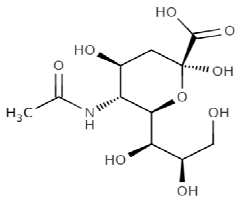
[81] *Jantscher-Krenn E., Zherebtsov M., et al. The human milk oligosaccharide disialyllacto-N-tetraose prevents necrotising enterocolitis in neonatal rats. Gut 61 (2012) 1417-1425.*

[82] *Jantscher-Krenn E., Marx C., et al. Human milk oligosaccharides are differentially metabolised in neonatal rat., Br. J. Nutr. 110 (2013) 640-650.*

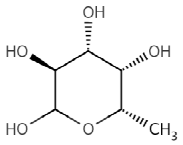
[83] *Rudloff S., Pohlentz G., et al. Urinary excretion of in vivo (1)(3)C-labelled milk oligosaccharides in breastfed infants. Br. J. Nutr. 107 (2012) 957-963.*

[84] *Wetzel W., Popov N., et al. Effect of L-fucose on brain protein metabolism and retention of a learned behavior in rats. Pharmacol. Biochem. Behav. 13 (1980) 765-771.*

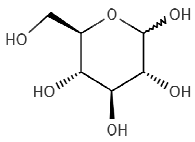
Se han identificado más de 150 HMOs en muestras de leche humana[85-87], conteniendo Lact en todas las estructuras identificadas hasta el momento.



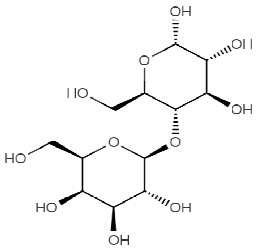
Sialic acid (SA)



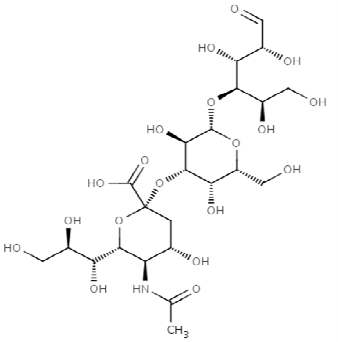
Fucose (Fuc)



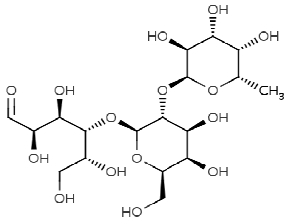
Glucose (Glu)



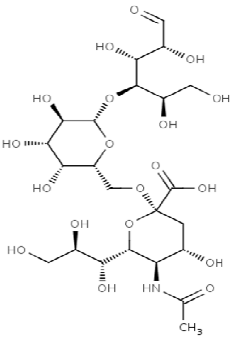
Lactose (Lact)



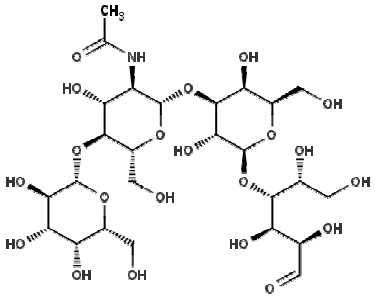
3'-sialyllactose (3'-SL)



2'-fucosyllactose (2'-FL)



6'-sialyllactose (6'-SL)



Lacto-N-neo-Tetraose (LNnT)

Figura 17. *Estructuras químicas de HMOs seleccionados (2'-FL, 3'-SL, 6'-SL y LNnT) y metabolitos (SA, Fuc, Lact y Gluc)*

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[85] *Kobata A., Structures and application of oligosaccharides in human milk. Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 86 (2010) 731-747.*

[86] *Wu S., Tao N., et al. Development of an annotated library of neutral human milk oligosaccharides. J. Proteome Res. 9 (2010) 4138-4151.*

[87] *Wu S., Grimm R., et al. Annotation and structural analysis of sialylated human milk oligosaccharides. J. Proteome Res. 10 (2011) 856-868.*

Como ya se ha mencionado con anterioridad, a los HMOs se les han atribuido diversas funciones biológicas, entre ellas destacan funciones anti-infecciosas e inmunológicas o prebióticas, lo que podría ser en parte debido a la presencia de estructuras fucosiladas[79,88-90]. El más abundante en la leche humana es la 2'-fucosillactosa (2'-FL), que representa el 30% del total de HMOs, siendo el sacárido predominante después de la Lact[91]. Curiosamente la 2'-FL no está presente en la leche de todas las mujeres y su concentración varía significativamente durante la lactancia y entre individuos. Otra característica interesante de la 2'-FL es su presencia en la leche de animales no humanos, que van desde monotremas (mamíferos de reproducción ovípara como el ornitorrinco) hasta los primates, por lo tanto, este HMO puede proporcionar pistas evolutivas de su función[92].

Aunque los mamíferos contienen estos oligosacáridos, la leche humana parece ser única en diversidad y concentración de estas estructuras. Hay dos características de los HMOs cuando se comparan con oligosacáridos de otras especies:

- La relación entre oligosacáridos fucosilados y no fucosilados es relativamente alta.

- Los oligosacáridos neutros están presentes en concentraciones más altas que los ácidos.

Sin embargo, hay HMOs que también se encuentran en la leche de otros mamíferos; por ejemplo, 3'-SL y 6'-SL están presentes en la leche bovina y de roedores, y se han detectado en fórmulas infantiles basadas en leche

[88] *Bode L., Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 22 (2012) 1147-1162.*

[89] *Kunz C., Rudloff S., et al., Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu. Rev. Nutr. 20 (2000) 699-722.*

[90] *Newburg D.S., Ruiz-Palacios G.M., et al. Human milk glycans protect infants against enteric pathogens. Annu. Rev. Nutr. 25 (2005) 37-58.*

[91] *Chaturvedi P., Warren C.D., et al. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11 (2001) 365-372.*

[92] *Urashima T., Saito T., et al. Oligosaccharides of milk and colostrum in non-human mammals. Glycoconj. J. 18 (2001) 357-371.*

bovina[93,94]. Por el contrario, la 2'-FL está ausente en la leche de roedor y sólo recientemente ha sido detectado en la leche bovina, aunque en concentraciones muy bajas[95]. La 2'-FL aparece notablemente temprano en la evolución de mamíferos como los monotremas[96], y también ha sido detectada en osos, focas, leones, elefantes, ballenas y chimpancés[97].

La leche de los animales domésticos de granja, como la leche bovina, ha sido el foco de interés ya que constituye la base para las fórmulas infantiles. Los OSLs en estos animales están presentes en baja concentraciones y sus estructuras moleculares son menos complejas que en otros mamíferos, como los humanos. Moléculas con Fuc suelen ser raras, siendo los sialiloligosacáridos dominantes[98,99]. Por ejemplo, la fucosilación está por debajo del 1% en leche bovina, mientras que los oligosacáridos sialilados llegan hasta el 70%[95,100]. La leche ovina también es sialilada principalmente, sin llegar a detectar moléculas fucosiladas[92]. Al igual que en la leche caprina, donde tampoco se detectan[101]. Los cerdos se utilizan como modelos para estudios de nutrición debido a las similitudes que presentan con los humanos[102]. Se han estudiado las estructuras de los OSLs en la especie porcina, predominando los sialilados. Se identifican seis OSLs fucosilados, aunque a muy baja concentración. La 2'-FL se encuentra

[93] *Martin-Sosa S., Martin M.J., et al. Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation. J. Dairy Sci. 86 (2003) 52-59.*

[94] *Sundekilde U.K., Barile D., et al. Natural variability in bovine milk oligosaccharides from Danish Jersey and Holstein-Friesian breeds. J. Agric. Food Chem. 60 (2012) 6188-6196.*

[95] *Aldredge D.L., Geronimo M.R., et al. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology (2013).*

[96] *Amano J., Messer M., et al. Structures of the oligosaccharides isolated from milk of the platypus. Glycoconjugate Journal 2 (1985) 121-135.*

[97] *Messer M., Gadiel P.A., et al. Carbohydrates of the milk of the platypus. Aust. J. Biol. Sci. 36 (1983) 129-137.*

[98] *Zivkovic A.M., Barile D. Bovine milk as a source of functional oligosaccharides for improving human health. Adv. Nutr. 2 (2011) 284-289.*

[99] *Urashima T., Taufik E., et al. Recent Advances in Studies on Milk Oligosaccharides of Cows and Other Domestic Farm Animals. Biosci. Biotechnol. Biochem. 77 (2013) 465-466.*

[100] *Tao N., DePeters E.J., et al. Bovine milk glycome. J. Dairy Sci. 91 (2008) 3768-3778.*

[101] *Martinez-Ferez A., Rudloff S., et al. Goat's milk as a natural source of lactosederived oligosaccharides: Isolation by membrane technology. International Dairy Journal 16 (2006) 173-181.*

[102] *Pond W.G., Boleman S.L., et al. Perinatal ontogeny of brain growth in the domestic pig. Proc. Soc. Exp. Biol. Med. 223 (2000) 102-108.*

en la leche porcina, pero no en el calostro[103]. Por otro lado, la leche de camello bactriano y el calostro se consumen en el Oriente Medio y Mongolia, y se han utilizado tradicionalmente como un producto saludable. Un estudio reciente caracterizó los OSLs de camella, encontrando que el calostro tenía particularmente una alta concentración en 3-fucosilactosa (3-FL)[104].

En resumen, la leche bovina contiene una concentración mucho menor de OSLs que la leche humana, y las estructuras fucosiladas apenas están presentes. Por lo tanto, las fórmulas infantiles podrían estar perdiendo los beneficios postulados proporcionados por estas biomoléculas encontradas en la leche humana. De ahí la necesidad de estudiar los OSLs para aportar más información sobre sus mecanismos de acción, biodisponibilidad y funciones bioquímicas con estudios preclínicos y posteriormente con estudios clínicos.

Con objeto de dar apoyo a diversos proyectos de investigación, se llevó a cabo la validación de métodos analíticos para la determinación cuantitativa de estas biomoléculas en muestras biológicas como suero y orina. Se diseñaron experimentos con ratas por la facilidad de manejo que presentan frente a otros modelos animales como el cerdo. Los experimentos se desarrollaron en las instalaciones que Abbott tiene en el CSIC de Granada.

1.2.2 Evidencias experimentales del comportamiento de los HMOs

Hay datos limitados sobre la absorción, distribución, metabolismo y excreción de glicanos solubles no digeribles. Para ejercer sus efectos beneficiosos prebióticos, es necesario que los HMOs no sean digeridos en el tracto intestinal superior, para llegar al colon intactos, donde pueden ser metabolizados por la microbiota intestinal o excretados en las heces[105,106]. Esos

[103] *Tao N., Ochonicky K. L., et al. Structural determination and daily variations of porcine milk oligosaccharides. J. Agric. Food Chem. 58 (2010) 4653-4659.*
[104] *Fukuda K., Yamamoto A., et al. Chemical characterization of the oligosaccharides in Bactrian camel (Camelus bactrianus) milk and colostrum. J. Dairy Sci. 93 (2010) 5572-5587.*
[105] *Engfer M.B., Stahl B., et al. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am. J. Clin. Nutr. 71 (2000) 1589-1596.*
[106] *Gnoth M.J., Kunz C., et al. Human milk oligosaccharides are minimally digested in vitro. J. Nutr. 130 (2000) 3014-3020.*

estudios *in vitro* indicaron que la 2'-FL, en particular, es resistente a la acción de las enzimas humanas y porcinas.

En las heces de los lactantes alimentados con leche materna se encontraron perfiles de HMOs similares a los de la leche de su madre; por ejemplo, se ha detectado 2'-FL y 3-FL en las heces de los bebés cuya madre presentaba estos HMOs en su leche, siendo analizados mediante cromatografía de líquidos de intercambio iónico[107] y por electroforesis capilar-espectrometría de masas[108,109]. Por el contrario, los oligosacáridos encontrados en las heces de los lactantes alimentados con fórmula estuvieron presentes en bajas concentraciones y eran estructuralmente diferentes a los de la leche humana.

Experimentos realizados empleando cromatografía de líquidos de alta resolución en capa fina (HPTLC), de cambio iónico y con espectrometría de masas, hallaron diversos HMOs intactos en la orina de los bebés alimentados con leche materna[110]. Sin embargo, en otros experimentos del mismo grupo de investigación encontraron que las orinas de los bebés alimentados con fórmula tenían un perfil mucho menos complejo, con sólo unos pocos componentes (principalmente 3'-SL y 6'-SL)[111]. Un informe reciente demuestra de manera concluyente la presencia de 2'-FL en la orina de bebés alimentados con leche materna[112]. Esta evidencia sugiere fuertemente que los HMOs son absorbidos y llegan a la circulación sistémica.

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[107] *Coppa G.V., Pierani P., et al. Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. Adv. Exp. Med. Biol. 501 (2001) 307-314.*

[108] *Albrecht S., Schols H.A., et al. CE-LIF-MS n profiling of oligosaccharides in human milk and feces of breast-fed babies. Electrophoresis. 31 (2010) 1264-1273.*

[109] *De Leoz M.L., Wu S., et al. A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. Anal. Bioanal. Chem. 405 (2013) 4089-4105.*

[110] *Rudloff S., Obermeier S., et al. Incorporation of orally applied (13)C-galactose into milk lactose and oligosaccharides. Glycobiology. 16 (2006) 477-487.*

[111] *Rudloff S., Pohlentz G., et al. Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. Acta Paediatr. 85 (1996) 598-603.*

[112] *Rudloff S., Kunz C. Milk oligosaccharides and metabolism in infants. Adv. Nutr. 3 (2012) 398S-405S.*

La combinación de los resultados obtenidos en experimentos *in vivo* e *in vitro* sugiere que aproximadamente del 1% al 2% de los HMOs ingeridos son absorbidos, llegando a la circulación sistémica y excretándose en la orina. Esto ha sido una especulación hasta hace muy poco tiempo; de hecho, recientemente se ha publicado un trabajo basado en el uso de la nanoHPLC-chip-Q-TOF-MS/MS donde demuestran la presencia de HMOs en plasma de niños, y se describen diferencias entre los alimentados con leche materna y con fórmulas[113]. Los investigadores encontraron HMOs neutros como lacto-N-tetraosa (LNT), LNnT, lacto-N-fucopentosa III (LNFP III) y 2'-FL. LNT, que es a menudo el más abundante HMO en la leche materna, se observó a baja concentración en el plasma de niños alimentados exclusivamente con fórmula. Además 2'-FL no se encontró en estos niños. Curiosamente 3'-SL y 6'-SL eran muy abundantes en el plasma de niños alimentados con leche materna.

El uso de modelos experimentales con roedores es frecuente para afrontar este tipo de estudios. Janster et al.[82] describieron en 2013 que la 3'-SL es el principal y predominante OSL presente en el suero y orina de rata. Los estudios realizados mediante HPLC con detección de fluorescencia y MS, demostraron que la 3'-SL predominaba sobre otros OSL en el suero y orina de crías, incluso alimentadas con fórmulas enriquecidas con otros OSL. Es decir, llegaron a la conclusión de que las ratas tenían un mecanismo de absorción específico para la 3'-SL. Además plantearon evaluar más profundamente este diseño experimental con ratas para encontrar modelos experimentales más cercanos al humano. Sin embargo, en contra de lo expuesto por el grupo de Janster, en esta Tesis Doctoral se presentan varias investigaciones con este modelo animal en rata en los que se desarrollan estudios de absorción con 2'-FL, 6'-SL y LNnT, obteniendo curvas de absorción típicas en todos ellos. Esta evidencia sugiere que puede existir un mecanismo de exportación desde el intestino a la sangre, que si bien no es selectiva exclusivamente para la 3'-SL sí que es cierto que algunos de ellos presentaron una absorción de mayor eficiencia que otros.

[113] *Ruhaak R.L., Stroble C., et al. Detection of milk oligosaccharides in plasma of infants. Anal. Bioanal. Chem. 406 (2014) 5775-5784.*

Nuestro grupo de Abbott Granada realizó un estudio en 2014 con una dieta suplementada con 2'-FL. Tras la administración de esta dieta los animales mostraron una mejora en la consolidación de la memoria y aprendizaje espacial y asociativo; es decir, la 2'-FL parece ejercer un efecto positivo en el aprendizaje y la memoria. Esta es la primera vez que un efecto sobre el desarrollo del comportamiento se describe para un OSL como complemento nutricional[114]. Sin embargo, la presencia de 2'-FL en el cerebro no se ha demostrado aún.

En resumen, a pesar de la influencia de 2'-FL en la mejora de las capacidades cognitivas que ya han sido demostradas, su mecanismo de acción sigue siendo desconocido. Ya sea porque 2'-FL pueda realmente llegar al cerebro, o si los efectos observados son el resultado de una acción indirecta. Con los métodos analíticos propuestos en esta Tesis Doctoral garantizamos añadir información con una investigación en profundidad en este campo bioquímico.

1.3 Leucina y β-hidroximetilbutirato

Son dos moléculas de gran interés biológico relacionadas clásicamente con el sistema músculo-esquelético. Para poder entender la importancia de estas biomoléculas hay que destacar que el HMB, metabolito de la Leucina (Leu), ha sido identificado como un compuesto con un papel particular en la nutrición. De hecho, algunos de los efectos descritos en la bibliografía atribuidos a la Leu, están siendo actualmente realacionados con este metabolito. El HMB se considera como un agente anti-catabólico asociado con la síntesis de proteínas y la atenuación de la degradación de las

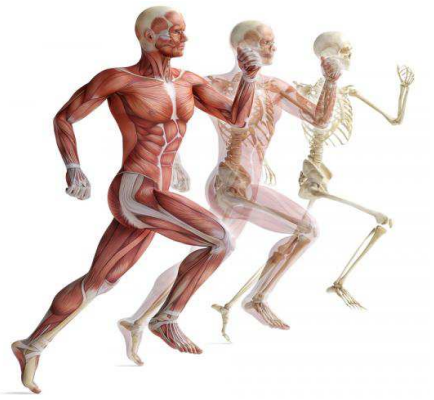

Figura 18. *Imagen del sistema músculo-esquéletico*

[114] *Vazquez E., Rueda R., et al. Methods for increasing brain functionality using 2-fucosyl-lactose. WO 2014043368 A1. 2014.*

mismas[115], y por lo tanto ejerce un efecto potenciador sobre la fuerza y la masa muscular en el deporte[116,117], así como en situaciones clínicas de aumento de la degradación de proteínas (caquexia), disminución de la tasa de síntesis de proteínas musculares (por inactividad), o la alteración de ambos (sarcopenia)[118].

Más allá de estos efectos, el HMB ha mostrado propiedades moduladoras inmunitarias *in vitro* sobre la inmunidad mediada por células, respuesta proliferativa de células mononucleares periféricas y linfocitos, la producción de citoquinas[119]. Así mismo, se ha visto que induce cambios positivos en el metabolismo oxidativo, la biogénesis mitocondrial y la oxidación de ácidos grasos[120]. Por último y más novedoso, nuestro grupo de Abbott Granada ha sido el único por el momento que ha relacionado al HMB con la función cognitiva, siendo este hallazgo parte del trabajo presentado en esta Tesis Doctoral.

La producción de nuevas proteínas es uno de los principales procesos que consume más energía en las poblaciones de células durante su crecimiento. La síntesis de proteínas en los tejidos es estimulado rápidamente después de la ingesta de nutrientes. Los aminoácidos son las fuentes más importantes para estimular la síntesis de proteínas del músculo esquelético, y la Leu parece mediar la mayoría de los efectos sobre el metabolismo proteico[121].

Desde un punto de vista químico, la Leu es un aminoácido esencial de cadena ramificada con fórmula HO_2C-CH-NH_2-CH_2-CH-$(CH_3)_2$. Por ser esencial, el

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[115] *Eley H.L., Russell S.T., et al. Attenuation of depression of muscle protein synthesis induced by lipopolysaccharide, tumor necrosis factor, and angiotensin II by beta-hydroxy-beta-methylbutyrate. Am. J. Physiol. Endocrinol. Metab. 295 (2008) 1409-1416.*

[116] *Portal S., Eliakim A., et al. Effect of HMB supplementation on body composition, fitness, hormonal profile and muscle damage indices. J. Pediatr. Endocrinol. Metab. 23 (2010) 641-650.*

[117] *Wilson J.M., Lowery R.P., et al. Beta-Hydroxy-beta-methylbutyrate free acid reduces markers of exercise-induced muscle damage and improves recovery in resistance-trained men. Br. J. Nutr. 110 (2013) 538-544.*

[118] *Fitschen P.J., Wilson G.J., et al. Efficacy of beta-hydroxy-beta-methylbutyrate supplementation in elderly and clinical populations. Nutrition 29 (2012) 29-36.*

[119] *Nunes E.A., Lomax A.R., et al. Beta-Hydroxy-beta-methylbutyrate modifies human peripheral blood mononuclear cell proliferation and cytokine production in vitro. Nutrition 27 (2010) 92-99.*

[120] *Stancliffe R.A., Eades M., et al. Role of mTOR and β-hydroxy-β-methylbutyrate (HMB) in leucine stimulation of muscle mitochondrial biogenesis and fatty acid oxidation. FASEB J. 25 (2011).*

[121] *Stipanuk M.H. Leucine and protein synthesis: mTOR and beyond. Nutr. Rev. 65 (2007) 122-129.*

cuerpo humano no puede sintetizarlo y debe ser ingerido, normalmente mediante proteínas. La Leu se utiliza en el hígado, tejido adiposo y tejido muscular. En estos dos últimos se usa mucho más, principalmente para la formación de los esteroles que cumplen funciones reguladoras, estructurales y hormonales[122].

Es sabido que la Leu disminuye el desgaste muscular y aumenta la síntesis de proteína en diferentes condiciones. Hay estudios que demuestran que el uso de Leu como aminoácido dietético tiene la capacidad de estimular la síntesis de proteína muscular y frenar la degradación de la misma[123]. Como suplemento dietético ha sido utilizada con este fin en ratas[124] y humanos de edad avanzada[125]. Sin embargo, hay resultados contradictorios cuando se comparan con otros estudios, donde parece ser que la suplementación con Leu a largo plazo no aumenta la masa muscular o fuerza en los hombres sanos de edad avanzada[126]. Parece que hay otros factores tales como el estilo de vida, edad, sexo, dieta, ejercicio, etc., que deberían tenerse en cuenta cuando se analiza los efectos de la Leu.

Por otro lado, se ha demostrado que la infusión de Leu en el cerebro de rata hace disminuir la ingesta de alimentos y por tanto el peso corporal a través de la activación de la vía de mTOR[127]. Hay que añadir que el exceso de Leu en el organismo puede ser tóxico, provocando delirio y daño neurológico; y, aunque esto no está demostrado, puede ser una causa de la pelagra, donde se presenta diarrea, dermatitis, demencia y muerte[128,129].

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[122] *Rosenthal J., Angel A., et al. Metabolic fate of leucine: A significant sterol precursor in adipose tissue and muscle. Am. J. Phys. 226 (2008) 411-418.*

[123] *Etzel M.R. Manufacture and use of dairy protein fractions. J. Nutr. 134 (2004) 996S–1002S.*

[124] *Combaret L., Dardevet D., et al. A leucine-supplemented diet restores the defective postprandial inhibition of proteasome-dependent proteolysis in aged rat skeletal muscle. J. Phys. 569 (2008) 489-499.*

[125] *Rieu I., Balage M., et. al. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. J. Phys. 575 (2006) 305-315.*

[126] *Verhoeven S., Vanschoonbeek K., et al. Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. Am. J. Clin. Nutr. 89 (2009) 1468-1475.*

[127] *Cota D., Proulx K., et al. Hypothalamic mTOR signaling regulates food intake. Science 312 (2006) 927–930.*

[128] *Hegyi J., Schwartz R.A., et al. Pellagra: Dermatitis, dementia, and diarrhea. Int. J. Dermatol. 43 (2004) 1–5.*

La investigación disponible sugiere que el HMB es uno de los metabolito de la Leu responsable, al menos en parte, de estas actividades. De hecho, uno de los efectos beneficiosos más relevantes del HMB es su propiedad de conservación muscular[130]. La ruta metabólica de la Leu donde están involucrados metabolitos como el HMB se conoce desde años atrás (Figura 19).

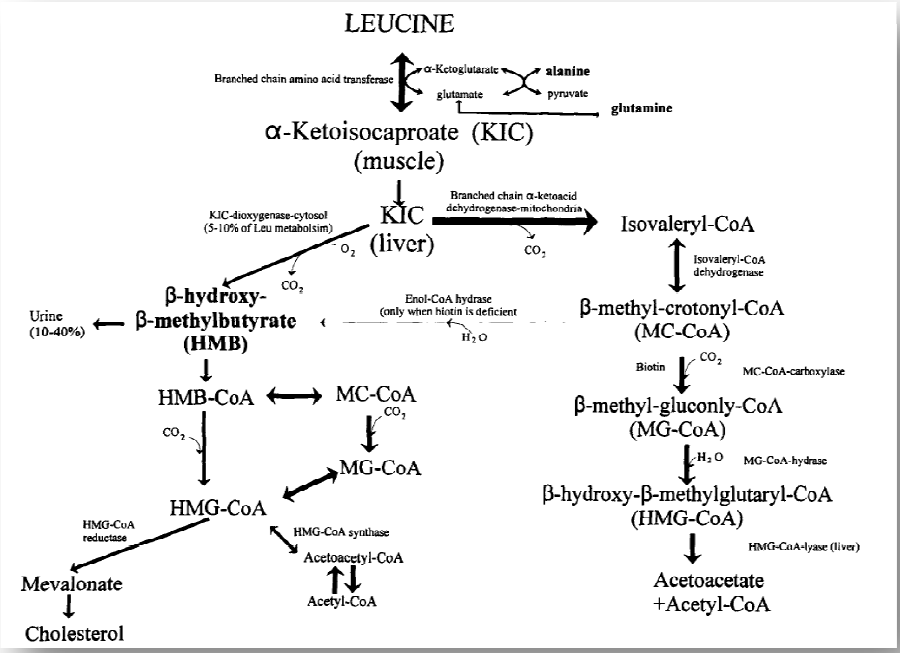

Figura 19. *Ruta metabólica de Leucina presentada por Nissen[131]. Descripción general de la leucina, del α-cetoisocaproato (KIC) y del HMB, en el metabolismo de mamíferos. Las enzimas y los principales co-factores se enumeran con cada reacción. El metabolismo de HMB se basa en datos isotópicos que sugieren que el el compuesto se convierte en β-hidroximetilglutaril-coenzimaA (HMG-CoA), en el citosol y en última instancia a colesterol*

[129] *Bapurao S., Krishnaswamy K. Vitamin B6 nutritional status of pellagrins and their leucine tolerance. Am. J. Clin. Nutr. 31 (1978) 819–824.*

[130] *Pinheiro C.H., Gerlinger-Romero F., et al. Metabolic and functional effects of beta-hydroxy-beta-methylbutyrate (HMB) supplementation in skeletal muscle. Eur. J. Appl. Physiol. 112 (2012) 2531-2537.*

[131] *Nissen S.L., Abumrad N.N. Nutritional role of the leucine metabolite β-hydroxy-β-methylbutyrate (HMB). Nutr. Biochem. 8 (1997) 300-331.*

El primer estudio que informó sobre los efectos de HMB[132] se publicó en 1996. Este estudio evaluó el efecto de diferentes concentraciones de HMB en la dieta para sujetos que entrenaban ejercicios de resistencia. Se demostró los efectos del HMB sobre la prevención de la proteólisis inducida por el ejercicio y/o daño muscular, contribuyendo a la gananacia en la función muscular.

Hay diversos estudios sobre los efectos del HMB en la fuerza y masa muscular[116,133-136]. Pero al igual que en la Leu, también existen resultados contradictorios que afirman que dichos efectos no son reales[137,138]. Es claro que la metodología, la dosis, el tipo y nivel de entrenamiento, así como el estado metabólico anterior puede justificar las diferencias encontradas en los estudios.

Como ya se ha mencionado con anterioridad, la importancia de estas dos biomoléculas radica en el papel especial que tienen en la nutrición. El HMB se considera como un agente anti-catabólico asociado con la síntesis de proteínas y la atenuación de la degradación de las mismas[115,139]; es un contribuyente a la síntesis de colesterol[131]; y agente inmunomodulador. Además, se han publicado cambios positivos en el metabolismo oxidativo, en la biogénesis mitocondrial y en la oxidación de ácidos grasos[120,140,141]. Por último, es de destacar que en los últimos años se está estudiando la función cognitiva del HMB.

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[132] *Nissen S., Sharp R., et al. Effect of leucine metabolite beta-hydroxy-beta-methylbutyrate on muscle metabolism during resistance-exercise training. J. Appl. Physiol. 81 (1996) 2095-2104.*

[133] *Wilson G.J., Wilson J.M., et al. Effects of beta-hydroxy-beta-methylbutyrate (HMB) on exercise performance and body composition across varying levels of age, sex, and training experience: A review. Nutr. Metab. 5 (2008) 1.*

[134] *Alon T., Bagchi D., et al. Supplementing with beta-hydroxy-beta-methylbutyrate (HMB) to build and maintain muscle mass: a review. Res. Commun. Mol. Pathol. Pharmacol. 111 (2002) 139-151.*

[135] *Slater G.J., Jenkins D. Beta-hydroxy-beta-methylbutyrate (HMB) supplementation and the promotion of muscle growth and strength. Sports Med. 30 (2000) 105-116.*

[136] *Palisin T., Stacy J.J. Beta-hydroxy-beta-Methylbutyrate and its use in athletics. Curr. Sports Med. Rep. 4 (2005) 220-223.*

[137] *Nunan D., Howatson G., et al. Exercise-induced muscle damage is not attenuated by beta-hydroxy-beta-methylbutyrate and alpha-ketoisocaproic acid supplementation. J. Strength Cond. Res. 24 (2010) 531-537.*

[138] *Ransone J., Neighbors K., et al. The effect of beta-hydroxy beta-methylbutyrate on muscular strength and body composition in collegiate football players. J. Strength Cond. Res. 17 (2003) 34-39.*

[139] *Lecker S.H., Solomon V., et al. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J. Nutr. 129 (1999) 227S-237S.*

[140] *Stancliffe R., Zemel M.B. Role of ß-hydroxy-ß-methylbutyrate (HMB) in leucine stimulation of muscle mitochondrial biogenesis. FASEB J. 26 (2012) 251.6.*

Por todas esas razones es de entender que Abbott esté interesada en este metabolito, teniendo diversas patentes de invención sobre distintas funcionalidades del mismo. Desde el punto de vista cognitivo se describen métodos para mejorar la función neural en un individuo, como un adulto mayor[142]. Los métodos incluyen composiciones nutricionales con HMB. Estas composiciones nutricionales proveen beneficios para las personas que tienen o pueden estar en riesgo de tener deterioro cognitivo, daño cognitivo y disfunción neuronal, por lo general como resultado de enfermedades cognitivas asociadas con enfermedades neurodegenerativas. De ahí el interés por estudiar el HMB como metabolito de la Leu en diversas situaciones y etapas de la vida haciendo uso de diseños experimentales específicos en cada caso.

1.4 Marcadores de oxidación

Figura 20. *Fotografía que refleja el envejecimiento con el tiempo*

Un sistema, ya sea biológico o un producto nutricional, envejece a través de procesos naturales o artificiales mediante los radicales libres que provienen del oxígeno y del nitrógeno, llamados especies reactivas de oxígeno (EROs) y de nitrógeno (ERNs)[143]. Es de elevado interés estudiar este tipo de procesos de envejecimiento, ya que la calidad de los productos de consumo puede verse afectada, generando compuestos indeseados que podrían influir en las funciones esenciales de un organismo vivo, incluidas las cognitivas[144-146].

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[141] *Zanchi N.E., Gerlinger-Romero F., et al. HMB supplementation: clinical and athletic performance-related effects and mechanisms of action. Amino Acids 40 (2011) 1015-1025.*

[142] *Rueda R., Ramirez M., et al. Methods for improving brain development and cognitive function using beta-hydroxy-beta-methylbutyrate. WO 2012112419 A1*

[143] *Valko M., Leibfritz D., et al. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39 (2007) 44-84.*

[144] *Bartzokis G. Age-related myelin breakdown: a developmental model of cognitive decline and Alzheimer's disease. Neurobiol Aging. 25 (2004) 5-28.*

[145] *Dröge W., Schipper H.M. Oxidative stress and aberrant signaling in aging and cognitive decline. Aging Cell. 6 (2007) 361–370.*

[146] *Mulero J., Zafrilla P., et al. Oxidative stress, frailty and cognitive decline. The Journal of Nutrition, Health & Aging. 15 (2011) 756-760.*

Esta fase del trabajo se ha centrado en productos nutricionales dirigidos a niños. Las fórmulas infantiles (FI) se consideran la mejor alternativa a la leche materna durante la lactancia cuando ésta no es posible o resulta insuficiente. En el recién nacido y durante el primer año de vida, los órganos encargados de la digestión de los alimentos y absorción de los nutrientes se encuentran en un proceso de maduración, por lo que para mantener un estado nutricional adecuado y un ritmo de crecimiento normal, la dieta debe adecuarse a la capacidad digestivo-metabólica y a las necesidades fisiológicas cambiantes a lo largo de la lactancia[147].

En base al Código Internacional de la Organización Mundial de la Salud (OMS) para la Comercialización de Sucedáneos de la Leche Materna, y las directivas publicadas por la Comunidad Europea (CE) relativas a los productos utilizados para la alimentación de los lactantes[148], se denominan preparados para lactantes a los productos alimenticios destinados a la alimentación especial de los lactantes durante los primeros 4 a 6 meses de vida, que satisfacen por sí mismos la necesidades nutritivas de este grupo de personas; y preparados de continuación, a los productos alimenticios destinados a este grupo de población a partir de los 6 meses de vida. A parte, la composición de las fórmulas comercializadas en España debe ajustarse a lo referido por la Reglamentación Técnico-Sanitaria específica relativa a los preparados para lactantes y preparados de continuación[149].

La alimentación durante las primeras etapas de la vida es fundamental para asegurar un buen desarrollo y crecimiento del lactante, además de la influencia que ejerce en su futuro estado de salud. Un óptimo estado de nutrición es vital debido a los efectos que tiene sobre el desarrollo físico, mental, emocional y psicomotor. Durante este periodo, se experimentan una

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[147] *Maldonado Lozano J., Gil Campos M. (2005). Nutrición del lactante. En: Tratado de Nutrición. Gil Hernández, A. (2ª Ed.) Acción Médica, España, Tomo 3.*

[148] *Directiva 2006/141/CE de la Comisión de 22 de diciembre de 2006 relativa a los preparados para lactantes y preparados de continuación y por la que se modifica la Directiva 1999/21/CE. Diario oficial de la Unión Europea. L 401, 1-39.*

[149] *Real Decreto 165/2014, de 14 de marzo, por el que se modifica el Real Decreto 867/2008, de 23 de mayo, por el que se aprueba la reglamentación técnico-sanitaria específica de los preparados para lactantes y de los preparados de continuación. BOE núm. 64. 23266-23269.*

serie de cambios antropométricos y en la composición de los tejidos corporales a causa de una disminución del contenido de agua y un aumento de la grasa y de las proteínas, además de algunos iones intracelulares, como el potasio, y extracelulares, como el calcio, que se deposita mayoritariamente en el tejido óseo.

Por todo ello, los alimentos infantiles deben proporcionar los nutrientes en cantidad suficiente para permitir un desarrollo y crecimiento óptimos, y prevenir enfermedades[150]. Diversos organismos internacionales se han encargado de realizar las recomendaciones que deben cumplirse en la elaboración de dichos productos. El Comité de Nutrición de la Academia Americana de Pediatría (AAPCON)[151] y, en nuestro medio, el Comité de Nutrición de la Sociedad Europea de Gastroentrología, Hepatología y Nutrición Pediátrica (ESPGHAN)[152] han dictado recomendaciones de carácter orientador. Todas ellas hacen referencia a consideraciones previas realizadas por la Comisión del Codex Alimentarius, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), la OMS y el Fondo de las Naciones Unidas para la Infancia (UNICEF)[153,154].

Atendiendo a éstas recomendaciones, el contenido en grasas, proteínas y carbohidratos es fundamental para satisfacer las necesidades energéticas. Además ciertos ingredientes como vitaminas liposolubles e hidrosolubles deben ser suplementados ya que ejercen funciones de vital importancia en el organismo. Ciertos nucleótidos se consideran nutrientes semiesenciales por lo que resulta imprescindible su aporte externo, al igual que algunos oligoelementos importantes, como el hierro, el cinc y el selenio.

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[150] *Guarino A., Guandalini S. The composition of infant formula: A worldwide approach. J. Pedatr. Gastr. Nutr. 41 (2005) 578-579.*

[151] *American Academy of Pediatrics. Committee on Nutrition. Commentary on breast-feeding and infant formulas, including proposed standards for formulas. Pediatrics 57 (1976) 278-282.*

[152] *Koletzko B., Baker S., et al. Global standard for the composition of infant formula: recommendations of an ESPGHAN coordinated international expert group. J. Pedatr. Gastr. Nutr. 41 (2005) 584-599.*

[153] *Codex Alimentarius Commission. Codex standards for foods for special dietary uses including foods for infants and children and related code of hygienic practice. Roma: Codex Alimentarius, FAO/WHO, 1988; 9 (Suppl 3).*

[154] *WHO/UNICEF. International code of marketing of breast milk substitutes. Geneva: WHO, 1981.*

En base a esto, la formulación, manipulación y almacenamiento de los alimentos infantiles son importantes para mantener la calidad nutricional y las propiedades fisicoquímicas de los mismos. Durante el almacenamiento, algunas reacciones e interacciones que se producen modifican las propiedades fisicoquímicas y nutricionales, tales como la cristalización de la lactosa, la oxidación lipídica[155], la Reacción de Maillard (RM) en la que se produce una pérdida de la biodisponibilidad de la lisina y una disminución de la digestibilidad proteica[156], así como las interacciones entre los micronutrientes y otros componentes[157].

1.4.1 Oxidación lipídica

La oxidación lipídica constituye uno de los principales problemas que afectan a la calidad de los alimentos procesados[158], por lo que la estabilidad de la fracción lipídica es muy importante desde el punto de vista nutricional, organoléptico y toxicológico[159].

Por tanto, el proceso de oxidación en las FI tiene especial atención, ya que al constituir la única fuente de nutrientes para la mayoría de los lactantes durante los primeros meses de vida, están expuestos a los efectos agudos y crónicos de los productos de oxidación lipídica, lo que a su vez influye en la digestibilidad de las proteínas y de los ácidos grasos esenciales, así como, la estabilidad de las vitaminas. Los productos de la oxidación lipídica inducen cambios patológicos en la mucosa del tracto gastrointestinal, inhibiendo la actividad de un gran número de enzimas y perturbando el metabolismo lipídico en el organismo[160].

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[155] *St. Angelo A.J., Vercellotti J., et al. Lipid oxidation in foods. Crit. Rev. Food Sci. 36 (1996) 175-224.*

[156] *Gonzales A.S.P., Naranjo G.B., et al. Available lysine, protein digestibility and lactulose in commercial infant formulas. Int. Dairy J. 13 (2003) 95-99.*

[157] *Nasirpour A., Scher J., et al. Baby foods: formulations and interactions (a review). Crit. Rev. Food Sci. 46 (2006) 665-681.*

[158] *Eriksson C.E. Lipid oxidation catalysts and inhibitors in raw materials and processed foods. Food Chem. 9 (1982) 3-19.*

[159] *Frankel E.N. Recent advances in lipid oxidation. J. Sci. Food Agr. 54 (1991) 495-511.*

[160] *Ziemlanski S., Budzynska-Topolowska J. Nutritional estimation of oxidized fats. Przemysł Spożywczy, 45 (1991) 98–100.*

Los lípidos de los alimentos están constituidos en su mayor parte por triglicéridos (TG) de ácidos grasos (AG), en su mayoría de cadena larga con número par de átomos de carbono, y una pequeña proporción de mono y diglicéridos, AG libres, esteroles y compuestos más complejos como fosfolípidos y glicolípidos. Son componentes alimenticios muy susceptibles a los procesos de oxidación, lo que conduce a la formación de otros muchos compuestos[161]. Hoy en día, la legislación para FI no especifica ninguna restricción en cuanto a la presencia ni el nivel de cualquier sustancia que pueda ser producto de la oxidación lipídica, aunque en la leche en polvo, los defectos de sabor se rigen en gran medida por la oxidación de las grasas debido a que algunos de sus efectos incluyen el desarrollo de sabores y olores desagradables, cambios en la textura, y una pérdida del valor nutritivo[162].

La degradación oxidativa de ácidos grasos insaturados (AGI), aquellos que poseen uno o más dobles enlaces en su cadena hidrocarbonada, contribuye significativamente a la reducción de la vida útil de muchos productos[163]. Durante la peroxidación de AGI, se genera una mezcla compleja de productos secundarios de oxidación lipídica (alcanos, alquenos, aldehídos, cetonas, etc.) que limitan su estabilidad durante el almacenamiento[164]. Además, los productos de peroxidación lipídica, tanto *in vivo* como en la dieta, parecen estar involucrados en el desarrollo de aterosclerosis, cáncer y procesos de envejecimiento[165]. Los ácidos grasos poliinsaturados (AGPI, también conocidos como PUFAs) presentan un número de insaturaciones comprendido entre 1 y 6 que permite clasificar a los AG en tres series: n-3, n-6 y n-9. Los AGPI de 18 átomos de carbono de las series n-3 y n-6 de conformación cis no pueden ser sintetizados por el organismo, por lo que sólo pueden ser ingeridos con la

[161] *Gardner H.W. Oxygen radical chemistry of polyunsaturated fatty acids. Free Radical Bio. Med. 7 (1989) 65-86.*

[162] *Allen J.C. Industrial aspects of lipid oxidation. Recent Advances in Chemistry and Technology of Fats and Oils. Eds. Elsevier, London, U.K (1987) 31-39.*

[163] *Eriksson C.E. Oxidation of lipids in food systems. Autoxidation of Unsaturated Lipids. Ed. Academic Press, London, U.K. (1987) 207-231.*

[164] *Van Zoeren-Grobben D., et al. Lipid peroxidation in human milk and infant formula: effect of storage, tube feeding and exposure to phototherapy. Acta Paediatrica. 82 (1993) 645-649.*

[165] *Rice-Evans C., Burdon R. Free radical-lipid interactions and their pathological consequences. Progress in Lipid Research. 32 (1993) 71-110.*

dieta. Dichos AG se consideran esenciales (AGE) y son los ácidos linoleico (18:3n-6) y α-linolénico (18:3n-3).

Otros compuestos insaturados, los esteroles, son alcoholes esteroídicos susceptibles a la oxidación. Se oxidan por un mecanismo de radicales libres de forma similar a los AGI, obteniéndose productos de oxidación del colesterol (COP) y de los fitosteroles (POP), que en conjunto se denominan productos de oxidación de los esteroles (SOP)[166].

Se conocen tres mecanismos diferentes de oxidación lipídica. Los AGI, en especial los AGPI, con posiciones alílicas muy reactivas, son capaces de reaccionar con relativa facilidad con el oxígeno molecular favoreciendo la oxidación lipídica. Aunque la oxidación enzimática y la fotoxidación tienen un papel destacado, el proceso más común e importante por el cual los AG reaccionan con el oxígeno formando radicales libres es la autoxidación lipídica[167,168]. La autooxidación es una reacción espontánea entre el oxígeno molecular y los AGPI, que conducen al deterioro oxidativo. Otro mecanismo de oxidación se produce mediante fotosensibilizadores y luz UV en presencia de oxígeno. La fotooxidación es una ruta alternativa que conduce a la formación de hidroperóxidos en lugar del mecanismo de radicales libres. El tercer mecanismo de oxidación se basa en la actividad lipooxigenasa y ciclooxigenasa, lo que se conoce por oxidación enzimática. La lipooxigenasa cataliza la inserción de una molécula de oxígeno en un AG según una reacción esteroespecífica. Produce compuestos aromáticos volátiles similares a los producidos durante la autooxidación. Por el contrario, la cicloxigenasa es una lipooxigenasa que incorpora dos moléculas de oxígeno a un AG para formar hidroperóxidos.

Existen determinados factores que influyen sobre la oxidación lipídica: el contenido y estructura de los AGI, ya que son el sustrato susceptible de sufrirla; la actividad de agua (relación entre la presión de vapor del aire alrededor de un

[166] *Sieber R. Oxidised cholesterol in milk and dairy products. Int. Dairy J. 15 (2005) 191–206.*
[167] *Frankel E.N. Lipid oxidation. Prog. Lipid Res. 19 (1980) 1-22.*
[168] *Porter N.A., Caldwell S.E., et al. Mechanisms of free radical oxidation of unsaturated lipids. Lipids. 30 (1995) 277-290.*

alimento y la presión de vapor del agua pura); el contenido de pro-oxidantes como riboflavina, metales de transición (cobalto, cobre, hierro, manganeso y níquel) y enzimas como lipooxigenasas y ascórbico-oxidasa; la presencia de antioxidantes naturales como los tocoferoles, que disminuye el grado de oxidación; la presión parcial de oxígeno o la radiación UV.

1.4.2 La reacción de Maillard (RM)

De las numerosas reacciones que pueden tener lugar durante el procesado de los alimentos, la RM es una de las que más se produce. Esta reacción se desarrolla también durante el almacenamiento, y los compuestos que origina se han utilizado como indicadores químicos de calidad. Descrita por primera vez por Louis-Camille Maillard en 1912, es la principal reacción de pardeamiento no enzimático y comienza con la condensación del grupo amino libre de un aminoácido, péptido o proteína con un grupo carbonilo, que procede de un azúcar reductor, aunque también puede proceder de la degradación del ácido ascórbico o de productos formados durante la oxidación lipídica. El primer esquema de todas las etapas que se producen en la RM fue propuesto por Hodge[169] en 1953. Ver Figura 21.

[169] *Hodge J. Chemistry of browning reactions in model systems. J. Agr. Food Chem. 1 (1953) 928-943.*

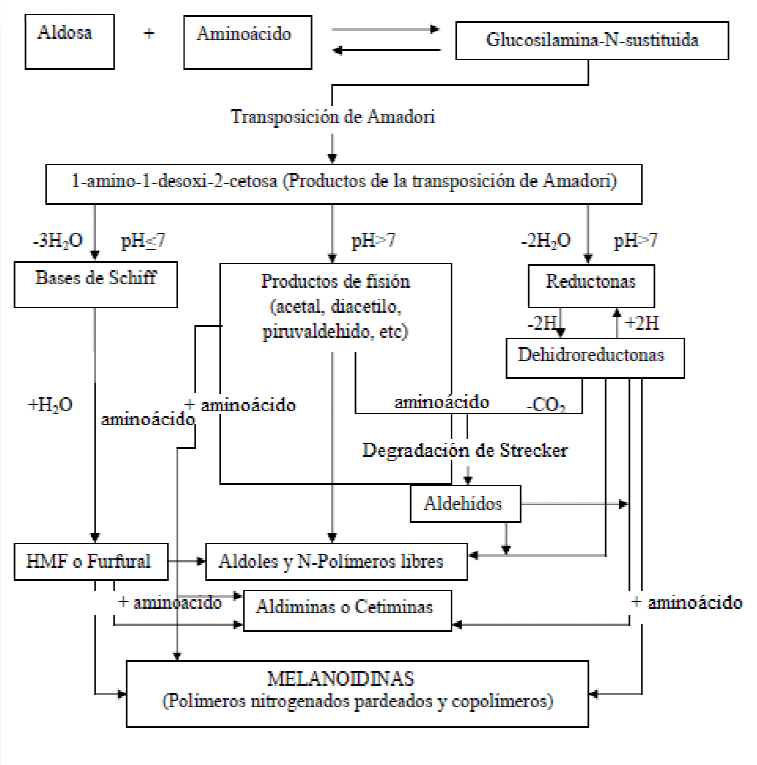

Figura 21. *Esquema de la RM (Hodge, 1953)*[170]

En productos de base láctea, los grupos amino proceden, principalmente, de los residuos de lisina de las proteínas lácteas. Parece ser que la actividad de los residuos de lisina de la caseína es mayor que la de las seroproteínas, siendo la k-caseína la más reactiva. El grupo imidazol de la histidina, el grupo indol del triptófano y el grupo guanidino de los residuos de arginina también pueden reaccionar, pero en menor medida[171]. Los azúcares que fundamentalmente reaccionan con los aminoácidos son los monosacáridos, glucosa y fructosa; y los disacáridos, maltosa y lactosa.

[170] *Martins S.I., Jongen W.M., et al. A review of Maillard reaction in food and implications to kinetic modelling. Trends Fod Sci. Tech. 11 (2000) 364-373.*

[171] *Oliver C.M., Melton L.D., et al. Creating proteins with novel funcionality via the Maillard reaction: A review. Crit. Rev. Food Sci. 46 (2006) 337-350.*

La reacción transcurre en tres etapas. En la etapa inicial, se produce una reacción de condensación irreversible entre el grupo carbonilo de un azúcar reductor y el grupo amino libre de un aminoácido, péptido o proteína. El compuesto formado pierde una molécula de agua y da lugar a una base de Schiff, que se transforma por ciclación en la correspondiente glicosilamina-N-sustituida. Al reordenamiento de una aldosilamina-N-sustituida para formar una 1-amino-1-deoxi-2-cetosa se le da el nombre de reordenamiento o transposición de Amadori, el más frecuente en los alimentos mientras que al reordenamiento de una cetosilamina-N-sustituida para formar una 2-amino-2-deoxialdosa se le conoce como reordenamiento de Heyns. En esta etapa se reduce el valor nutritivo ya que en los compuestos de Amadori la lisina no está disponible.

En las etapas avanzadas, se produce la degradación de los compuestos de Amadori que puede ocurrir por dos vías principales. A pH inferior a 7, la enolización que afecta a los carbonos C1 y C2 favorece la formación de 1,2-eneaminol (vía 1,2-E); mientras que a pH alcalino, la enolización que afecta a los carbonos C2 y C3 da lugar a la formación de 2,3-enediol (vía 2,3-E). Además, el compuesto 1-amino-1-deoxi-2-cetosa, que es una amina secundaria, puede reaccionar con otra molécula de azúcar en una segunda RM para formar una dioxicetosa-amina.

En la vía 1,2-E, el compuesto 1,2-eneaminol pierde el grupo hidroxi del C3 con la consiguiente desaminación del C1. Se forma 3-deoxiosona que por deshidratación se convierte en un compuesto dicarbonilo insaturado, a partir del cual, mediante ciclaciones, nuevas deshidrataciones y translocaciones de los dobles enlaces se originan diferentes compuestos como 2-furfural, 5-hidroximetilfurfural (HMF), furfurialcohol, pirralinas, lisilpirralinas, imidazolonas y pentosidinas. En la vía 2,3-E, el compuesto 2,3-enediol se desamida y forma un producto intermedio, 1-metil-2,3-dicarbonilo-(1-deoxiosona), cuya rotura da lugar a una variedad de productos de fisión que incluyen reductonas, otros dicarbonilos y aldehídos.

En productos lácteos, la degradación del producto de Amadori ocurre, principalmente, por la ruta 2,3-E y se originan compuestos como carboximetil-

lisina (CML), ácido eritrónico, 3-furanona, β-piranona, ciclopentenonas, maltoles, galactosil-isomaltoles; que a su vez pueden seguir reaccionando[172].

En presencia de grupos amino, las β-piranonas, ciclopentenonas y galactosilisomaltoles condensan fácilmente con productos que contienen nitrógeno como acetilpirroles, betaínas, maltosinas y furanona-amina; algunos de los cuales pueden reaccionar con proteínas para formar compuestos por uniones de entrecruzamiento. Además, se han identificado compuestos como histidinoalanina (HAL), imidazol-lisina, pentodil-lisina y pirrolopiridinas.

Los compuestos dicarbonilo producidos en las vías anteriores, mediante la degradación de Strecker, pueden reaccionar con aminoácidos para dar aldehídos y dióxido de carbono (CO_2). Se forman nuevos compuestos carbonilo que pueden reaccionar entre sí, con aldehídos o con grupos amino y producir compuestos volátiles aromáticos. La mayoría de compuestos dicarbonilo reactivos en la degradación de Strecker se produce en la vía 2,3-E por lo que valores de pH más altos favorecen su degradación, así como la producción de CO_2 y compuestos volátiles.

Las etapas finales de la RM son complejas y poco conocidas, conducen a la conversión de los precursores de bajo peso molecular como furfurales, productos de fisión y reductonas en melanoidinas; pigmentos pardos de alto peso molecular que contienen nitrógeno, pero cuya estructura está poco definida. Se considera que las melanoidinas se originan por condensaciones de grupos aldol y por polimerizaciones entre grupos aldehído y amino.

Entre los factores que afectan al desarrollo de la RM destacan el estado físico-químico del producto, así como, la naturaleza y concentración de los compuestos que reaccionan ya que los compuestos de bajo peso molecular suelen ser más reactivos debido a un menor impedimento estérico; también influye la naturaleza del grupo amino; el pH y la presencia de agua y de ciertas sales como las de cobre o hierro o los fosfatos.

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[172] *Van Boekel M.A.J.S. Effect of heating on Maillard reactions in milk. Food Chem. 62 (1988) 403-414.*

2. TÉCNICAS EMPLEADAS PARA LA DETERMINACIÓN DE BIOMOLÉCULAS

Se entiende por biomolécula a aquella que forma parte de los seres vivos. En la tabla 1 se muestra la clasificación de dichas moléculas (Macarulla y colaboradores)[173] .

Tabla 1. *Clasificación según Macarulla y colaboradores.*

Orgánicas	**Inorgánicas**
Carbohidratos o Azúcares (simples y complejos): Glucosa, Lactosa, Almidón, Celulosa, etc.	**Gases**: O_2, N_2, CO_2, etc.
Lípidos: Ácidos grasos, Fosfolípidos, Cerebrósidos, etc.	**Aniones**: (HCO_3^-), Cl^-, (SO_4^-), etc.
Proteínas: Insulina, Hemoglobina, Fibrinógeno, etc.	**Cationes**: Na^+, K^+, (NH_4^+), etc.
Ácidos Nucleicos: DNA, RNA, y sus componentes	
Metabolitos intermedios: Ácido acético, Etanol, Urea, etc.	

La Química Analítica dispone de un amplio abanico de técnicas que se podrán seleccionar para el análisis de biomoléculas en función de las distintas propiedades físico-químicas que poseen dichas moléculas. En el caso de muestras complejas, como son las biológicas, se han aplicado una gran diversidad de técnicas, aunque las más habituales en la actualidad son las cromatográficas acopladas a sistemas de detección de índole muy diversa, principalmente la detección por espectrofotometria UV-Vis y fluorescencia, detección electroquímica y detección por espectrometría de masas.

[173] *Macarulla J., Marino A., et al. Bioquímica Cuantitativa. Barcelona, España: Editorial Reverté S.A. (1988) 20p.*

2.1 Evolución Histórica

En los últimos años, se ha producido una espectacular evolución en el campo de la Química Analítica donde destacan eventos o cambios notables como:

- ✓ El progresivo desplazamiento de métodos químicos tradicionales (volumetría y gravimetría) por técnicas instrumentales.

- ✓ El desarrollo casi "explosivo" de las técnicas de separación como medios de análisis, incluyendo la cromatografía de gases y de líquidos, y la electroforesis, que han resultado ser los más versátiles.

- ✓ La disminución del uso de las espectroscopias ópticas, que aunque siguen siendo parte integral de los laboratorios de análisis e investigación, hoy en día no aportan una excesiva innovación.

- ✓ De un modo análogo ocurre con las técnicas electroquímicas (polarografía, potenciometría, amperometría, etc.).

- ✓ El alcance de la madurez en la espectrometría de masas, la resonancia magnética nuclear, la absorción atómica y la espectroscopia basada en plasma, ya que nos aportan elevada certeza y especificidad en la identificación de compuestos o elementos y su determinación a niveles muy bajos o en muestras muy complejas.

- ✓ La introducción de microprocesadores, ordenadores y softwars específicos para el control de instrumentos y procesamiento de datos.

- ✓ Los avances en automatización que permiten diversas posibilidades uniendo distintas técnicas y metodologías.

- ✓ Las técnicas híbridas o hifenadas (GC-MS, LC-MS, etc.) incluso en laboratorios de rutina, por las ventajas innumerables que tienen, potenciando sus ventajas individuales de manera sinérgica.

- ✓ La aparición de las técnicas ómicas dirigidas al estudio de nuevos biomarcadores como proteómica, lipidómica o metabolómica. Los avances que en los últimos años se están produciendo en estas tecnologías está suponiendo el descubrimiento paralelo de nuevos biomarcadores de utilidad clínica en el diagnóstico, pronóstico y tratamiento de numerosas enfermedades.

2.2. Análisis de Biomoléculas

La metodología utilizada actualmente para la determinación de biomoléculas es muy variada e incluye la utilización de diferentes técnicas analíticas. Cada una de ellas aporta una serie de ventajas e inconvenientes que deben ser valoradas detenidamente a la hora de seleccionar el sistema de medida a utilizar en cada caso. No obstante, la determinación de estos bioanalitos en muestras biológicas de origen animal, a las bajas concentraciones que suelen encontrarse, implica la necesidad de desarrollar métodos de análisis muy sensibles y selectivos. Las técnicas de análisis que se aplican hoy en día para la detección de biomoléculas pueden dividirse en dos grandes grupos: técnicas no cromatográficas y técnicas cromatográficas.

2.2.1 *Técnicas no cromatográficas*

Desde finales de la década de los 50 y comienzos de los 60, los investigadores han aprendido a caracterizar, aislar y manipular los componentes moleculares de las células y los organismos. Hay multitud de métodos y técnicas que han ido mejorando con el tiempo y que hoy día siguen siendo ampliamente utilizados. A continuación se enumeran los más habituales.

- ➢ Métodos para la determinación de **carbohidratos**: análisis elemental por técnicas de combustión; análisis proximal de Weende (1860); gravedad

específica; refractometría; polarimetría; métodos para reductores; detección cualitativa con espectrofotometría o prueba de Molish; detección cuantitativa con espectrofotometría o ensayo de Benedict (azúcares reductores); ensayo de Barfoed (monosacáridos reductores); ensayo de Saliwanoff (Ceto-azúcares como la Fructosa); ensayo de Bial (pentosas); Kits enzimáticos basados en el uso de la espectrofotometría para determinar fructooligosacáridos y galactooligosacáridos.

- Métodos para la determinación de **lípidos**: método gravimétrico de Rose-Gottlieb; método del butirómetro de Gerber; índice de color; densidad; punto de fusión; humedad; acidez; índice de saponificación; impurezas; índice de peróxidos; etc.

- Métodos para la determinación de **proteínas**: método del Biuret; método de Lowry; métodos de unión a colorantes; fotometría (Nanodrop®); inmunoturbidimetría e inmunonefelometría; turbidimetría; nefelometría; inmunodifusión; ELISA; PCR. Frecuentemente estas últimas técnicas requieren de protocolos que incluyen el aislamiento y purificación de las proteínas, para detectarlas y/o caracterizar su estructura y función. Incluso se han modernizado con sistemas computacionales donde se facilitan los métodos de trabajo.

- Métodos para la determinación de **ácidos nucleicos**: método enzimático o método de los dedeóxidos de Sanger; método de Maxam y Gilbert.

- Métodos para la determinación de **metabolitos intermedios** como urea, etanol, etc.: biosensores basados en la conductimetría o en reacciones redox. En los últimos tiempos se están utilizando como transductor fluorímetros, espectrofotómetros IR y UV, equipos RMN, etc. Biosensores típicos son los que miden etanol, ácido úrico, colesterol, glucosa o sacarosa.

- Métodos para la determinación de **gases**: pH, pCO_2 y pO_2, CO_2, el exceso de base y la saturación de O_2 de la hemoglobina; determinación de monóxido

de carbono (CO) por cooximetría; reacción redox para la determinación de CO con el método de Gettler y Freimuth, al igual que la microdifusión en cámaras de Conway.

- Métodos para la determinación de **iones**: absorción atómica; fotometría de llama, etc.

Esos métodos son sólo algunos ejemplos de muchos que son llevados a cabo en diversos laboratorios desde clínicos, toxicológicos, forenses hasta de investigación en general.

2.2.2 Técnicas cromatográficas

Las técnicas cromatográficas son de gran aplicación hoy en día en el campo del análisis de biomoléculas. Las mas empleadas son la de gases, la de líquidos y recientemente la de fluidos supercríticos.

A. Cromatografía de gases (CG)

Debido a su simplicidad, sensibilidad y efectividad en la separación de componentes, la CG es una de las herramientas más importantes en Química Analítica. La CG es una técnica útil para la separación y análisis de compuestos orgánicos, con puntos de ebullición de hasta 300 °C y que sean térmicamente estables o que puedan llegar a serlo tras una reacción de derivación. Se utiliza en muchos análisis de rutina en todos los sectores industriales o medicinales, siendo muy útil para la determinación de O_2 disuelto, N_2, CO_2 y CO en sangre; análisis de contaminantes del aire; etanol en sangre; aceites esenciales y productos alimenticios por ejemplo. En las figuras siguientes se muestra un esquema y un equipo de CG.

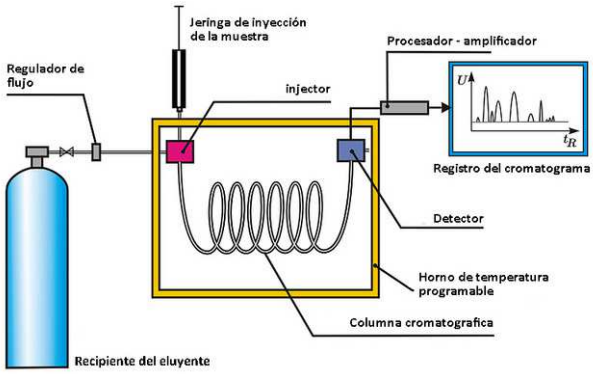


Figura 22. *Esquema general de un cromatógrafo de gases. Biotecnofil. Versión traducida al español por Marta Bretó, del trabajo original de Dubaj*

Figura 23. *Cromatógrafo de Gases, modelo 7890A GC System. Equipado con automuestreador de la Serie 7693, de Agilent*

Como sistemas de detección acoplados al cromatógrafo de gases para la detección de biomoléculas, se suelen emplear el detector de ionización de llama (FID), el detector de conductividad térmica (TCD) y el detector selectivo de masas (MSD).

Sin embargo, la CG es una técnica analítica que presenta grandes desventajas en cuanto al análisis de biomoléculas. Muchas de ellas son muy poco volátiles e inestables, lo que sumado a la complejidad habitual de las muestras objeto de estudio, convierte a los procedimientos de trabajo en laboriosos con diversas etapas de extracción o limpieza para eliminar interferentes, hidrólisis de naturaleza diversa o reacciones de derivatización

que no siempre tienen una alta eficiencia. Por este motivo, la técnica de elección hoy en día suele ser la cromatografía de líquidos.

B. Cromatografía de Líquidos

Es la técnica de separación cromatográfica de biomoléculas por excelencia y la seleccionada para los trabajos desarrollados en la presente Tesis Doctoral. Tiene una serie de ventajas que la hacen más versátil y funcional para llevar a cabo el objetivo de este trabajo. Son varias las modalidades de este tipo de cromatografía utilizadas en la actualidad. En la tabla 2 se muestra un resumen y las características básicas que las diferencian.

Tabla 2. *Principales diferencias entre las distintas modalidades de LC.*

Columna Cromatográfica	**UHPLC**	**HPLC**	**Micro-LC**	**LC-Capilar**	**Nano-LC**
Diámetro interno (mm)	2.1	3-4.5	0.4-0.8	0.18-0.32	0.075-0.1
Longitud (cm)	3-15	5-300	5-25	5-25	5-15
Tamaño partícula (µm)	< 2	3-40	3- 5	3-5	3- 5
Flujo fase móvil (mL min^{-1})	0.2-0.6	0.2-2.5	0.01-0.1	0.001-0.01	0.0001-0.001
Máxima presión (psi)	15000	5000	10000	5000	10000

La presente Tesis Doctoral ha sido desarrollada en su totalidad empleando la modalidad de UHPLC (cromatografía de líquidos de ultra-resolución), en concreto se ha empleado un equipo de UPLC® de la casa comercial de Waters (Figura 24).

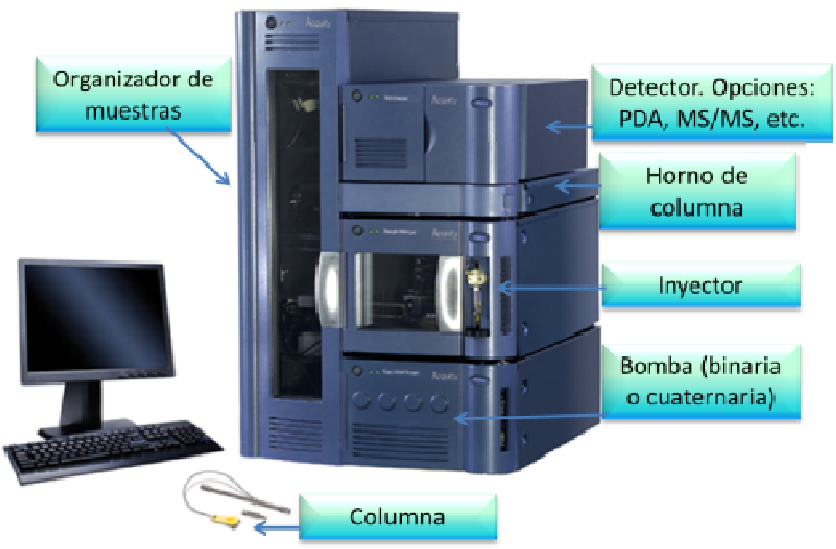


Figura 24. *UPLC® de la casa comercial Waters*

La principal ventaja del uso de esta técnica radica en la miniaturización del sistema cromatográfico incluyendo el tamaño de partícula, hecho que produce una mejora notable de la eficacia y resolución de la separación cromatográfica. De hecho se consigue aumentar la eficacia de la separación aumentando los platos teóricos desde los 15.000-25.000, que suele encontrarse en un HPLC convencional, hasta los 100.000 platos teóricos que se consigue en UHPLC[174]. Los picos cromatográficos suelen ser mucho más estrechos y aumentan su altura o intensidad de señal, con lo cual la sensibilidad del método desarrollado mejora de una forma notable.

La aplicación en el campo de las biomoléculas de esta técnica se extiende a la determinación cuantitativa y cualitativa de aminoácidos y proteínas, ácidos nucleicos, vitaminas, carbohidratos, plaguicidas en muestras biológicas, esteroides, compuestos organometálicos, etc.

Como sistemas de detección, los más usados son los detectores de UV-visible, de fluorescencia (FD), de índice de refracción, evaporativo de luz

[174] *Desmet, G., Cabooter, D., et al. Future of high pressure liquid chromatography: Do we need porosity or do we need pressure? J. Chrom. A. 1130 (2006) 158-166.*

dispersa (ELSD), de conductividad, electroquímico (ECD), y actualmente, por su elevada selectividad, entre otras importantes ventajas, el detector de espectrometría de masas (MS).

En los métodos analíticos puestos a punto para la determinación de biocompuestos a lo largo del desarrollo de la presente Tesis Doctoral, el detector empleado ha sido el de espectrometría de masas. En concreto, la espectrometría de masas en tándem (MS/MS) de tipo triple cuadrupolo (QqQ). También se ha hecho uso de la detección mediante espectrofotometría de UV-vis con fotodiodo array (PDA) y la espectrometría de masas de alta resolución de tiempo de vuelo (TOF). A continuación se presenta un breve resumen para entender en qué se basan las técnicas de detección seleccionadas que han permitido desarrollar los métodos analíticos de esta Tesis Doctoral.

- ***Detectores basados en la espectroscopía de Absorción UV-Vis***

Muchas moléculas pueden absorber radiación en esta zona del espectro electromagnético, por lo que este detector se puede considerar universal. Este comportamiento presenta la ventaja de poder resolver una gran cantidad de problemas analíticos, pero también es un inconveniente en aquellos casos en los que se requiere selectividad. Aunque la detección UV-Vis es muy robusta, en los análisis cualitativos y de identificación de moléculas, dado que no proporciona información estructural, no permite identificar de manera inequívoca compuestos aunque se dispongan de patrones comerciales, ya que habitualmente existen interferentes cuyas características espectrales son similares a las presentadas por el analito. Sin embargo, su sencillez, rapidez y relativo bajo coste hacen que su uso esté ampliamente extendido.

En esta Tesis Doctoral, el detector PDA, ha sido acoplado en línea con UHPLC y con el espectrómetro de masas para dar información complementaria en la metodología desarrollada en el capítulo dedicado al estudio de marcadores de oxidación en un sistema biológico.

- ***Detectores basados en la espectrometría de masas***

En los últimos años este sistema de detección ha ganado popularidad principalmente por la selectividad que presenta. Cuando se acopla a una técnica separativa, como HPLC o UHPLC, este detector proporciona una segunda dimensión de separación, ya que tras la separación de los analitos en la técnica cromatográfica, se produce una segunda separación de los iones en el espectrómetro de masas en función de su relación *m/z*. Existen distintos sistemas de ionización y analizadores de masas. En esta Tesis Doctoral, se han empleado los sistemas de detección QqQ y TOF con ionización en ambos casos mediante electrospray (ESI).

Sistema de ionización. Ionización por electrospray (ESI). Esta interfase es muy eficaz en compuestos polares, lábiles y/o con bajo peso molecular, como son las biomoléculas seleccionadas para su estudio en esta Tesis Doctoral.

El proceso de formación del electrospray se lleva a cabo a presión atmosférica. La muestra en estado líquido, procedente del UHPLC en nuestro caso, es conducida a través de un capilar de acero inoxidable de pequeño diámetro cuyo extremo se encuentra sometido a un potencial eléctrico elevado (3-4 kV). Con la ayuda de un gas nebulizador, generalmente N_2, que fluye a través de un tubo coaxial al capilar por donde es transportada la muestra, y gracias al campo eléctrico formado por la aplicación del potencial en el capilar principal, la muestra forma una serie de microgotas cargadas eléctricamente. El disolvente de las microgotas formadas se va evaporando en un proceso conocido como desolvatación (ver Figura 25). En el proceso de ionización se pueden formar iones con una o más cargas por lo que utilizando analizadores de masas que trabajan con un rango de valores de m/z limitado es posible detectar compuestos con pesos moleculares elevados. La ionización se puede llevar a cabo en modo positivo o negativo. En el modo positivo, se podrán formar iones múltiplemente protonados $[M+nH]^{n+}$, donde n es el número de protones cargados positivamente en la molécula. Del mismo modo, es posible también la formación de aductos con iones sodio, litio, potasio, amonio, etc. En

el modo negativo, se observa normalmente la desprotonación de las moléculas, pudiéndose formar también iones múltiplemente desprotonados $[M\text{-}nH]^{n-}$.

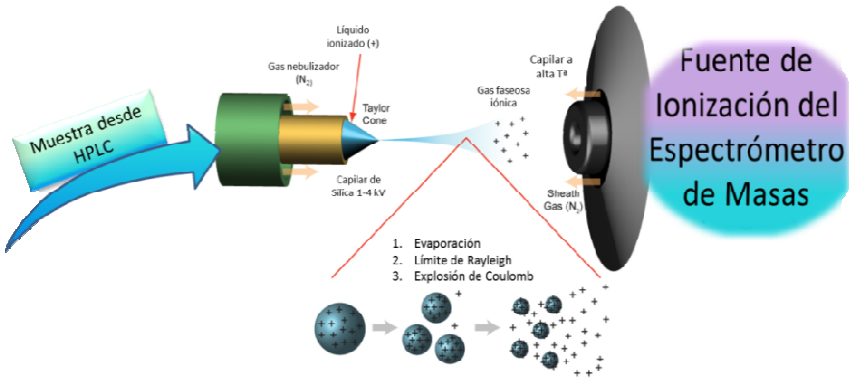


Figura 25. *Esquema básico del modo de ionización por electrospray positivo (ESI+) en un espectrómetro de masas*

En el acoplamiento al cromatógrafo de líquidos, el principal problema que debe solventarse es que el flujo de la fase móvil suele ser demasiado elevado y no puede introducirse en la mayoría de los casos directamente en el espectrómetro de masas. Generalmente los flujos de fase móvil en la separación cromatográfica oscilan entre 0.2-0.8 mL min^{-1}, pero en algunos casos pueden emplearse flujos que alcanzan los 3 mL min^{-1}. A pesar de que las interfases ESI comerciales admiten flujos comprendidos entre 1-1000 µL min^{-1}, el flujo recomendado a la entrada del espectrómetro de masas es menor (200-500 µL min^{-1}) dependiendo del analizador. La solución tradicional a este inconveniente ha sido el empleo de divisores de flujo entre el cromatógrafo de líquidos y el espectrómetro de masas, donde sólo parte del eluyente es introducido en el analizador de masas. En el caso de UHPLC no existe este inconveniente, ya que los flujos utilizados suelen ser adecuados para ser directamente introducidos en el analizador de masas. En la siguiente figura se muestra el diseño específico de una interfase típica de ESI del equipo utilizado en esta memoria de Tesis.

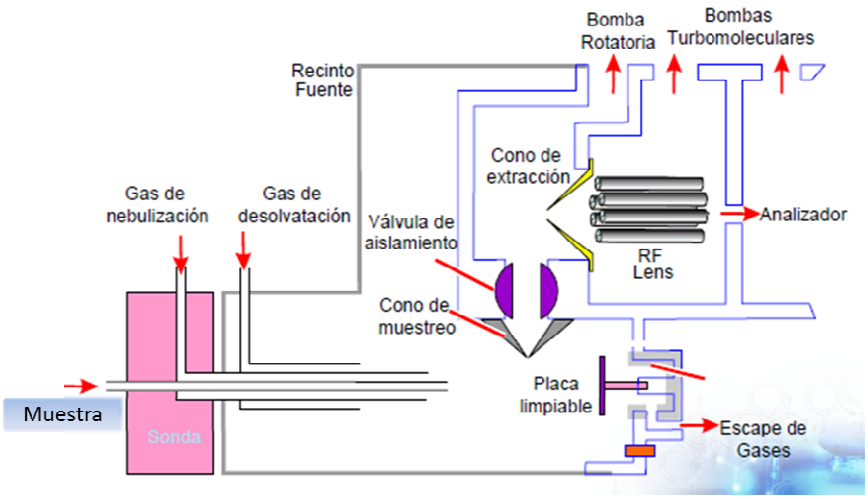


Figura 26. *Esquema de un sistema de ionización ESI de la casa comercial Waters*

Las características generales de ESI son:

- Los compuestos de bajo peso molecular forman generalmente iones monocargados por pérdida o ganancia de un protón.
- Biomoléculas de alto peso molecular y polímeros forman iones multicargados.
- La fragmentación es muy escasa o nula.
- Se pueden formar aductos o multímeros. Los aductos de disolvente y NH_4^+ (M+18), Na^+ (M+23) y K^+ (M+39) son muy comunes.

Analizadores de masas. Detector de triple cuadrupolo (TQD). Una vez que los iones procedentes de la técnica separativa (UHPLC en nuestro caso) se transforman en fase gaseosa en la interfase, son dirigidos hacia el interior del analizador de masas. Estos analizadores de masas permiten la separación, detección y cuantificación de los analitos de interés con un grado de sensibilidad y selectividad muy elevado, proporcionando información sobre su

masa molecular. En el desarrollo experimental de esta memoria, se han utilizado los analizadores de triple cuadrupolo y de tiempo de vuelo

El cuadrupolo consta de 4 cilindros de molibdeno paralelos. Los rodillos están equidistantes de un eje central. Una combinación de voltajes de dc (corriente continua) y rf (radiofrecuencia, una frecuencia de corriente alterna) se aplican a los rodillos. Rodillos opuestos tienen la misma polaridad (positiva o negativa), rodillos adyacentes tienen polaridad opuesta. La combinación de voltajes permite a los iones ser filtrados en función de relación m/z. A unos voltajes dados, solo un valor m/z tiene un trayectoria estable a lo largo de la longitud de los rodillos hasta el detector. Debido a la forma en que trabajan, los cuadrupolos son comúnmente denominados como filtros de masas.

En la Figura 27 se aprecia como los iones entran a través de un pequeño orificio en el centro del cuadrado definido por las varillas y luego siguen ciertas trayectorias basadas en los valores de m/z. En un momento dado, solo los iones de interés con m/z específicos y cuyos parámetros de voltaje han sido fijados tienen trayectorias estables y pueden pasar con éxito a través de las varillas de cuadrupolo al detector. Todos los otros iones con distinto valor del m/z a estudiar tienen trayectorias inestables y se filtran sin llegar al detector.

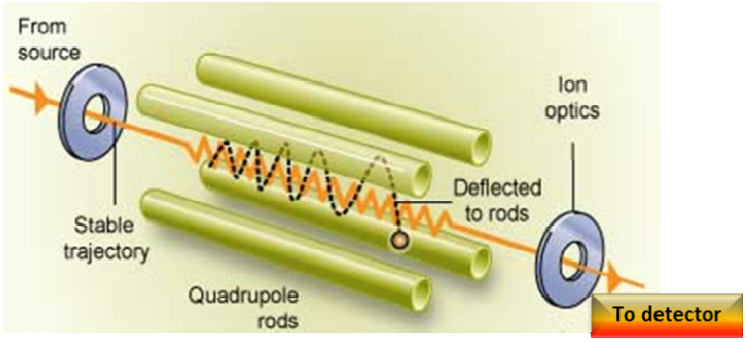


Figura 27. *Solo valores m/z específicamente fijados pueden pasar con éxito*

Es importante mencionar que dependiendo del fabricante y modelo, en lugar de cuadrupolos se pueden encontrar hexapolos u octopolos, aunque el término cuadrupolo es aceptado de forma generalizada. De hecho, el equipo

empleado en esta Tesis Doctoral incluye hexapolos. Adicionalmente algunos equipos incluyen un prefiltro situado antes del primer cuadrupolo. Se trata de un cuadrupolo muy corto fabricado del mismo material y diámetro que el filtro de masas. La ventaja es que la contaminación procedente de la fuente se deposita en este prefiltro en lugar de en el cuadrupolo.

El uso de un único cuadrupolo es útil para llevar a cabo análisis en los que se requiere un barrido completo de masas, por ejemplo, cuando no se sabe qué hay exactamente en la muestra. Sin embargo, debido a las limitaciones que presenta este sistema, actualmente dada la necesidad de confirmar fehacientemente la identidad de los analitos estudiados, el uso de los equipos de cuadrupolo simple ha disminuido notablemente, siendo sustituidos por espectrómetros de masas de triple cuadrupolo (QqQ); donde el Q1 se utiliza para el aislamiento de iones precursores, el Q2 funciona como una celda de colisión para el ion seleccionado y el Q3 analiza los iones producto del ion precursor fragmentado en el Q2. En este caso además de los modos de barrido de masas SCAN y SIM propios de un cuadrupolo simple, se añaden nuevas modalidades de gran interés analítico (Figura 28).

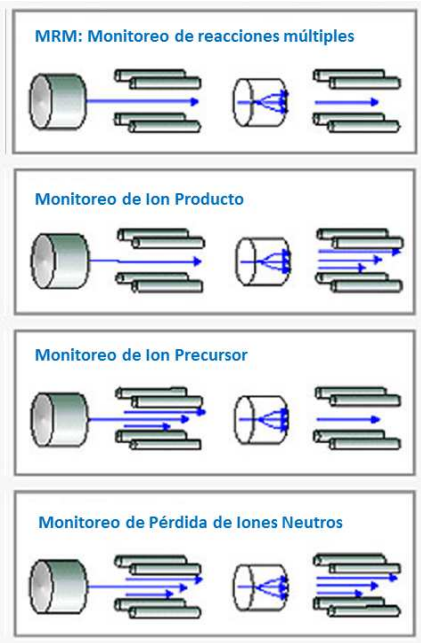


Figura 28. *Tipos de análisis en un TQD*

Por último, una vez separados, los iones entran en el **sistema de detección (fotomultiplicador)**. En el equipo empleado en la presente investigación, los iones a su salida del filtro de masas encuentran un dínodo de conversión donde se transforman en electrones. Estos electrones colisionan después con fósforo que, cuando se excita, emite fotones. Los fotones chocan con un fotocátodo en el frente del fotomultiplicador para producir electrones siendo amplificada la señal a continuación. El proceso se produce a bajo vacío permanente para prevenir la contaminación y controlar la eficacia.

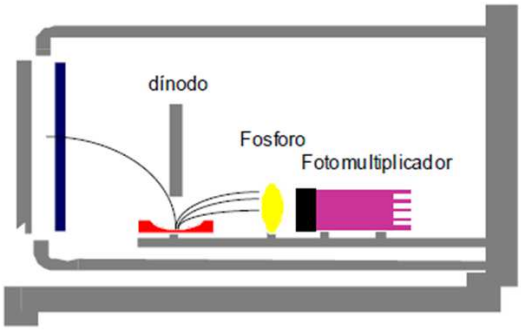


Figura 29. *Detector típico de un TQD. Fotomultiplicador ópticamente alineado con un fósforo emisor de luz y con un dínodo de conversión de alto voltaje para conseguir alta sensibilidad tanto en modo positivo como en modo negativo*

Analizadores de masas. Detector de tiempo de vuelo (TOF). Este analizador separa los iones según la distinta velocidad que adquieren en el interior del tubo de vuelo en función de su relación m/z. En primer lugar, los iones son extraídos de la cámara de ionización y dirigidos hacia el tubo de vuelo, lugar en el que se aceleran por aplicación de un campo electrostático que les aporta una elevada energía cinética. Los iones de mayor m/z "volarán" a menor velocidad que los de menor m/z a lo largo del tubo de vuelo, consiguiéndose su separación. La resolución entre los iones de diferente m/z será mayor cuanto mayor sea la longitud del tubo de vuelo del espectrómetro de masas (habrá una mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energía cinética de los iones. La siguiente figura muestra el esquema del interior de un espectrómetro de masas TOF.

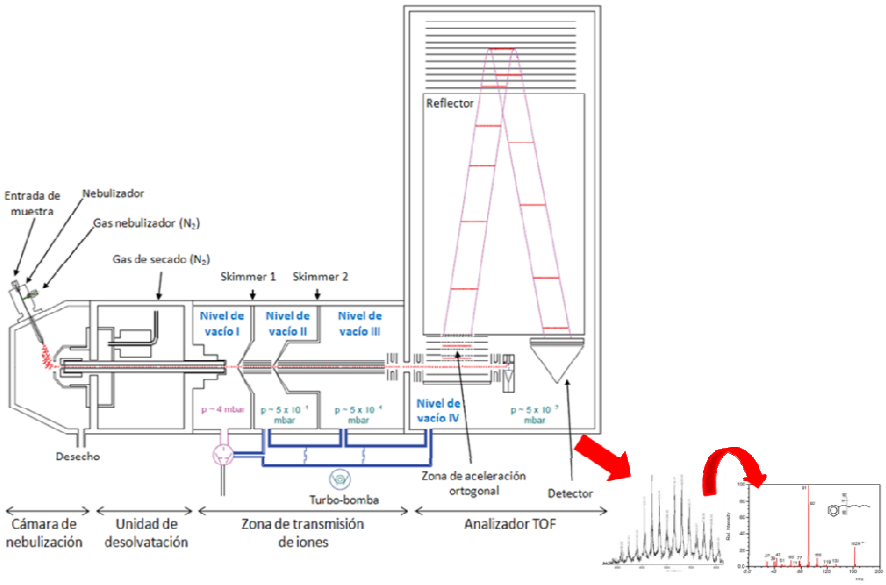


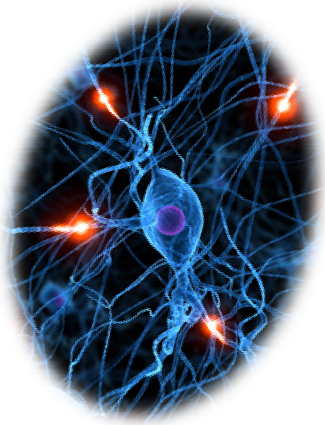
Figura 30. *Analizador de masas TOF con forma de V*

Los iones formados en la cámara de nebulización son atraídos hacia la unidad de desolvatación y la atraviesan conducidos a través de un capilar de vidrio. Esta unidad de desolvación consta, además del capilar de vidrio, de un calentador de gas de secado. Esta zona conecta la cámara de ionización, que se encuentra a presión atmosférica con la zona de transmisión de iones, que se encuentra a alto vacío. La zona de transferencia óptica o transmisión de iones está formada por tres zonas de alto vacío diferenciadas y separadas entre sí por dos skimmers. Los iones son transportados a lo largo de la zona de transferencia por dos hexapolos, y las lentes son las encargadas de focalizarlos y dirigirlos hacia el analizador TOF propiamente dicho.

Las principales ventajas de este detector es que es un detector rápido, sensible, de gran resolución, que proporciona valores de masa muy exactos. Además la combinación de los valores de masas exactas que proporciona junto con la de la distribución isotópica, permite la determinación de la fórmula molecular del analito, facilitando en gran medida la identificación. Dependiendo de la casa comercial y modelos, especificaciones típicas de los analizadores TOF son: rango de masas de 50-3.000 m/z; resolución de 10.000-50.000 FWHM, y una exactitud de 0.2-5 ppm.

CAPÍTULO I

Determinación de Neurotransmisores, Metabolitos y Precursores



I.1 OBJETIVOS

El objetivo principal del trabajo presentado en este primer capítulo es dar apoyo analítico a los proyectos relacionados directamente con la función cognitiva para determinar neurotransmisores y compuestos relacionados por ser metabolitos o derivados. Los objetivos específicos fueron los siguientes:

- ✓ Proponer metodología de buenas características analíticas para la determinación de 17 neurocompuestos mediante cromatografía de líquidos-espectrometría de masas en tándem (LC-MS/MS) en muestras de microdializado de cerebro de rata y en pez cebra.

- ✓ Validar los métodos analíticos propuestos en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación aplicables.

- ✓ Aplicar las metodologías desarrolladas en muestras reales en animales sometidos a condiciones naturales y/o bajo manipulación nutricional.

I.2 ESTRATEGIAS DE TRABAJO SEGUIDAS

En las últimas décadas algunas técnicas novedosas han permitido realizar mediciones neuroquímicas que han mejorado nuestra comprensión de la relación entre la química en el sistema nervioso central (SNC) y el estado conductual, cognitivo y emocional de un organismo. La neurotransmisión anormal se ha vinculado a una amplia gama de enfermedades y problemas como la depresión, la dependencia de las drogas, la esquizofrenia, las enfermedades degenerativas y otras muchas[175-178]. La medida directa de los neurotransmisores *in vivo* en el espacio extracelular del SNC de un animal es una herramienta esencial para estos experimentos. Estas determinaciones presentan una gran cantidad de dificultades relacionadas con la complejidad y delicadeza del tejido. En la actualidad, la ciencia ha progresado y son muchas las técnicas surgidas para analizar estos mensajeros neuronales, todavía existen limitaciones para determinarlos con precisión por los cambios tan rápidos que sufren y la heterogeneidad con la que se producen[179]. Por otro lado, aunque es posible medir la concentración *in vitro* de los neurotransmisores, mediante el análisis de las células en cultivo y en tejidos preparados *post-mortem* de partes específicas del cerebro, estos experimentos conllevan una serie de problemas y dificultades relacionados con la complejidad de las muestras y la presencia de componentes inestables en el tiempo.

El primer trabajo expuesto en este capítulo se ha centrado en el estudio *in vivo* de la concentración de neurocompuestos endógenos, que se encuentran presentes en el líquido intersticial del cerebro de un animal, con objeto de analizar su evolución cuando se administra un ingrediente específico, ya que la concentración de estos compuestos puede ser modificada a través de la

[175] *Clausius N., Born C., et al. The relevance of dopamine agonists in the treatment of depresión. Neuropsychiatrie 23 (2009) 15-25.*
[176] *Berridge K.C., Robinson T.E., et al. Dissecting components of reward: 'liking', 'wanting', and learning. Current Opinion in Pharmacology 9 (2009) 65-73.*
[177] *Gunduz-Bruce H. The acute effects of NMDA antagonism: from the rodent to the human brain. Brain Research Reviews 60 (2009) 279-286.*
[178] *Lewis S.J.G., Barker R.A. Understanding the dopaminergic deficits in Parkinson's disease: Insights into disease heterogeneity. Journal of Clinical Neuroscience 16 (2009) 620-625.*
[179] *Schultz K.N., Kennedy R.T. Time-resolved microdialysis for in vivo neurochemical measurements and other applications. Annual Review of Analytical Chemistry 1 (2008) 627-661.*

nutrición, entre otros factores. Los neurocompuestos estudiados fueron: betaina, glutamina, glutamato, ácido γ-aminobutírico, fosfocolina, colina, acetilcolina, glicerofosfocolina, 5'-citindifosfocolina, dopamina, norepinefrina, serotonina, tirosina, epinefrina, triptófano y ácido 5-hidroxiindolacético en microdializados obtenidas directamente del cerebro de un animal vivo.

La microdiálisis es una técnica ampliamente usada en neurociencia, siendo una de las pocas técnicas que permite la cuantificación de neurotransmisores, péptidos y hormonas para estudiar el comportamiento animal en respuesta a diferentes estímulos como el desempeño de tareas, la administración de drogas local o sistémicamente, o la ingesta de nutrientes[180]. La técnica requiere la inserción de una pequeña sonda de microdiálisis en un área específica del cerebro. Esta sonda está diseñada para imitar un capilar de sangre y consiste en un eje con una membrana de fibra semipermeable que está hueca en su punta, quedando conectada a un tubo de entrada y a otro de salida. Esta técnica permite la medición continua de sustancias de pequeño peso molecular, como son la mayoría de neurotransmisores que existen en el espacio intersticial.

Una vez obtenida la muestra, es necesario disponer de métodos analíticos capaces de separar los distintos neurotransmisores y ser lo suficientemente sensibles para su cuantificación en estos microdializados, ya que la concentración de la mayoría de neuroquímicos en el espacio extracelular es del orden de trazas. Además, la resolución temporal de un analito en un microdializado es inversamente proporcional al volumen recogido. Por tanto, los métodos de análisis deben proporcionar límites de detección y cuantificación por debajo de la mínima concentración que se espera obtener en el dializado, y deben requerir menos volumen de muestra que el usado en el protocolo estandarizado de microdiálisis.

Por otro lado, las técnicas de limpieza o pasos intermedios con pipeteo no son recomendables por el mínimo volumen recogido y la baja concentración que existe de estos analitos. Además, se debe tener en cuenta que el propio

[180] *Chefer V.L., Thompson A.C., et al. Overview of brain microdialysis. Current Protocol Neuroscience (2009) Chapter 7:Unit 7.1.*

medio de perfusión contiene iones inorgánicos que pueden interferir con el método de cuantificación empleado[181].

El segundo trabajo se ha centrado en el mismo tipo de determinación pero en este caso en muestras de pez cebra, para estudiar la evolución a lo largo del ciclo de la vida de este animal de los neurocompuestos mencionados anteriormente además de la agmatina, que ha sido definido como neurotransmisor en los últimos tiempos[182].

La estrategia seguida para alcanzar los objetivos fijados se basó en las siguientes etapas:

A. Proceso de microdiálisis. Con objeto de recibir el entrenamiento oportuno en la técnica de microdiálisis y diseñar los experimentos pertinentes, inicialmente fue necesaria una colaboración externa por parte del grupo de investigación del Dr. Miguel Alfonso Pallares, Profesor Titular del Área de Fisiología del Departamento de Biología Funcional y Ciencias de la Salud, Vigo (España). Los investigadores colaboradores realizaron las pertinentes explicaciones teóricas y prácticas para aprender los procedimientos de trabajo. Realizado este periodo de aprendizaje, la tecnología se implantó, con algunas modificaciones, en los laboratorios que Abbott dispone en las instalaciones del CSIC en Granada, España.

B. Análisis mediante cromatografía de líquidos-espectrometría de masas. Se desarrolló una nueva metodología para la determinación de todos los analitos seleccionados en un único análisis, con el objetivo final de obtener la máxima información posible en un único paso. Se realizó una extensa búsqueda bibliográfica y se realizaron los ensayos necesarios para la optimización del nuevo método y su validación:

[181] *Zapata A., Chefer V.L., et al. Detection and quantification of neurotransmitter in dialysates. Current Protocol Neuroscience (2009); Chapter 7:Unit7.4.*

[182] *Reis D.J., Regunatjan S. Is agmatine a novel neurotransmitter in brain? Trends Pharmacol Sci. 21 (2000) 187-193.*

- ***Separación cromatográfica.*** Cromatografía de líquidos de ultra-resolución (Ultra Performance Liquid Chromatography, UPLC®). Se estudió y optimizó la metodología teniendo en cuenta todas las posibles combinaciones de fases móviles, aditivos, fases estacionarias y otros factores que pueden afectar a la separación cromatográfica de los analitos seleccionados.

- ***Detección mediante espectrometría de masas.*** Se optimizaron los parámetros y requerimientos implicados en esta tecnología en un espectrómetro de masas de triple cuadrupolo (TQD).

- ***Validación del método.*** Se llevó realizó en términos de sensibilidad, selectividad y exactitud (precisión y veracidad)[183], según las recomendaciones de las guías internacionales adecuadas para la validación de métodos bioanalíticos[184].

C. Aplicación del método a la determinación de los compuestos de interés en muestras de microdializados de cerebro de rata. Una vez optimizado el método, se realizaron experimentos con animales vivos, administrando vía oral ingredientes o sustancias específicas y siguiendo la evolución de los compuestos de interés en el fluido intersticial del cerebro.

D. Aplicación del método a la determinación de los compuestos de interés en muestras de pez cebra. Haciendo uso del mismo método cromatográfico optimizado para microdializados, se desarrolló una segunda aplicación para la determinación 17 neurocompuestos, incluyendo la agmatina, en pez cebra. El objetivo del experimento fue ofrecer información relacionada con el perfil de neurotransmisores, metabolitos y precursores a lo largo del ciclo de la vida de este animal como herramienta para futuros estudios en los que se use este animal como modelo.

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[183] *ISO 5725-1:1994. Accuracy (trueness and precision) of measurement methods and results -- Part 1: General principles and definitions.*

[184] *Guidance for Industry, Bioanalytical Method Validation, U.S. 2001. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM).*

I.3 ESTADO ACTUAL DE LA INVESTIGACIÓN. ANTECEDENTES BIBLIOGRÁFICOS

Con objeto de cuantificar el interés de la investigación científica sobre los biocompuestos seleccionados, se ha llevado a cabo una revisión bibliográfica profunda en diversos buscadores como Scifinder, Scopus, Web of Science o Google Scholar, que nos ha permitido conocer el "estado del arte" en este campo de investigación. En este sentido, es de destacar el elevado interés general que despiertan los neurotransmisores en diversas áreas de investigación. En la figura I.1, se muestra cómo los trabajos publicados sobre neurotransmisores han experimentado un crecimiento a lo largo de los últimos 50 años, con más de 100.000 documentos encontrados en la base Scopus.

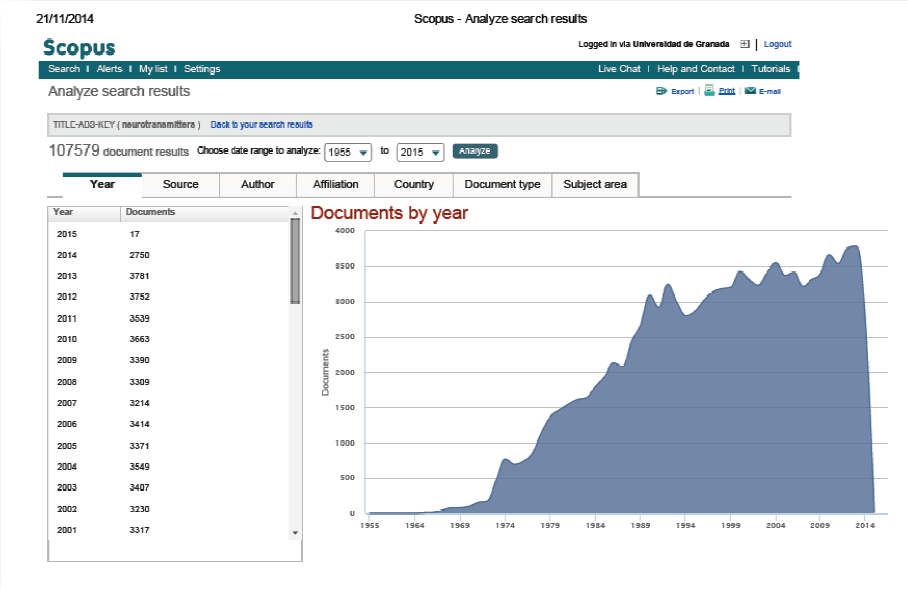


Figura I.1. *Informe obtenido en Scopus donde se refleja el número de trabajos publicados entre 1955 y 2014 sobre neurotransmisores*

Si se acota la búsqueda con términos específicos como "Neurotransmitters determination" se encuentra igualmente un interés creciente (Figura I.2), con más de 2000 documentos encontrados.

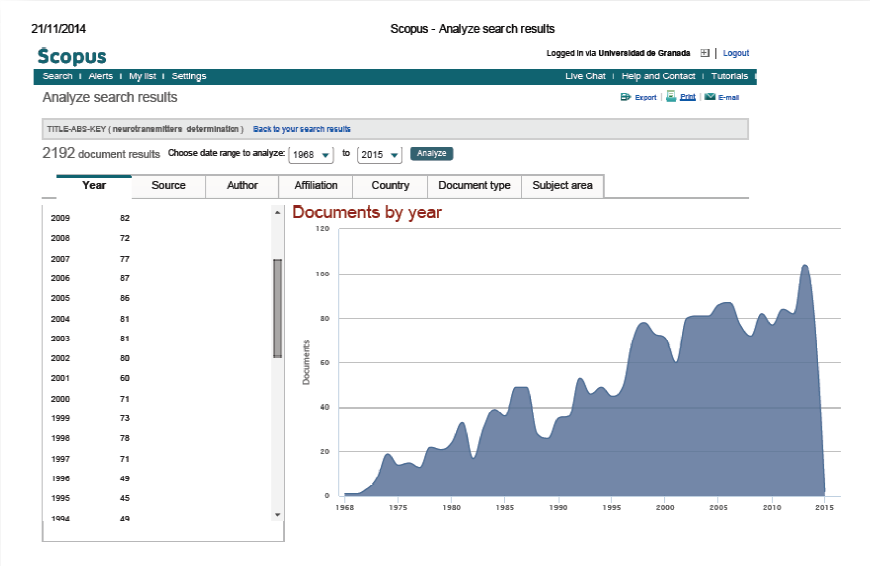


Figura I.2. *Informe obtenido en Scopus del número de trabajos publicados entre 1955 y 2014 sobre "neurotransmitters determination"*

Además podemos conocer los porcentajes de publicaciones por áreas (Figura I.3), siendo bioquímica, neurociencia y medicina las áreas en las que mas interés despiertan estos biocompuestos. También podemos evaluar el tipo de documento publicado (Figura I.4). Las publicaciones de artículos originales son claramente mayoritarias en más del 75%, frente a otros tipos de documentos como libros, conferencias o revisiones.

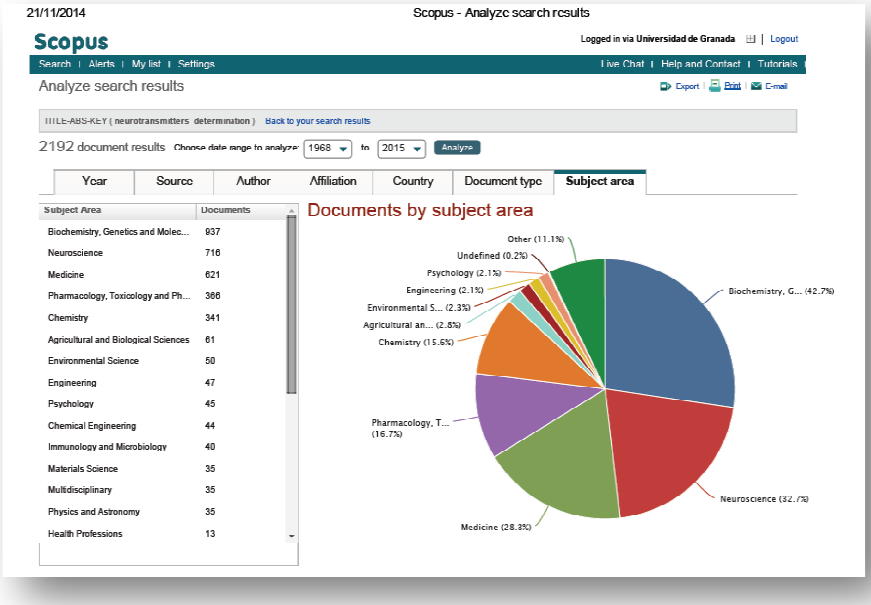


Figura I.3. *Informe obtenido en Scopus con la búsqueda "neurotransmitters determination" para conocer los % de trabajos publicados por áreas de investigación*

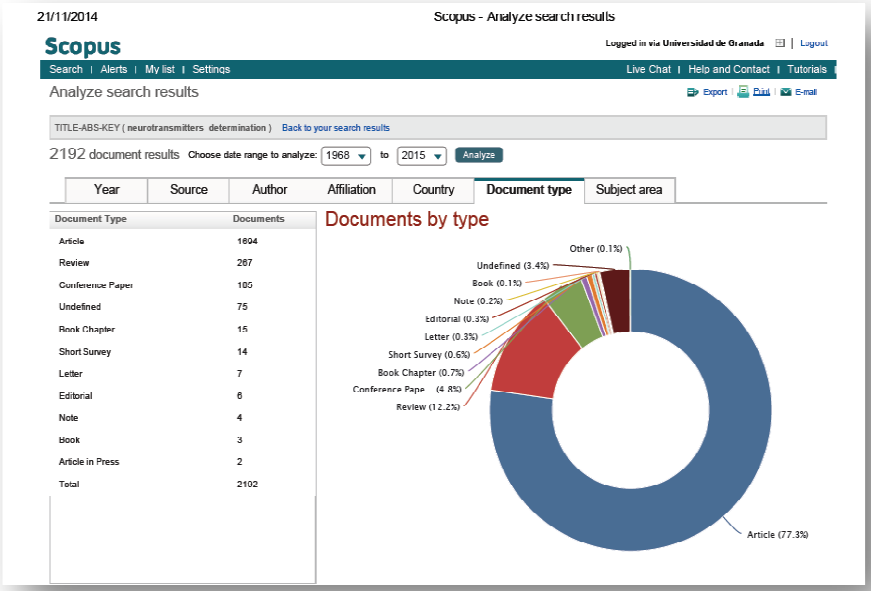


Figura I.4. *Informe obtenido en Scopus con la búsqueda "neurotransmitters determination" para conocer el tipo de documento publicado. El tipo artículo o manuscrito es el mayoritario con un 77%*

I.4 ANÁLISIS DE NEUROTRANSMISORES, METABOLITOS Y PRECURSORES MEDIANTE UHPLC-MS EN TANDEM

En esta Tesis se ha desarrollado metodología analítica para determinar los neurotransmisores, metabolitos y derivados anteriormente presentados. En una primera fase, el trabajo se centró en la determinación cuantitativa de 16 neurocompuestos en muestras del líquido intersticial del cerebro de un animal vivo (roedor) a través de la técnica de microdiálisis. Posteriormente en un segundo trabajo de investigación, se determinaron esos 16 neurocompuestos mas uno adicional (Agm) a lo largo del ciclo de la vida del pez cebra.

I.4.1 Cuidado animal

En el primer trabajo, todos los procedimientos relacionados con la manipulación animal han sido conformes con las guías institucionales y las leyes y políticas sobre el Cuidado y Uso de Animales de Laboratorio (RD 2101-2005, 86/609/CEE). Los animales estaban ubicados en las instalaciones que Abbott Laboratories dispone en el CSIC de Granada, conforme la ley mencionada anteriormente (Figura I.5).

Figura I.5. *Alojamiento de los animales en las instalaciones del CSIC, Granada*

En el segundo trabajo, con pez cebra, los animales fueron suministrados por Neuron Bio (Granada, España). El mantenimiento y cuidado de los peces se realizó de acuerdo a la Certificación de Calidad bajo el Sistema de Gestión de Certificación UNE 166002: 2006 para I+D (Figura I.6).

Figura 27. *Instalaciones de cuidado de pez cebra*

I.4.2 Técnicas de microdiálisis

Los experimentos comenzaron con la intervención quirúrgica de las ratas, que fueron anestesiadas para insertar estereotáxicamente la cánula-guía en una zona específica del cerebro. Esta cánula proporciona el soporte para la introducción de la sonda de microdiálisis posteriormente. La implantación de la sonda en un área específica del cerebro se realiza con la ayuda de un aparato de estereotaxia, que es un dispositivo mecánico que inmoviliza totalmente la cabeza del animal anestesiado, permitiendo, con gran precisión, alcanzar cualquier punto del cerebro. Se fija el cráneo del animal con ayuda de unos soportes insertados en los conductos auditivos externos, y para la absoluta inmovilización de la cabeza se fijan los incisivos superiores en una barra horizontal. Una vez colocado apropiadamente el animal en el aparato estereotáxico y usando como puntos de referencia los puntos de corte entre las suturas craneales (llamados Bregma o Lambda), se puede localizar cualquier zona o estructura anatómica del cerebro mediante el cálculo de sus coordenadas estereotáxicas registradas en un atlas estereotáxico. Para cada

área cerebral, el atlas proporciona medidas exactas en las tres direcciones del espacio, con respecto a los puntos estereotáxicos de referencia. La sonda de microdiálisis, o cualquier otro dispositivo que se desee introducir en el cerebro, se coloca en el micromanipulador del aparato estereotáxico, el cual permite desplazar el dispositivo en las tres direcciones del espacio hasta localizar la zona del cerebro en la que se quiera implantar (Figuras I.7 y I.8).

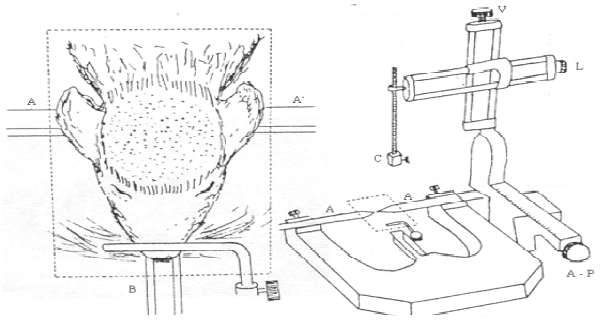


Figura I.7. *Esquema de la colocación del animal en el aparato estereotáxico (Fundamentos de la microdiálisis y HPLC, Universidad de Vigo)*

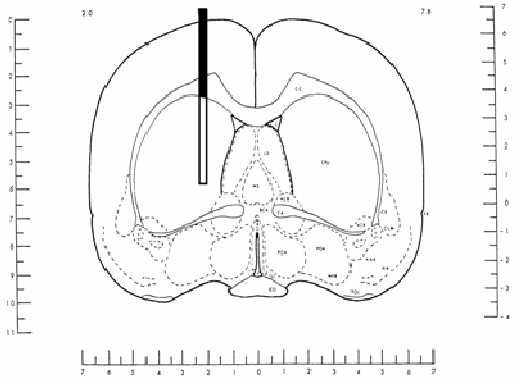


Figura I.8. *Corte sagital con inserción de la sonda de microdiálisis de acuerdo al atlas estereotáxico (Fundamentos de la microdiálisis y HPLC, Universidad de Vigo*

Una vez anestesiado el animal, se coloca en el aparato de estereotaxia y se realiza un corte longitudinal en la piel del cráneo. Tomando como punto de referencia el Bregma (punto de la superficie del cráneo situado entre las suturas sagital y coronal) y, usando el atlas estereotáxico, se establecen las

coordenadas del punto exacto de la zona para implantar la sonda y se accede a ese punto practicando un pequeño orificio en el cráneo del animal e introduciendo la cánula hasta la profundidad de la coordenada vertical deseada. Una vez colocada, ésta se fija al cráneo con cemento acrílico (típico en las clínicas dentales). Es necesario esperar al menos 24 h para la recuperación del animal porque la colocación de la cánula causa un ligero daño en el tejido neuronal, afectando principalmente al flujo sanguíneo, a la barrera hematoencefálica y a los procesos de neurotransmisión, los cuales se han comprobado que se recuperan transcurrido este tiempo[185-187]. Por el contrario, un tiempo de espera superior a tres días implica que se origine un proceso de gliosis alrededor del lugar de implantación de la cánula, pudiendo disminuir la recuperación de los neurotransmisores que se estén analizando[188,189]. El aspecto de una rata después de la intervención quirúrgica se muestra en la Figura I.9.

Figura 28. *Rata tras la intervención quirúrgica*

[185] *Imperato A., Di Chiara G. Trans-striatal dialysis coupled to reverse phase high performance liquid chromatography with electrochemical detection: A new method for the study of the in vivo release of endogenous dopamine and metabolites. J. Neurosc. 4 (1984) 966-977.*

[186] *Westerink B.H.C., De Vries J.B. (1988). Characterization of in vivo dopamine release as determined by brain microdialysis after acute and subchronic implantations: methodological aspects. J. Neurochem. 51 (1988) 683-687.*

[187] *Benveniste H., Diemer N.H. Cellular reactions to implantation of a microdialysis tube in the rat hippocampus. Acta Neuropathol. 74 (1987) 234-238.*

[188] *Benveniste H., Hüttemeier P.C. Microdialysis- Theory and application. Progr. Neurobiol. 35 (1990) 195-215.*

[189] *Fibiger H.C. Physiological relevance: a fundamental goal of brain microdialysis. Commentary on Di Chiara et al. Estimation of in-vivo neurotransmitter release by brain microdialysis: the issue of validity. Behav. Pharmacol. 7 (1996) 661-662.*

Durante el tiempo de recuperación se coloca un protector en la cánula-guía sustituyendo a la sonda de microdiálisis. Esto permitirá que la sonda no sufra ni modifique sus características durante el período que no se está realizando el experimento de microdiálisis, evitando así los problemas de obturación, disminución en la recuperación y otros problemas (Figuras I.10 y I.11).

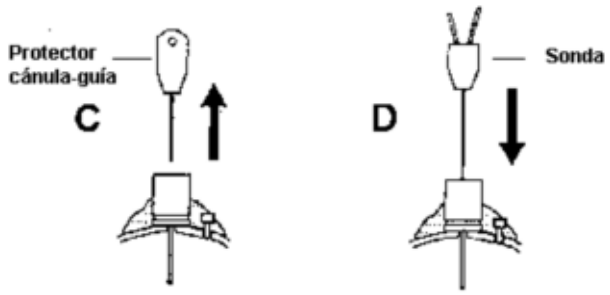


Figura I.10. *Esquema de la cánula-guía con el protector (C) y la sonda (D).*
(Fundamentos de la microdiálisis y HPLC, Universidad de Vigo)

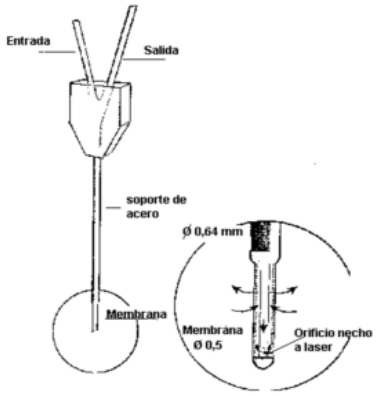


Figura I.11. *Esquema de la sonda de microdiálisis*
(Fundamentos de la microdiálisis y HPLC, Universidad de Vigo)

En el extremo superior de la sonda se conecta todo el sistema de tuberías que perfunde el líquido cerebroespinal artificial, que entra y sale del cerebro a través de los poros de cierto tamaño de la sonda. Los analitos presentes en el fluido extracelular del cerebro se recogen al pasar el líquido cerebroespinal artificial lentamente a través de la sonda (Figura I.12).

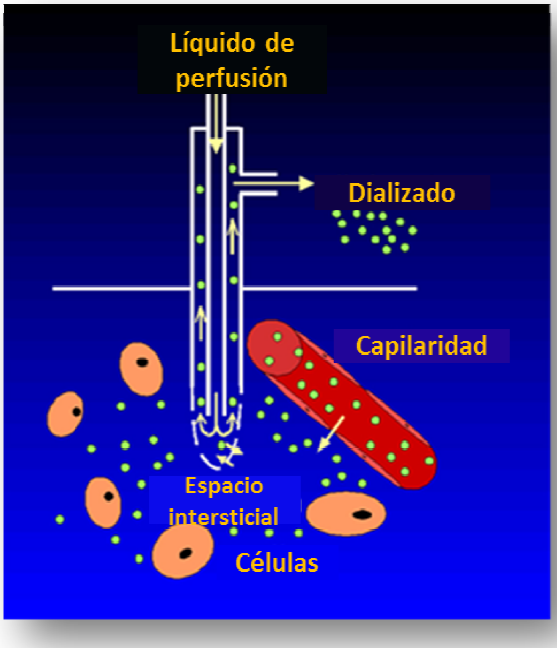


Figura I.12. *Esquema teórico del proceso de microdiálisis*

Con la técnica de microdiálisis no se recoge el 100% de las sustancias presentes en el espacio extracelular, ni las sustancias administradas difunden por completo al medio. Esto depende de factores como el tipo de membrana, el tamaño de la sustancia, su permeabilidad a través de la membrana y el flujo de perfusión. Así para una determinada sustancia, trabajando a flujos elevados, la difusión será baja, en cambio si se disminuye el flujo de perfusión, la difusión del analito aumentará. Se debe alcanzar un compromiso que permita la obtención de un volumen suficiente en el tiempo requerido.

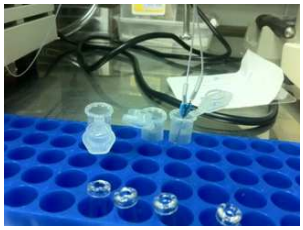
En el inicio del experimento se comprueba el rendimiento de la sonda de microdiálisis *in vitro*, calculando el % de recuperación de cada analito de interés. Este concepto indica la cantidad de la sustancia que atraviesa una sonda bajo determinadas condiciones experimentales, es decir, este término es la relación entre la concentración de una sustancia medida en el dializado recogido y su concentración en el medio exterior a la membrana de microdiálisis. Para calcular la recuperación con una determinada sonda, se coloca ésta en un tubo

tipo eppendorf con una disolución que contiene todos los analitos de interés a una concentración conocida. Después de perfundir la sonda con líquido cerebroespinal artificial (aCSF) o medio Ringer, se recogen las muestras y se determina la concentración de cada sustancia en dichos dializados. La recuperación de la sonda para cada sustancia se determina calculando el porcentaje que representan la cantidad medida en los dializados de la sustancia, respecto a la cantidad presente en el medio externo a la sonda de esa misma sustancia. Este ensayo se realiza a tres niveles de concentración. Se suelen obtener valores típicamente aceptables en el rango del 10% al 30%.

Una vez verificado el funcionamiento de la sonda de microdiálisis con la que se realizará el experimento, se procede a insertarla en la cánula-guía implantada en el animal ya recuperado y despierto. El animal está dispuesto en un habitáculo giratorio que permite relativamente el libre movimiento. Esto resulta de gran ayuda para evitar que el sistema de tuberías gire y se entrecruce, evitando el estrangulamiento del propio animal por las tuberías conectadas en su cabeza.

El equipo consta de una bomba peristáltica que introduce, a un determinado flujo el aCSF o medio Ringer a través del sistema de tuberías en la sonda de microdiálisis. En la salida de la sonda existe otra tubería que conecta directamente al microcolector de fracciones automático donde se colocan los viales y es recogido el dializado cada cierto tiempo predeterminado. En la Figura I.13 se muestra el equipo de microdiálisis usado.

A



B

C

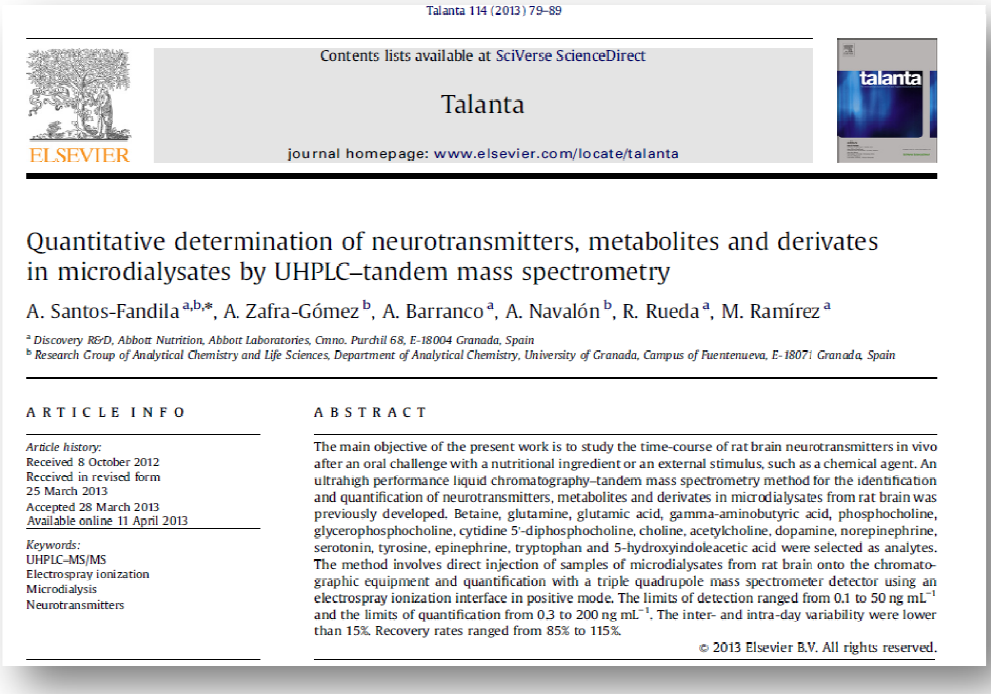
D

E

Figura 29. *Equipo de microdiálisis: A) sistema de bombeo; B) sonda de microdiálisis y viales de muestra; C) colector de fracciones; D) habitáculo giratorio; E) típica configuración del sistema de microdiálisis completo*

I.4.3 Determinación de Neurocompuestos en Microdializado de Cerebro de un Roedor (Publicación I)

En este apartado se presenta el trabajo publicado en la revista internacional **Talanta** de la editorial Elsevier (Figura I.14).



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Quantitative determination of neurotransmitters, metabolites and derivates in microdialysates by UHPLC–tandem mass spectrometry

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ABSTRACT

The main objective of the present work is to study the time-course of rat brain neurotransmitters in vivo after an oral challenge with a nutritional ingredient or an external stimulus, such as a chemical agent. An ultrahigh performance liquid chromatography–tandem mass spectrometry method for the identification and quantification of neurotransmitters, metabolites and derivates in microdialysates from rat brain was previously developed. Betaine, glutamine, glutamic acid, gamma-aminobutyric acid, phosphocholine, glycerophosphocholine, cytidine 5′-diphosphocholine, choline, acetylcholine, dopamine, norepinephrine, serotonin, tyrosine, epinephrine, tryptophan and 5-hydroxyindoleacetic acid were selected as analytes. The method involves direct injection of samples of microdialysates from rat brain onto the chromatographic equipment and quantification with a triple quadrupole mass spectrometer detector using an electrospray ionization interface in positive mode. The limits of detection ranged from 0.1 to 50 ng mL^{-1} and the limits of quantification from 0.3 to 200 ng mL^{-1}. The inter- and intra-day variability were lower than 15%. Recovery rates ranged from 85% to 115%.

Figura I.14. *Quantitative determination of neurotransmitters, metabolites and derivates in microdialysates by UHPLC–tandem mass spectrometry. Talanta 114 (2013) 79–89 (factor de Impacto, 3.511)*

Quantitative determination of neurotransmitters, metabolites and derivates in microdialysates by UHPLC–tandem mass spectrometry

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ABSTRACT

The main objective of the present work is to study the time-course of rat brain neurotransmitters *in vivo* after an oral challenge with a nutritional ingredient or an external stimulus, such as a chemical agent. An ultrahigh performance liquid chromatography–tandem mass spectrometry method for the identification and quantification of neurotransmitters, metabolites and derivates in microdialysates from rat brain was previously developed. Betaine, glutamine, glutamic acid, gamma-aminobutyric acid, phosphocholine, cytidine 5′-diphosphocholine, glycerophosphocholine, choline, acetylcholine, dopamine, norepinephrine, serotonin, tyrosine, epinephrine, tryptophan and 5-hydroxyindolacetic acid were selected as analytes. The method involves the direct injection of samples of microdialysates from rat brain onto the chromatographic equipment and quantification with a triple quadrupole mass spectrometer detector using an electrospray ionization interface in positive mode. The limits of detection ranged from 0.1 to 50 ng mL^{-1} and the limits of quantification from 0.3 to 200 ng mL^{-1}. The inter- and intra-day variability was lower than 15%. Recovery rates ranged from 85% to 115%.

Keywords:

UHPLC–MS/MS; Electrospray ionisation; Microdialysis; Neurotransmitters

Highlights:

- Multi-analyte method for the determination of different families of neurotransmitters.
- Direct injection of microdialysates into the LC-MS/MS system (in vivo experiments).
- Validation of the method: quality parameters, accuracy (trueness and precision).
- Influence of stimulant compounds injected locally or given orally.

1. Introduction

Synaptic transmission between neurons is the mechanism by which information is transmitted in the central nervous system. Most of this transmission occurs through the release and activity of neurotransmitters, which can be classified according to their chemical structure and activity into: i) monoamines, ii) aminoacids, iii) peptides and hormones. Other compounds that can also work in neuronal communication, such as nitric oxide, ATP or adenosine, do not belong to any of the above chemical groups.

Microdialysis is a widely used technique in neuroscience and is one of the few that permit quantification of neurotransmitters, peptides, and hormones in the behaving animal [1] in response to different stimuli: performance of task, administration of drugs systemically or locally, and intake of a nutrient. The microdialysis technique requires the insertion of a small microdialysis probe into an area of the brain. The probe is designed to mimic a blood capillary and consists of a shaft with a semipermeable hollow fiber membrane at its tip, which is connected to inlet and outlet tubing. This technique enables the continuous measurement of small-molecular-weight substances like most neurotransmitters from the interstitial space.

Sensitive analytical methods are needed for the separation and quantification of neurotransmitters in microdialysates. First, the concentration

of most neurochemicals in the extracellular space is very low. In addition, the temporal resolution of a microdialysate analyte is inversely related to the volume. Therefore, the analytical methods should provide detection limits below the lowest concentration expected in the dialysate and should require less sample volume than the one used in the microdialysis protocol. Moreover, pipetting or sample clean-up techniques are often impossible. Finally, the perfusion medium itself contains inorganic ions that may interfere with the quantification method employed [2].

The classical methods for the measurement of neurotransmitters depended on their chemical structure, for example, liquid chromatography with electrochemical detection for catecholamines, or liquid chromatography with fluorescent detection for amino acids [2]. This fact limited the simultaneous determination of different neurotransmitters in microdialysates due to the low sample volume. Perry and Kennedy published a five-year review of analytical techniques for the determination of neurotransmitters, and also grouped the methods by chemical structure [3]. They included a special section on 'multiplexing' or multi-analyte monitoring and highlighted the opportunity of observing interactions of neurotransmitter systems and detecting changes that were not anticipated by the original hypothesis. They concluded that only a limited number of methods detect analytes from different categories of neurotransmitters. Only two out of six methods detected more than 4 compounds of different families within the nanomolar range. The first one was able to detect 21 analytes related to the metabolism of tyrosine, tryptophan and glutamic acid [4]. The second one detected six compounds in extracellular brain fluid [5]. A more recent article reported a method to profile the neurologically related metabolites of multiple principal transmitter pathways in the rat brain [6].

A number of methods have been developed previously for the determination of these compounds in biological matrices using a wide range of techniques, reagents, additives, derivatization procedures, equipment, and detectors as mass spectrometry in several modes, among other detectors. Liquid chromatography tandem mass spectrometry with electrospray

ionization in positive or negative mode (HPLC-ESI-MS/MS) [7, 9, 11-12, 15, 17-18, 20, 23-24, 26, 28-29]; HPLC coupled to atmospheric pressure chemical ionization mass spectrometry (HPLC-APcI-MS) [8, 22, 25]; HPLC coupled to thermospray-mass spectroscopy (HPLC-TS/MS) [10]; HPLC with electrochemical detection (HPLC/ECD) [10, 16, 19, 21, 27]; ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) [13-14]; HPLC with fluorescence detector [25, 30]; HPLC coupled to single-quadrupole mass spectrometer (HPLC-MS) [31]; HPLC with ultraviolet-visible detector (HPLC-UV) [31] and gas chromatography with mass spectroscopy (GC/MS) [10], have been used. However, the previous methods have several shortcomings in comparison to the proposed method. Those methods are limited by the number of compounds that can be analyzed simultaneously, and they are not applicable to the matrices for which the proposed method was developed, and they are restricted by higher limits of detection (LODs). Recent methods that rely on modern analytical techniques like liquid chromatography with tandem mass spectrometry generally have lower LODs, but are restricted to only a few analytes [11, 13-14]. Attempts to improve LODs by derivatization have been done, but the procedures are tedious and time consuming [13]. Recent methods that rely on modern analytical techniques like liquid chromatography with tandem mass spectrometry generally have lower LODs, but are restricted to only a few analytes. Attempts to improve LODs by derivatization have been done, but the procedures are tedious and time consuming.

In the present work, an ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS) method for the quantification of compounds of different families of neurotransmitters within the ultra trace range was developed and validated. The method consists of a multi-analyte approach for the measurement of neuromediators with chemical structures related to aminoacids, including tyrosine (Tyr), glutamine (Gln), glutamic acid (Glu), gamma-aminobutyric acid (GABA) and tryptophan (Trp); and monoamines, including acetylcholine (AcCh) and metabolites such as choline (Cho), glycerophosphocholine (GPCho), cytidine 5′-diphosphocholine (CDPCho), phosphocholine (PCho), betaine (Bet); catecolamines such as dopamine (DA), epinephrine (E), norepinephrine (NE); indolamines such as

serotonin (SE), and its metabolite 5-hydroxyindolacetic acid (5-HIAA). This novel method of multi-analyte detection has the advantage of measuring a higher number of compounds at the same time with acceptable LODs and higher selectivity than methods based on LC with electrochemical, ultraviolet or fluorescence detection. The method was subsequently applied to microdialysis experiments for testing the influence of a stimulant compound injected locally or a carbohydrate given orally.

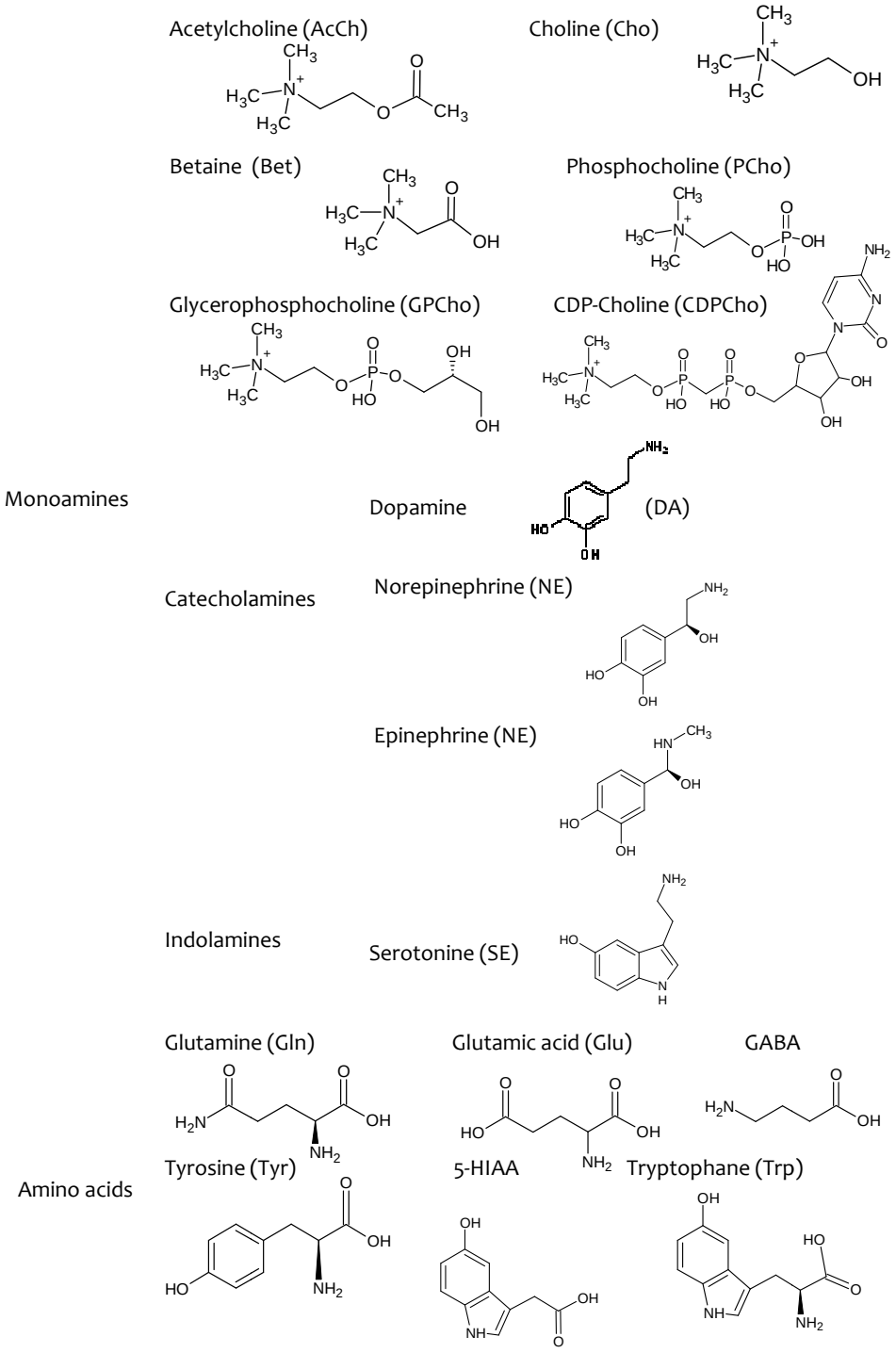


Fig. 1. Chemical structures of the compounds of interest

2. Materials and methods

2.1 Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Bet, Gln, Glu, GABA, PCho, GPCho, CDPCho, Cho, AcCh, DA, NE, SE, Tyr, E and 5-HIAA were supplied by Sigma-Aldrich (Madrid, Spain). Eserine from Sigma-Aldrich (Spain). Methanol, acetonitrile, sodium thiosulfate, ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid, heptafluorobutyric acid (HFBA) and formic acid (LC-MS grade) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) or Ringer solution was purchased from Harvard Apparatus (Holliston, Massachusetts, USA).

A stock solution was prepared by weighing 0.01 g of each compound into a 10 mL flask, except CDPCho and PCho, for which 0.1 g were weighed. Then, 1 mL of concentrated formic acid (98-100%, v/v), and water up to the final volume were added. The solution remained stable for at least one month at 4 °C. An intermediate solution (N° 1) was prepared by diluting 50 µL of the stock solution to 10 mL in water. A second intermediate solution (N° 2) was prepared by diluting 100 µL of solution N° 1 to a final volume of 2 mL with aCSF.

Work standards for calibration purposes, named WS1, WS2, WS3, WS4, WS5 and WS6, respectively, were prepared by taking 3 µL, 5 µL, 40 µL, 200 µL, 400 µL and 800 µL from intermediate solution N° 2 and diluting to a final volume of 1 mL with aCSF. Each vial received 20 µL of formic acid before of the final volume. Two more solutions were prepared from intermediate solution N° 1 by diluting 100 µL and 250 µL to a final volume of 1 mL with aCSF, and also adding 20 µL of concentrated formic acid to each one. They were named, WS7 and WS8, respectively. The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS4) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment.

2.2 *Apparatus and software*

An UPLC® Acquity from Waters (Milford, MA, USA) equipped with a binary pump, vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector was used. The chromatograph was connected on-line to a triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. The following chromatographic columns were tested: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 µm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 µm particle size), and Acquity UPLC HSS T3 (2.1 mm × 100 mm i.d., 1.8 µm particle size) from Waters. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis.

Auxiliary apparatuses were: analytical balance with a precision of 0.1 mg, vortex-mixer, maximum recovery LC vials and screw caps from Waters, and eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom).

2.3 *Animal manipulation*

Sprague-Dawley rats (Charles River, France) weighing 400-500 g were used. The animals were kept at constant room temperature (22 ± 2 °C) and 45-55% humidity under a regular 12-hour light/dark schedule. Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101-2005, 86/609/CEE).

Rats were anesthetized intraperitoneally with a mixture of ketamine–rompun (2:1, v/v) and commercially supplied probes (4 mm long membrane, PAN. 30KDa MW, BASI, USA) were stereotaxically inserted into the left lateral hippocampus area (-5.6 mm anterior bregma, 4.4 mm lateral, -7.5 mm from the dura mater) according to the coordinates described in a stereotaxic atlas for rats. The animal was allowed to recover from surgery for at least three days.

On the day of the experiment, the rat was implanted with the brain microdialysis probe that was inserted into the guide cannula. The probe was perfused with aCSF containing 7 µM of eserine at a constant rate of 2 µL min^{-1} to inhibit acetylcholinesterase. The animal was left in the cage with space for relatively free movement. A bolus dose of a specific ingredient was given by gavage 100 min after starting the experiment and sampling was continued for another 220 min. Microdialysate samples were automatically collected every 20 min directly into vials and frozen at -80 °C until analysis. All studies included a 20 min washout period prior to collecting the dialysates. Six dialysate samples were then collected and defined as basal samples.

Each probe was individually tested in vitro to assess the recovery of the analytes. A solution containing all the compounds in concentrations within the lineal dynamic range was prepared. The probe was immersed in the solution and the system was set up to simulate the experimental conditions in the animals. Serial samples were collected every 20 minutes, analyzed and the recovery calculated. The average recovery for each compound should be higher than 20%. Every probe that did not comply with this requirement was discharged. In one experiment, the animals were administered with KCl directly into the inflow of the probe as an excitatory compound able to induce neurotransmitter release, especially AcCh [32]. In a second experiment, the animals received a glucose bolus by oral gavage (1g/kg/body weight).

2.4 Sample preparation

Artificial cerebrospinal fluid is commonly used when sampling brain interstitial fluid. This solution closely matches the electrolyte concentrations of cerebrospinal fluid. It is commercially prepared in high purity water and analytical grade reagents, micro-filtered and sterile. Final ion concentrations in this solution were (in mM): Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155. Samples (typically 40 µL) of microdialysate obtained from *in vivo* studies were placed in maximum recovery vials. Two microliters of formic acid were added and then mixed for 10 s on a vortex mixer.

2.5 *Liquid chromatographic conditions*

Chromatographic separation was performed using an UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size) from Waters. The standards and samples were separated using a gradient mobile phase consisting of a mixture of water, acetonitrile, HFBA and formic acid in a proportion of 900:100:1:1 (v/v/v/v) as solvent A, and 0.1% (v/v) of formic acid in acetonitrile as solvent B. Gradient conditions were: 0.0-1.0 min, 5 to 50% B; 1.0-2.0 min, 50 to 95% B; 2.0-3.0 min, 95% B; and back to 5% B in 1.0 min. Flow rate was 0.4 mL min^{-1}, injection volume 5 µL (in partial loop mode), the column temperature was maintained at 25 °C, the sample temperature at 8 °C and total run time was 4 min. Weak solvent was a mixture of 427.5 mL of water and 72.5 mL of acetonitrile, and strong solvent was pure acetonitrile. These solutions were stable for at least one week at room temperature.

2.6 *Mass spectrometric conditions*

ESI was performed in positive ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1-1.0 mg L^{-1}). Electrospray ionization spray voltage was 3000 V. Nitrogen was used as desolvation gas at 800 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 350 °C. Argon (99.999% purity) was used as collision gas at an approximate rate of 0.13 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the two functions are Inter Channel Delay: 5 ms and InterScan Time: 5 ms.

Table 1. MS parameters for ionisation with ESI + in MRM mode.

*Function 1 (0.0 to 1.0 min)				
Compound	Parent (Da)	Daughter (Da)	Cone (V)	Collision (eV)
Bet	118.0	57.9	35	22
		58.9	35	18
Gln	147.0	83.9	18	15
		130.0	18	10
Glu	148.1	83.9	18	15
		130.1	18	10
GABA	104.0	86.9	18	10
		68.9	18	15
PCho	184.0	86.1	30	20
		124.8	30	20
GPCho	258.0	104.1	30	17
		125.0	30	30
CDP-Cho	489.0	184.0	35	35
		264.0	35	25
*Function 2 (0.8 to 3.0 min)				
Compound	Parent (Da)	Daughter (Da)	Cone (V)	Collision (eV)
Cho	104.0	44.9	35	15
		59.9	35	15
AcCh	146.0	60.0	23	10
		86.9	23	15
DA	153.9	90.9	20	20
		137.0	20	10
NE	170.0	106.9	12	20
		152.0	12	8
SE	177.0	115.0	15	25
		160.0	15	10
Tyr	182.1	136.0	18	14
		165.0	18	10
E	183.9	107.0	15	22
		166.0	15	10
5-HIAA	192.0	146.1	25	17
		91.1	25	37
Trp	205.1	188.1	18	10
		146.0	18	18

* Dwell time (ms): 20; Delay (ms): 5

2.7 Quality parameters and definitions

Several quality parameters of the method have been assessed namely linearity, selectivity, accuracy and sensitivity. The concepts and definitions are as follows:

Linearity is defined by the intercept of the calibration curve (a), slope (b) and determination coefficient (R^2) in addition to P-value for lack-of-fit test ($\%P_{lof}$).

Selectivity. The specificity of the method was determined by comparing the blank chromatograms with those corresponding to the samples. Blank samples were aCSF without adding any component to the matrix.

Accuracy: precision and trueness. Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank aCSF samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolating from the standard calibration curve. Recoveries (R) were calculated by comparing the interpolated amounts to the theoretical amounts, spiked amounts. To evaluate the precision of the method, the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound (see Table 3). Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and were repeated for five days to determine inter-day variability.

Sensitivity. Limit of detection (LOD) and limit of quantification (LOQ) are two fundamental aspects that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. The LOD is the minimum amount of analyte detectable in the sample while the LOQ is the minimum amount that can be quantified. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives.

In this work, these parameters were calculated from the signal-to-noise ratio (LODs signal-to-noise ratio = 3, LOQ signal-to-noise ratio = 10) injecting several solutions of decreasing amounts of the compounds of interest spiked into blank aCSF samples.

3. Results and Discussion

3.1 Liquid chromatographic analysis

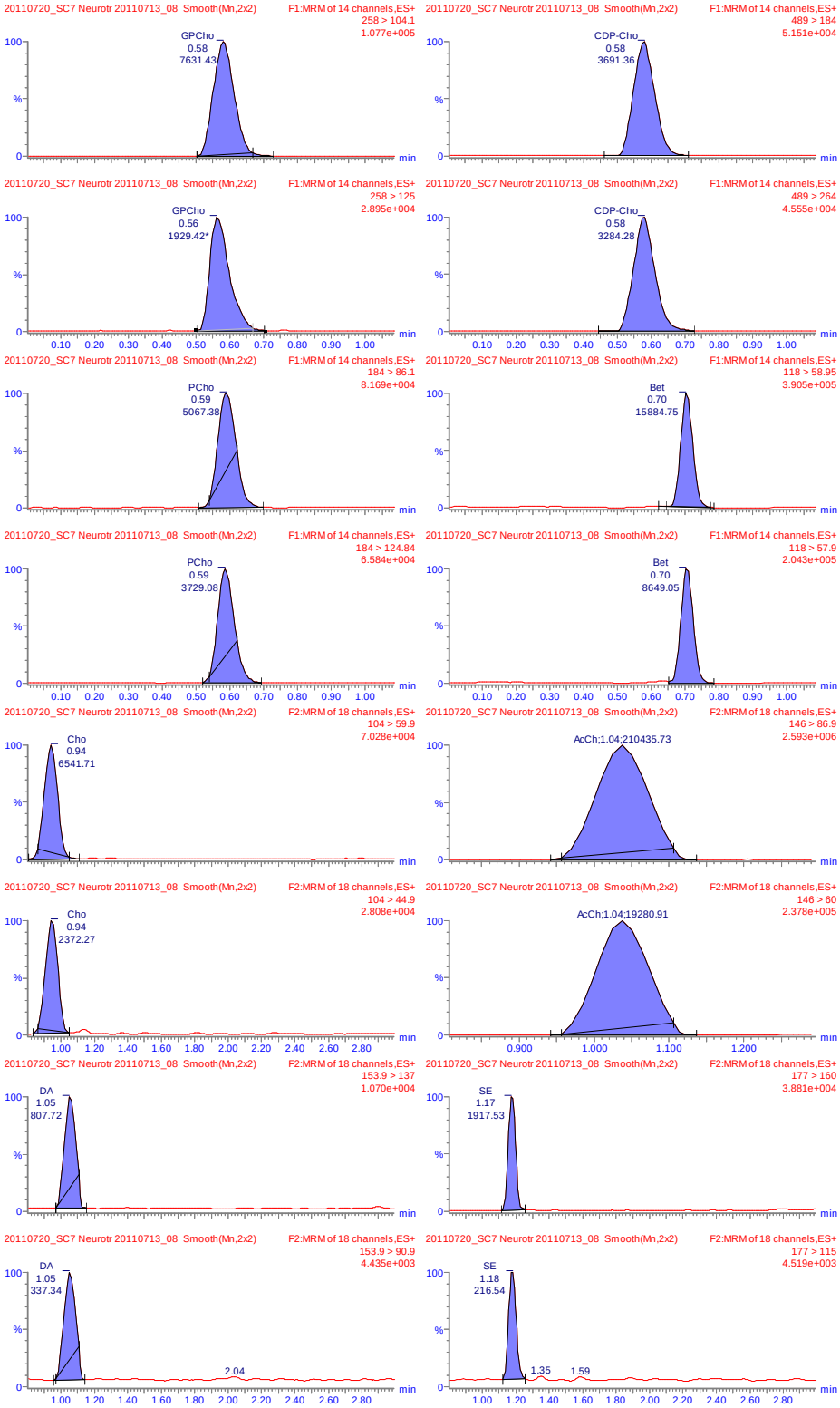
Initial experiments were designed to evaluate chromatographic conditions similar to those described previously in the literature [7-11, 14, 17, 31], but in this case, for an UPLC® instrument. Three liquid chromatography columns were tested: an Acquity UPLC BEH C18, an Acquity UPLC BEH HILIC, and an Acquity UPLC HSS T3. All three columns provided good resolution for the analytes. However, the BEH C18 column provided the best resolution in the shortest amount of time. Therefore, this column was selected. Different mobile phases were also studied in order to optimize the separation and peak shapes. With that objective in mind, two organic solvents (methanol and acetonitrile) commonly used in reversed-phase liquid chromatography were evaluated. Acetonitrile gave better results than methanol in terms of resolution and peak shape. Moreover, based on some of the previous methods mentioned above, several additives were evaluated (ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid, formic acid, HFBA) in order to improve the analytical signal and the resolution of the chromatographic peaks. The best separation was obtained using a mobile phase composed of water, acetonitrile, HFBA and concentrated formic acid in a proportion of 900:100:1:1 (v/v/v/v) as solvent A and 0.1% (v/v) of formic acid in acetonitrile as solvent B. The linear gradient described previously was used.

To improve the sensitivity of the method, a study was performed to evaluate the possibility of increasing the injection volume and to evaluate different modes of injection. Injection volumes ranging from 1 to 10 µL were studied. An extra broadening of the peaks was observed at volumes higher than 5 µL, therefore, this volume was selected. An investigation of injection

mode showed that full loop required a greater amount of sample compared to partial loop. Therefore, partial loop was chosen. The stability of the standards was also studied, which included an investigation of different additives to extend the expiration. Sodium thiosulfate, acetic acid, formic acid and several mixtures of acetonitrile, methanol and water were tested [15]. The results showed that a 10% (v/v) solution of formic acid in water was the most appropriate additive to prepare the stock solution. Microdialysate samples were prepared with 7 µM of eserine, to prevent the degradation of AcCh by inhibiting acetylcholinesterase. There is no agreement on the literature about the use of acetylcholinase inhibitors or the type of compound used [16, 28]. Nonetheless, we preferred to prevent degradation as no interferences were evident in treated samples.

3.2 *Mass spectrometric analysis*

The MS/MS detection was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and combined electrospray and atmospheric pressure ionization source (ESCI) interfaces in positive and negative modes were evaluated. ESI interface in positive mode was selected because it showed higher sensitivity for all compounds of interest. The response of two daughter ions (two reactions), one for quantification and the other for identification or confirmation, were monitored. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Figure 2 shows chromatograms of a standard mixture of compounds.


20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 258 > 104.1 1.077e+005
GPCho 0.58 7631.43
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 489 > 184 5.151e+004
CDP-Cho 0.58 3691.36
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 258 > 125 2.895e+004
GPCho 0.56 1929.42*
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 489 > 264 4.555e+004
CDP-Cho 0.58 3284.28
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 184 > 86.1 8.169e+004
PCho 0.59 5067.38
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 118 > 58.95 3.905e+005
Bet 0.70 15884.75
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 184 > 124.84 6.584e+004
PCho 0.59 3729.08
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F1:MRM of 14 channels,ES+ 118 > 57.9 2.043e+005
Bet 0.70 8649.05
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 104 > 59.9 7.028e+004
Cho 0.94 6541.71
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 146 > 86.9 2.593e+006
AcCh;1.04;210435.73
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 104 > 44.9 2.808e+004
Cho 0.94 2372.27
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 146 > 60 2.378e+005
AcCh;1.04;19280.91
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 153.9 > 137 1.070e+004
DA 1.05 807.72
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 177 > 160 3.881e+004
SE 1.17 1917.53
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 153.9 > 90.9 4.435e+003
DA 1.05 337.34
2.04
20110720_SC7 Neurotr 20110713_08 Smooth(Mn,2x2) F2:MRM of 18 channels,ES+ 177 > 115 4.519e+003
SE 1.18 216.54
1.35
1.59
min

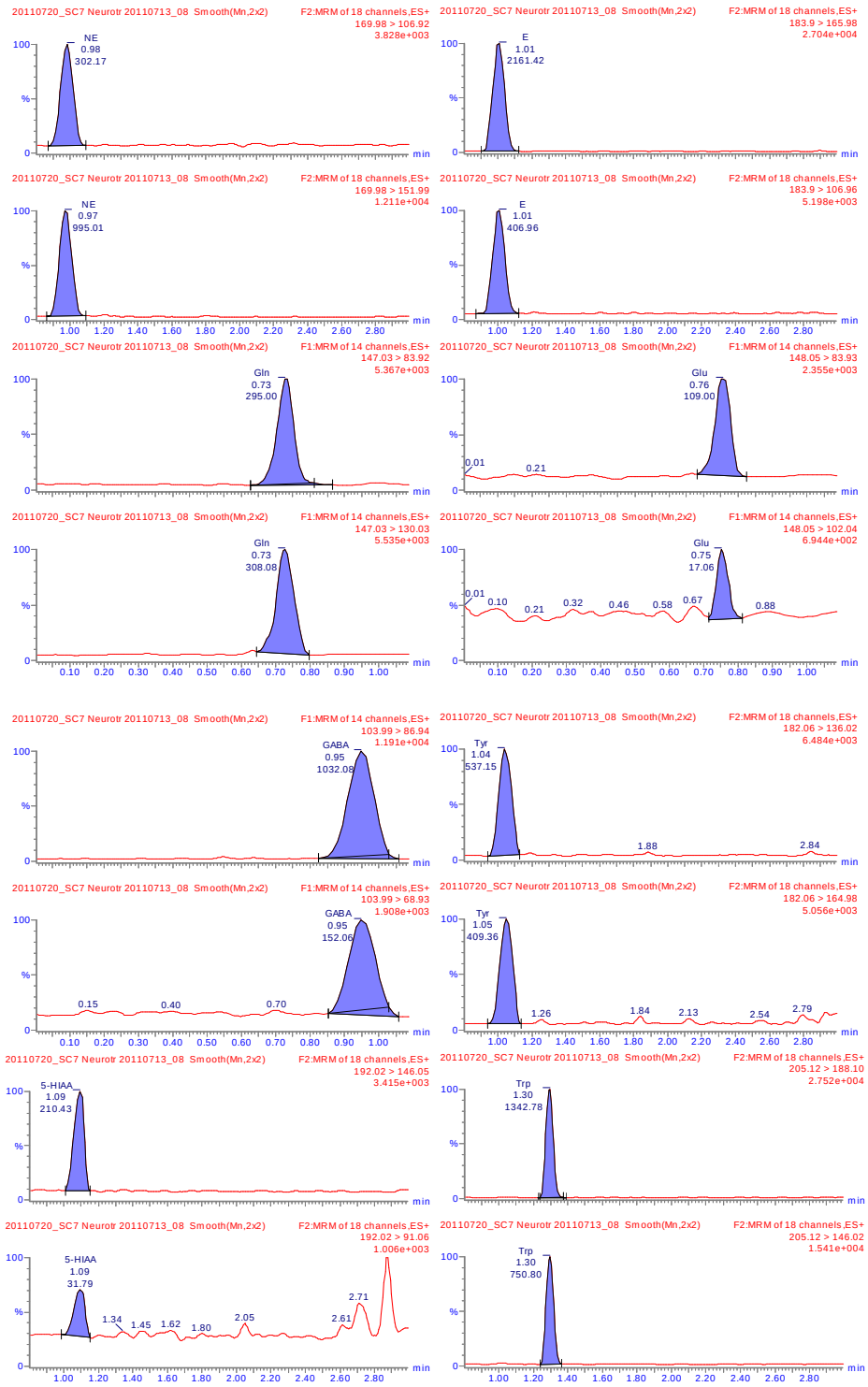


Fig. 2. Chromatogram of each analyte standard in aCSF (two transitions). The first transition was used for quantification and the second for confirmation.

3.3 Analytical performance

A calibration curve was obtained for each compound by injecting 5 µL of different standard solutions (prepared in aCSF) at concentrations ranging from 20-5000 ng mL^{-1} for GPCho, 200-5000 ng mL^{-1} for CDPCho, 50-5000 ng mL^{-1} for PCho, 20-2000 ng mL^{-1} for Bet, 3-1000 ng mL^{-1} for Cho, 0.3-1000 ng mL^{-1} for AcCh, 5-1000 ng mL^{-1} for DA and SE, 50-1000 ng mL^{-1} for NE, Gln, Glu and GABA, 20-1000 ng mL^{-1} for E, 3-1500 ng mL^{-1} for Tyr, 50-2000 ng mL^{-1} for 5-HIAA and 10-1500 ng mL^{-1} for Trp. Analytical performance was evaluated according to the recommendations of Analytical Methods Committee [33]. The *lack-of-fit* test was applied to two replicates and three injections of each standard (five concentration levels). The results are summarized in Table 2.

A quality control standard (WS4) was injected after every 20 injections to assure the validity of the calibration curve. The predicted value expected to not exceed ± 15% of the theoretical value.

Table 2. Analytical and statistical parameters.

	n	a	b (mL ng^{-1})	R^2 (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})	$\%P_{lof}$
GlyPCho	5	-32.8	2.482	99.8	5	20	20-5000	22.9
CDP-Cho	5	-25.3	0.770	99.2	50	200	200-5000	98.9
PhCho	5	-80.7	2.180	99.8	20	50	50-5000	12.8
Bet	5	-25.7	5.133	99.3	10	20	20-2000	49.1
Cho	5	79.1	31.97	99.1	1	3	3-1000	99.2
AcCho	5	285	283.7	99.8	0.1	0.3	0.3-1000	93.2
DA	5	33.3	3.270	99.7	2	5	5-1000	91.4
SE	5	-16.8	7.711	99.8	2	5	5-1000	93.4
NE	5	9.10	0.502	99.1	20	50	50-1000	51.7
E	5	-28.8	5.759	99.9	5	20	20-1000	62.1
Gln	5	2.60	0.633	99.9	20	50	50-1000	45.4
Glu	5	-14.0	0.405	99.2	20	50	50-1000	31.7
GABA	5	-12.6	1.675	99.5	20	50	50-1000	97.3
Tyr	5	-7.80	1.478	99.4	1	3	3-1500	77.7
5-HIAA	5	-17.0	0.683	99.5	20	50	50-2000	89.4
Trp	5	-34.7	4.365	99.3	3	10	10-1500	91.4

n, calibration levels; a, intercept; b, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; $\%P_{lof}$, P-value for lack-of-fit test.

3.4 Method validation

Validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [34].

Linearity. The values obtained for R^2 ranged from 99.1% for Cho to 99.9 % for Gln, and P_{lof} values were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

Selectivity. No interferences from endogenous substances were observed at the retention times of each respective analyte (Figure 2), which eluted at 0.55 min, 0.57 min, 0.58 min, 0.70 min, 0.73 min, 0.75 min, 0.92 min, 0.94 min, 0.97 min, 1.01 min, 1.04 min, 1.04 min, 1.05 min, 1.09 min, 1.17 min and 1.30 min for GPCho, CDPCho, PCho, Bet, Gln, Glu, GABA, Cho, NE, E, AcCh, Tyr, DA, 5-HIAA, SE, and Trp respectively. This finding suggested that the LC-MS/MS conditions provided sufficient selectivity.

Accuracy: precision and trueness. Recoveries and repeatability and within-laboratory reproducibility are summarized in Table 3. The recoveries were between 85% and 115% in all cases. Relative standard deviation values (RSD) were between 0.6% and 18.2%. The highest RSDs were for NE and 5-HIAA at 18.2 and 18%, respectively. This was due to the fact that their concentrations were close to or below the limit of quantification (LOQ). Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, where it should not deviate by more than 20%. Precision and trueness data indicated that the methodology to determine the target compounds in microdialysates from rat brain is highly accurate and precise, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Sensitivity. The calculated LODs (signal-to-noise ratio = 3) are in the range from 0.1 ng mL^{-1} for AcCh to 50 ng mL^{-1} for CDPCho, and the corresponding LOQs (signal-to-noise ratio = 10) ranged from 0.3 ng mL^{-1} to 200 ng mL^{-1}, respectively. The values obtained are shown in Table 2.

Table 3. Recovery, precision and accuracy of target compounds in microdialysate samples.

		Intra-day					Inter-day				
	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n
GPCho	335.3	317.4	16.6	94.7	5.2	3	372.7	43.5	111.2	11.7	15
	670.6	622.9	32.3	92.9	5.2	3	607.5	19.6	90.6	3.2	15
	1342.0	1291.7	90.9	96.3	7.0	3	1174.6	90.8	87.6	7.7	15
CDPCho	420.2	359.9	8.8	85.6	2.4	3	369.7	25.4	88.0	6.9	15
	840.4	801.7	91.2	95.4	11.4	3	715.6	58.9	85.1	8.2	15
	1680.0	1435.5	49.3	85.4	3.4	3	1448.3	55.4	86.2	3.8	15
PCho	264.1	246.9	23.5	93.5	9.5	3	260.7	10.7	98.7	10.7	15
	528.2	499.2	57.2	94.5	11.5	3	496.3	29.5	93.9	5.9	15
	1056.0	975.5	46.5	92.3	4.8	3	1021.0	94.3	96.6	9.2	15
Bet	77.3	77.4	6.6	100.2	8.6	3	86.7	11.4	112.2	13.2	15
	154.6	145.9	18.4	94.4	12.6	3	132.4	9.1	85.7	6.9	15
	309.2	267.1	27.0	86.4	10.1	3	262.8	21.1	85.0	8.0	15
Cho	36.6	33.5	2.0	91.5	5.9	3	36.0	2.3	98.2	6.5	15
	73.2	66.1	4.0	90.2	6.0	3	65.7	6.5	89.6	9.9	15
	146.4	127.9	15.0	87.2	11.7	3	125.9	5.1	85.9	4.0	15
AcCh	157.6	160.5	18.1	101.8	11.3	3	167.7	14.9	106.4	8.9	15
	315.2	306.8	34.8	97.3	11.3	3	336.7	38.1	106.8	11.3	15
	630.4	575.9	28.1	91.3	4.9	3	577.8	36.9	91.6	6.4	15
DA	36.6	36.2	5.3	98.8	14.7	3	35.0	5.0	95.6	14.4	15
	73.2	83.3	6.3	113.6	7.6	3	68.8	9.9	93.9	14.4	15
	146.4	135.6	19.5	92.5	14.4	3	148.5	19.4	101.3	13.0	15
SE	36.6	35.6	4.7	97.2	13.2	3	34.6	4.0	94.4	11.6	15
	73.2	80.1	0.5	109.2	0.6	3	73.0	10.8	99.5	14.8	15
	146.4	139.4	20.1	95.1	14.5	3	166.0	24.0	113.2	14.5	15
NE	77.3	82.8	15.0	107.2	18.2	3	75.5	8.7	97.6	11.6	15
	154.6	163.0	14.5	105.5	8.9	3	147.1	21.0	95.2	14.3	15
	309.2	304.1	13.6	98.4	4.5	3	320.9	34.8	103.8	10.9	15
E	50.7	55.8	4.8	109.9	8.6	3	53.1	6.5	104.8	12.2	15
	101.4	97.0	13.4	95.6	13.8	3	112.0	16.2	110.4	14.5	15
	202.8	203.3	29.0	100.2	14.2	3	218.8	28.6	107.8	13.1	15
Gln	52.4	45.6	0.6	87.1	1.2	3	51.5	6.8	98.2	13.2	15
	104.8	100.5	2.3	95.9	2.2	3	92.5	6.6	88.3	7.2	15
	209.6	191.1	18.7	91.2	9.8	3	184.8	12.8	88.2	6.9	15

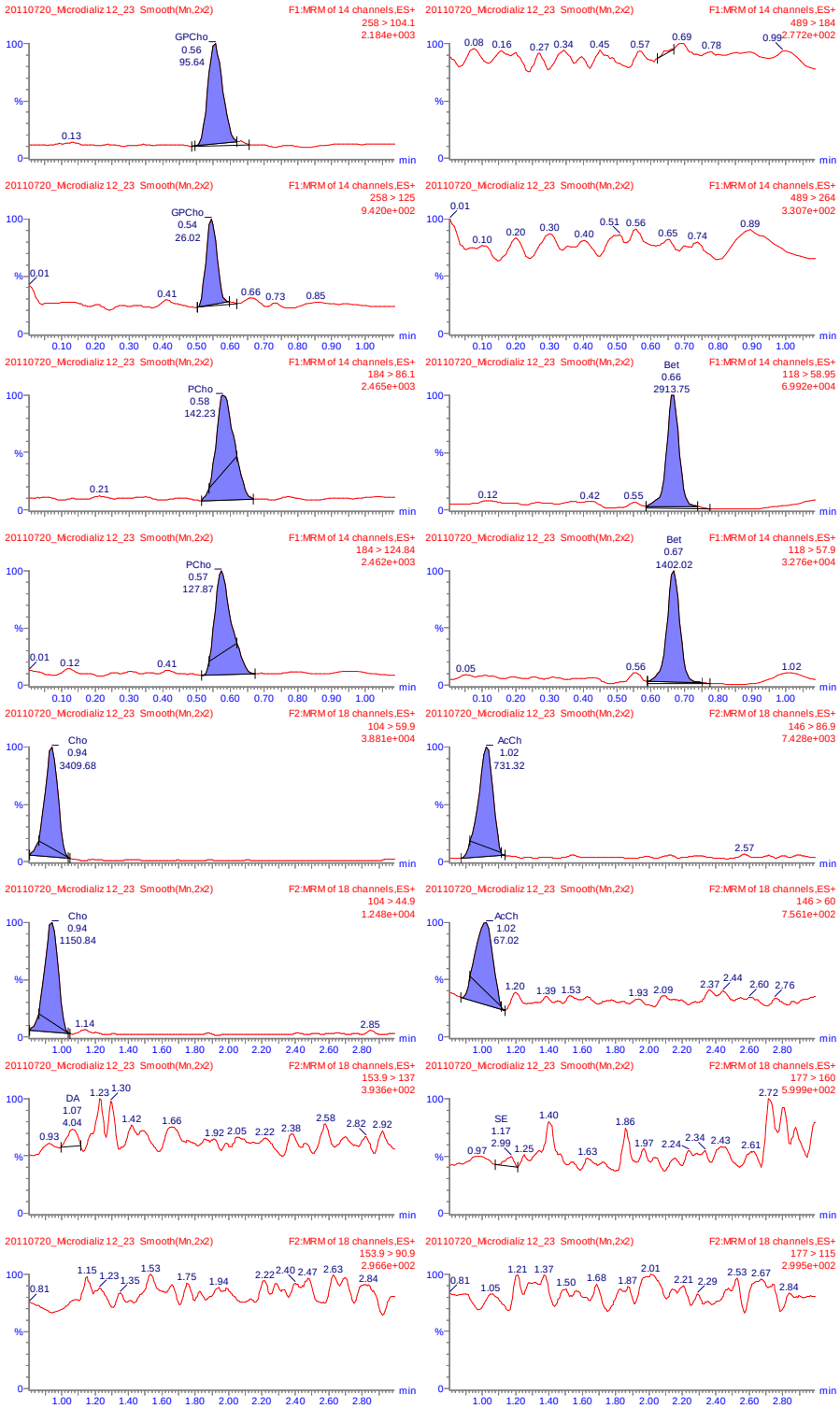
Table 3 (cont). Recovery, precision and accuracy of target compounds in microdialysate samples.

	Intra-day						Inter-day				
	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n
Glu	57.1	57.9	5.1	101.3	8.9	3	52.6	5.4	92.1	10.2	15
	114.2	106.8	5.4	93.5	5.0	3	96.9	14.6	84.8	15.0	15
	228.4	228.8	33.5	100.2	14.6	3	201.2	30.8	88.0	15.3	15
GABA	60.6	54.9	6.8	90.6	12.4	3	55.9	6.9	92.2	12.3	15
	121.2	124.9	13.7	103.1	10.9	3	120.6	12.2	99.6	10.1	15
	242.4	254.9	6.8	105.2	2.7	3	242.4	37.0	100.0	15.2	15
Tyr	57.1	65.5	9.5	114.6	14.4	3	54.5	7.5	95.4	13.7	15
	114.2	126.0	13.9	110.3	11.0	3	130.6	18.7	114.3	14.3	15
	228.4	229.5	29.4	100.5	12.8	3	211.0	20.3	92.3	9.6	15
5-HIAA	77.27	73.4	7.5	95.0	10.2	3	68.6	10.1	88.7	14.7	15
	154.5	157.3	28.4	101.8	18.0	3	164.2	14.3	106.2	8.7	15
	309.0	317.2	14.2	102.6	4.5	3	301.375	25.3	97.5	8.4	15
Trp	52.4	59.9	8.7	114.4	14.6	3	54.6	7.3	104.2	13.4	15
	104.8	117.0	13.2	111.7	11.3	3	108.3	10.5	103.4	9.7	15
	209.6	238.3	6.5	113.8	2.7	3	204.0	30.3	97.4	14.9	15

[a] Mean value; SD, standard deviation; R, % recovery; RSD, relative standard deviation; n, number of determinations

3.5 Application of the method

Samples of microdialysates from rat brain were collected following the protocol previously described. The main objective of the work was to determine the time-course of neurotransmitters when different ingredients (individually or together) were supplied to the animals orally by gavage or locally into the brain. Figure 3 shows chromatograms obtained for a microdialysate sample. Under the experimental conditions, AcCh, Cho, GPCho, PCho, Bet, Tyr, Gln, Glu and Trp were detected. Figure 4 and Figure 5 show examples of the time-course of some neurotransmitters when two different stimuli are used. Only those neurotransmitters that were related to the stimuli or that changed during the protocol are shown.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2.184e+003
GPCho
0.56
95.64
0.13
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
489 > 184
2.772e+002
0.08 0.16 0.27 0.34 0.45 0.57 0.69 0.78 0.99
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
258 > 125
9.420e+002
GPCho
0.54
26.02
0.01 0.41 0.66 0.73 0.85
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
489 > 264
3.307e+002
0.01 0.10 0.20 0.30 0.40 0.51 0.56 0.65 0.74 0.89
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
184 > 86.1
2.465e+003
PCho
0.58
142.23
0.21
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
118 > 58.95
6.992e+004
Bet
0.66
2913.75
0.12 0.42 0.55
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
184 > 124.84
2.462e+003
PCho
0.57
127.87
0.01 0.12 0.41
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F1:MRM of 14 channels,ES+
118 > 57.9
3.276e+004
Bet
0.67
1402.02
0.05 0.56 1.02
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
104 > 59.9
3.881e+004
Cho
0.94
3409.68
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
146 > 86.9
7.428e+003
AcCh
1.02
731.32
2.57
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
104 > 44.9
1.248e+004
Cho
0.94
1150.84
1.14
2.85
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
146 > 60
7.561e+002
AcCh
1.02
67.02
1.20 1.39 1.53 1.93 2.09 2.37 2.44 2.60 2.76
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
153.9 > 137
3.936e+002
DA
1.07
4.04
0.93 1.23 1.30 1.42 1.66 1.92 2.05 2.22 2.38 2.58 2.82 2.92
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
177 > 160
5.999e+002
SE
1.17
2.99
0.97 1.25 1.40 1.63 1.86 1.97 2.24 2.34 2.43 2.61 2.72
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
153.9 > 90.9
2.966e+002
0.81 1.15 1.23 1.35 1.53 1.75 1.94 2.22 2.40 2.47 2.63 2.84
20110720_Microdializ 12_23 Smooth(Mn,2x2)
F2:MRM of 18 channels,ES+
177 > 115
2.995e+002
0.81 1.05 1.21 1.37 1.50 1.68 1.87 2.01 2.21 2.29 2.53 2.67 2.84
100
%
0
min
1.00 1.20 1.40 1.60 1.80 2.00 2.20 2.40 2.60 2.80
0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90 1.00

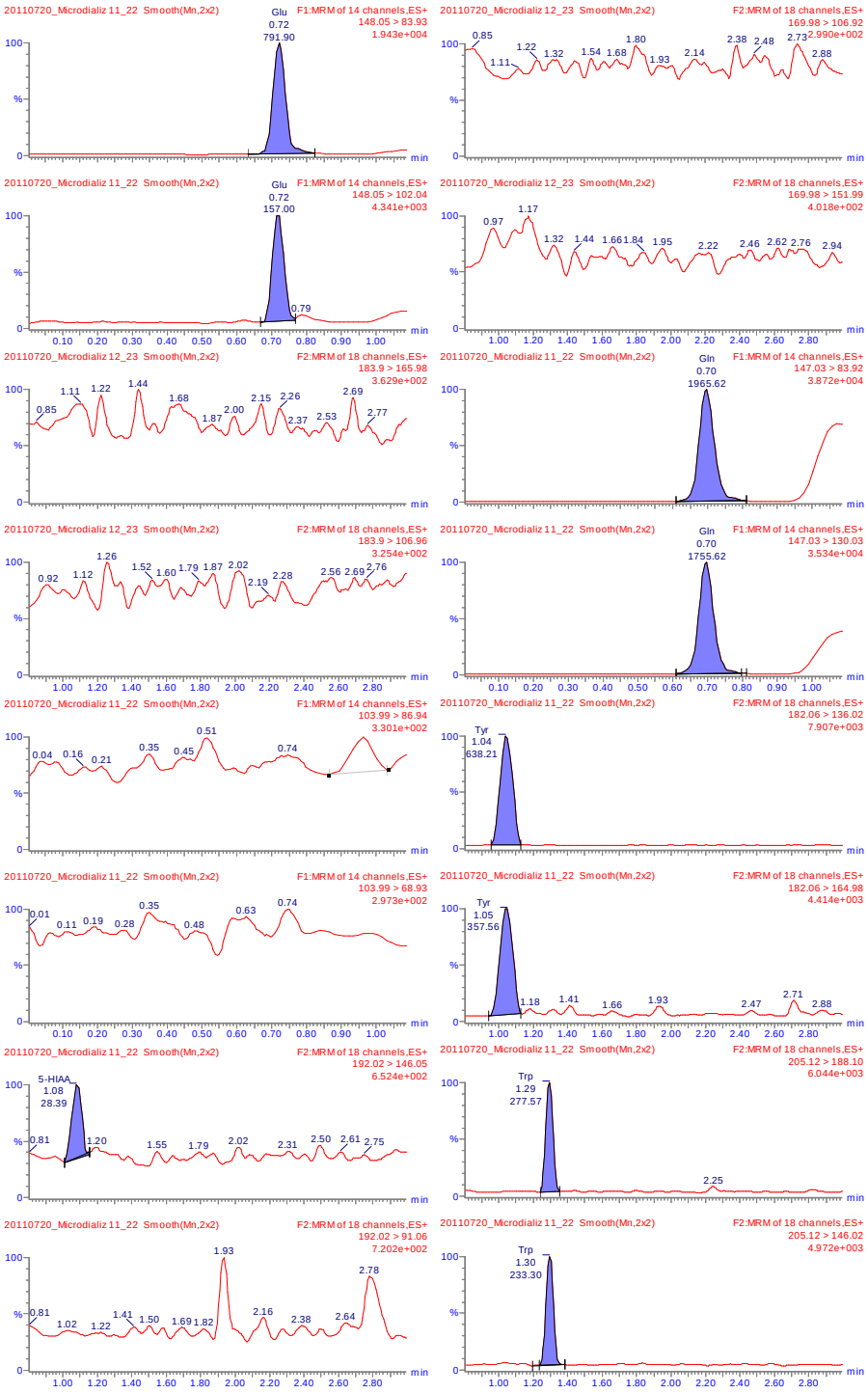

Fig. 3. Chromatograms of each analyte in a rat microdialysate sample (two transitions). The first transition of each analyte was used for quantification and the second for confirmation.

Figure 4 displays the progress of AcCh, SE, Cho, PCho and GPCho in the brain of an animal for 200 min, during which 100 mM KCl was added to the perfusion media at 140 min after the beginning of the experiment. It is known that KCl stimulates specific areas of the brain to release AcCh and SE [34, 35]. As expected, AcCh and SE displayed a rapid increase 20 min after the addition of the KCl, with a peak at 180 min and only 40 min after the addition. Moreover, SE also increased at zero time likely due to the stress of the animal after manipulation and/or to the stress of the tissue after the insertion of the probe. Similarly, Cho, PCho, and GPCho also increased at the beginning of the experiment although they were not affected by the addition of KCl and remained stable over time.

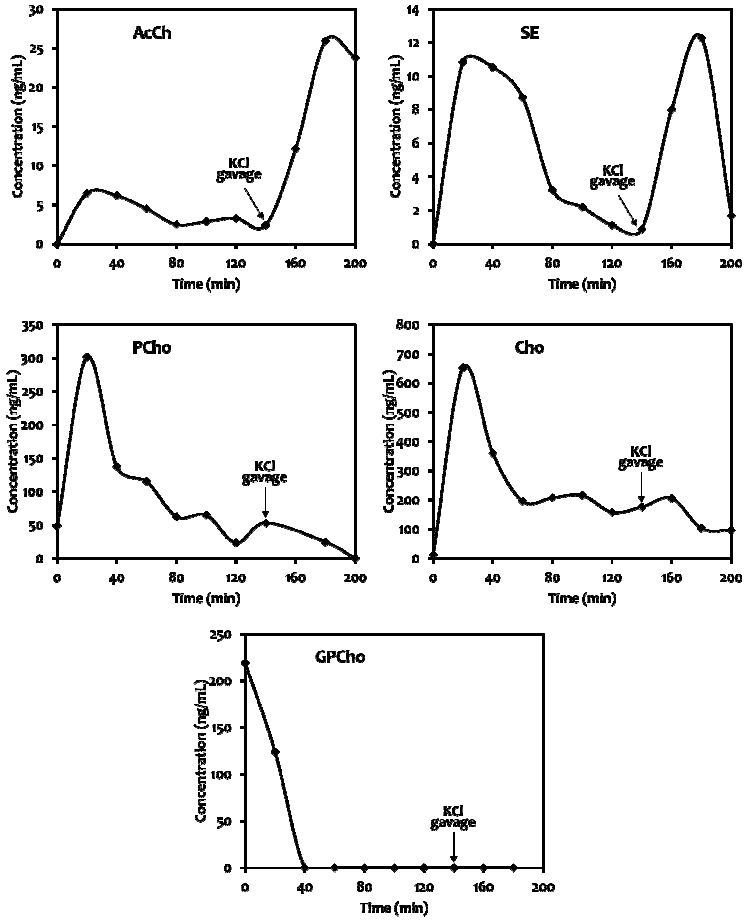

Fig. 4. Time-course of acetylcholine and other metabolites in rat hippocampus after the addition of KCl.

Figure 5 shows AcCh and Cho overflow from rat hippocampus over 260 min. The data are expressed as % of basal release (mean of samples 60, 80 and 100 min as a basal reference). Rats received a gavage of D-glucose (1g/kg body weight) at the end of sample 100 min. A two-fold increase in AcCh overflow was observed 40 min after the gavage, but it progressively returned to baseline values over the ensuing 2 h period. Choline showed a decrease of two-thirds of the baseline concentration at 60 min after the gavage, but gradually returned to baseline level over the ensuing 40 min.

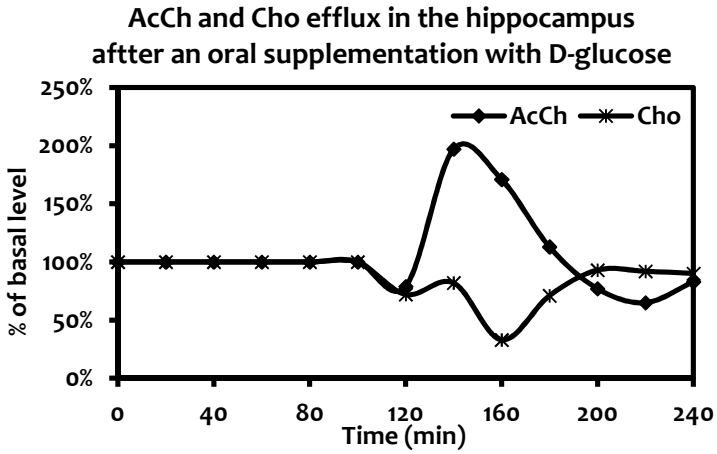

Fig. 5. Time-course of acetylcholine and choline in rat hippocampus after an oral bolus of D-glucose (1.0g / kg of body weight). Values are expressed as % of basal release (mean of samples 60, 80 and 100 min as a basal reference).

The observed increase in AcCh levels agrees with the fact that AcCh synthesis is directly dependent on glucose supply (glucose is critical for the production of acetyl-CoA, a precursor of acetylcholine) [36]. In fact, there are a number of hypotheses about the physiological bases of the memory-improving action of glucose. Glucose sources could alleviate localized deficits in extracellular glucose in the hippocampus. Extracellular brain glucose concentrations vary with neuronal activity, indicating that glucose may be critical in modulating memory functioning. Moreover, it has been reported that hippocampal AcCh release increases in rats during a spatial task [37]. AcCh is rapidly hydrolyzed to free Cho and acetate into synapses by the enzyme, acetylcholinesterase. This process terminates the physiologic actions of the

neurotransmitter [38]. The observation of a decrease in the concentration of Cho, after an increase of AcCh, could be explained as a high activation of the reuptake process to replenish the intracellular levels of AcCh. Most of the free Cho liberated by the intrasynaptic hydrolysis of AcCh is taken back into its nerve terminal of origin by the high-affinity Cho transporter, and either reacetylated to form AcCh or phosphorylated for ultimate conversion to membrane PCho [39].

4. Conclusions

The proposed method is a powerful tool for the simultaneous determination of 16 compounds, including neurotransmitters of different chemical families and related metabolites. With this improvement, several previous methods have been combined into a single method, which minimizes the time and total cost of the analysis. The simplicity and sensitivity of the new method makes it possible to do an *in vivo* study of changes in chemical signals in the brain of an animal. Therefore, it is helpful in studies of neurotransmitters released in the brain when different ingredients or external agents are administered or when specific situations or activities are monitored, such as learning, memorizing, stress situations, etc. In summary, the proposed method provides opportunities to study all of these components in wide range of situations.

Acknowledgments

The authors are indebted to all the participants, without whom this work would not have been possible. We are grateful to the team of technicians of Abbott which helped in the tasks of care and maintenance of animals and in the surgical process that allowed the microdialisis experiments, especially María Luisa Jiménez López. We are also grateful to Dr. Miguel Alfonso Pallares (Department of Functional Biology and Health Sciences, University of Vigo) and his group for teaching us microdialysis procedures and sharing their knowledge. Finally, authors are grateful to Dr. Dustin Starkey (Senior Scientist,

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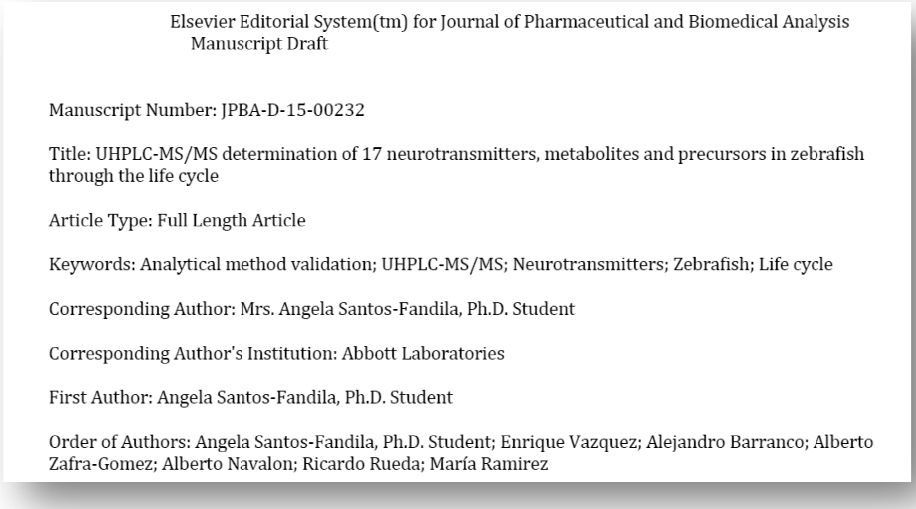
I.4.4 Determinación de Neurocompuestos en Pez Cebra (Publicación II)

En este apartado se presenta el trabajo de investigación desarrollado para la determinación de diferentes neurocompuestos en pez cebra en las distintas etapas de la vida del animal. El trabajo ha sido enviado a la revista de **Journal of Pharmaceutical and Biomedical Analysis** de la editorial Elsevier con fecha 05/02/2015.



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Title: UHPLC-MS/MS determination of 17 neurotransmitters, metabolites and precursors in zebrafish through the life cycle

Article Type: Full Length Article

Keywords: Analytical method validation; UHPLC-MS/MS; Neurotransmitters; Zebrafish; Life cycle

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Figura I.15. *UHPLC-MS/MS determination of 17 neurotransmitters, metabolites and precursors in zebrafish through the life cycle. Draft of Journal of Pharmaceutical and Biomedical Analysis (factor de Impacto, 2.829)*

UHPLC–MS/MS determination of 17 neurotransmitters, metabolites and precursors in zebrafish through the life cycle

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ABSTRACT

An ultrahigh performance liquid chromatography–tandem mass spectrometry method for the identification and quantification of neurotransmitters, metabolites and precursors at different stages in zebrafish life was developed. Betaine, glutamine, glutamic acid, gamma-aminobutyric acid, phosphocholine, glycerophosphocholine, cytidine 5′-diphosphocholine, choline, acetylcholine, dopamine, norepinephrine, serotonin, tyrosine, epinephrine, tryptophan, 5-hydroxyindolacetic acid and agmatine were selected as analytes. The method consisted of a deproteinization using methanol and formic acid, subsequent injection onto the chromatographic equipment and quantification with a triple quadrupole mass spectrometer detector using positive mode electrospray ionization. The limits of detection ranged from 0.02 to 11 ng mL^{-1} and the limits of quantification from 0.1 to 38 ng mL^{-1} depending on the analyte. The inter- and intra-day variability was lower than 15%. Recovery rates ranged from 85% to 115%. The method was applied to the measurement of these compounds in zebrafish from early stages of development to adulthood and showed the time-course of neurotransmitters and others neurocompounds through the life cycle.

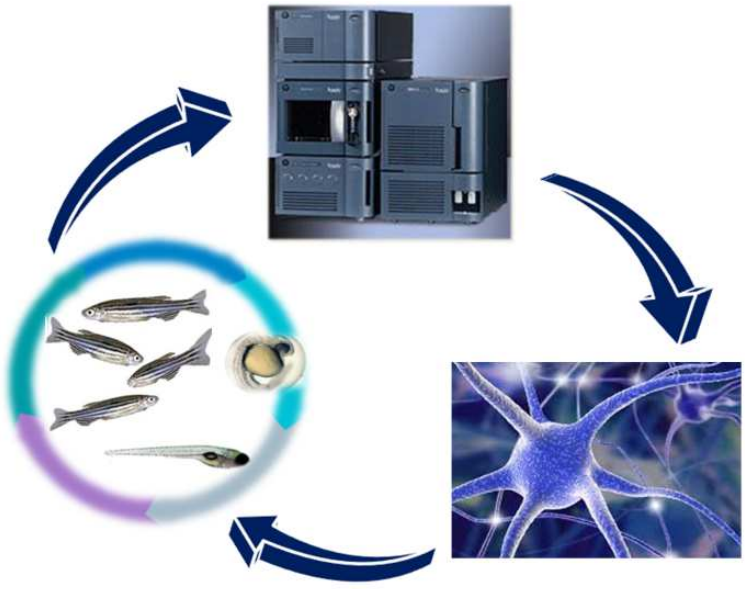
Keywords:

Analytical method validation; UHPLC-MS/MS; Neurotransmitters; Zebrafish; Life cycle

Highlights:

- Determination of 17 neurotransmitters, metabolites and precursors using UHPLC-MS/MS.
- The sample treatment is a simple deproteinization using methanol and formic acid.
- Validation of the method: quality parameters, accuracy (trueness and precision).
- Analysis of compounds in zebrafish from early stages of development to adulthood.
- The time-course of the neurocompounds through the life cycle of the animal is showed.

Graphical Abstract:



1. Introduction

Since its introduction as an experimental model to study development and genetics in 1981 [1], the zebrafish has become a promising tool in many research areas. Drug discovery studies usually go from screening in cell cultures to *in vivo* effects in rodent models or superior mammals, and to clinical studies in humans. This model provides an intermediate step between cell culture and rodents, allowing the study of complex processes such us organ development. The simplicity of evaluating morphological, biochemical and physiological information at all stages of life and the ability to evaluate the brain and nervous system in early stages of development have made the zebrafish an excellent model to study chemical exposure in general and neurotoxicity in particular [2-6]. Recently, this model has been also used for the study of nutritional ingredients such as vitamin E or docosahexaenoic acid (DHA) [7-9], as well as to study human diseases including cognitive decline and Alzheimer [10]. Zebrafish shows genetic and anatomic conservation with both mice and humans and a high degree of genetic homology [11,12]. In addition, the role of different excitatory and inhibitory neurotransmitter systems in the zebrafish has been reviewed by Rico et al. showing that all human neurotransmitters are preserved in zebrafish with similar biological roles [13,14]. Advantages such as easy management, cost reduction, similarity to the human genome, similarity of the neurotransmitters system, etc. have made the zebrafish a very interesting model in research projects aimed at deepening into the knowledge of neurotransmitters. For these reasons, the zebrafish is gaining popularity as an excellent model to complement translational biomedical topics and specifically neuroscience research [15-24].

Neurotransmitters are biochemical messengers that are involved in the transmission of the synaptic information. They are directly responsible for the transfer of information from one neuron to another nearby; and also they participate in different functions related with signals between neurons and other cells in the body. Neurotransmitters are involved in brain development, maintenance of normal brain functionality and cognitive decline. In fact, changes in neurotransmitter levels produce regional selective changes

indirectly via their trophic actions during development [25]. Moreover, neurotransmitter levels are altered in psychiatric disorders [26] and Alzheimer's disease [27-29]. In the present work, an ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS) method for the quantification of compounds of different families of neurotransmitters and neurocompounds within the ultra trace range was developed and validated. The method consists of a multi-analyte approach for the measurement of neuromediators with chemical structures related to aminoacids, including tyrosine (Tyr), glutamine (Gln), glutamic acid (Glu), gamma-aminobutyric acid (GABA) and tryptophan (Trp); and monoamines, including acetylcholine (AcCh) and metabolites such as choline (Cho), glycerophosphocholine (GPCho), cytidine 5′-diphosphocholine (CDPCho), phosphocholine (PCho), betaine (Bet); catecolamines such as dopamine (DA), epinephrine (E), norepinephrine (NE); indolamines such as serotonin (SE), and its metabolite 5-hydroxyindolacetic acid (5-HIAA). In addition, agmatine (Agm), a derivative of L-arginine that is an endogenous neuromodulator [30], was also included.

A number of methods have been developed for the determination of these compounds in biological matrices using a wide range of techniques, reagents, additives, derivatization procedures, equipment, and detectors as mass spectrometry in several modes, among other detectors. However, those methods are limited by the number of compounds that can be analyzed simultaneously, and to our knowledge they are not applicable to the matrix for which the proposed method was validated (zebrafish). This novel method of multi-analyte detection has the advantage of measuring a higher number of compounds at the same time with acceptable LODs and higher selectivity than methods based on LC with electrochemical, ultraviolet or fluorescence detection previously proposed in the scientific literature.

The main objective of the present work was to apply the validated method for the measurement of 17 neurocompounds in zebrafish during the life cycle in the segmentation period (embryos of 24 hours post fecundation -hpf-), pharyngula period (embryos of 48 hpf), hatching period (larvaes of 72 hpf),

early larvae (120 hpf) and mature larvaes (15 days post fecundation -dpf-), juvenile (30 dpf) and adult zebrafish (3 months post fecundation -mpf-).

2. Materials and methods

2.1. Materials

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Bet, Gln, Glu, GABA, PCho, GPCho, CDP-Cho, Cho, AcCh, DA, NE, SE, Tyr, E, 5-HIAA and Agm were supplied by Sigma-Aldrich (Madrid, Spain). Eserine and Trizma®-HCl from Sigma-Aldrich. EtOH, MeOH, MeCN, $CHCl_3$, IPA, HFBA and FA additives of LC-MS grade, were purchased from Scharlab (Barcelona, Spain). Stock Solution and Work Standard for calibration were prepared following the protocols of the published article above mentioned [30], with the exception of Intermediate Solutions and Work Solution which were prepared with 1% FA in MeOH. A solution of 0.1 N Tris-HCl (pH 7.40) was prepared for use in the measurement of protein.

2.2. Instrumentation

In these experiments, an UPLC® Acquity from Waters (Milford, MA, USA) equipped with the same options as in our previous work mentioned above, was used. The chromatograph was also connected on-line to a triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. Auxiliary apparatuses were: Thermomixer® Comfort from Eppendorf (Hamburg, Germany), microfuge MIKRO 200R from Hettich (Tuttlingen, Germany), Analytical balance with a precision of 0.1 mg AE200 from Mettler Toledo (Toledo, Spain), eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom), pipette Microman® from Gilson (Middleton, USA), Pasteur glass pippetes, vortex-mixer, spectrofotometer NanoDrop® 2000c de Thermo Scientific (Wilmington, DE, USA) and maximum recovery LC vials and screw caps from Waters.

2.3. Animal manipulation

Zebra fishes were obtained from Neuron Bio (Granada, Spain). The species used for this assay was the zebrafish (Danio rerio), AB strain. The maintenance and care of the reproductive and adult fish was performed according Quality Certification under R&D Management System Certification UNE 166002: 2006 standard. Only embryos and larvae without any external abnormalities were used for the assays. They were placed in a microtube, placing five individual with 200 µL of lysis buffer (Tris-HCl 0.1 M containing 10 µM of BW284c5 from Sigma-Aldrich to inhibit acetylcholinesterase activity). Embryos/larvae were mechanically homogenized to be used to determine the neurotransmitter levels. Samples were stored frozen at -80 °C until analysis. And on the other hand similar procedures were carried out with one individual in each microtube of 15 dpf larva, 30 dpf juvenile and 3 mpf adult fish. Homogenized samples were processed following the description bellow for all the stages of development, except for 3 months adults where the head was dissected from the body and both samples were processed separately.

2.4. Sample preparation

Samples included the following stages of zebrafish: embryo 24 hpf, embryo 48 hpf and embryo 72 hpf; larva 96 hpf, larva 120 hpf and larva 15 dpf; alevin 1 mpf and adult 3 mpf, in which was analyzed the body and brain separately. The homogenates were made of 5 animals, except the three last stages in which only 1 animal was included per homogenate. A n=5 were determined in each stage. The homogenates were prepared in 200 µL 0.1 M Tris-HCl buffer containing 10 µM BW284c5 (Sigma-Aldrich) to inhibit acetylcholinesterase activity. Samples were stored frozen at -80 °C until analysis. Before analysis, samples were defrosted at room temperature and then vigorous stirred and homogenized. Aliquots of 25 µL were taken and placed into 1.5 mL eppendorf, where 175 µL of 1% FA in MeOH were added. After stirring for 5 minutes at 8 °C 1300 rpm in Thermomixer®, samples were centrifuged at 10 °C and 14000 rpm for 5 minutes more. Finally, the supernatant was pipetted off using a Pasteur pipette and placed into UHPLC vials maximum

recovery. For the samples of alevin and adult body, a further dilution was performed: 20 µL of the first dilution were placed in a clean vial maximum recovery and 380 µL of 1% FA in MeOH were added.

2.5. *Liquid-chromatographic mass spectrometric analysis*

Chromatographic separation was performed as follows. UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size) from Waters; a gradient mobile phase consisting of a mixture of water, MeCN, HFBA and FA in a proportion of 900:100:1:1 (v/v/v/v) as solvent A, and 0.1% (v/v) of FA in MeCN as solvent B; 0.0-1.0 min, 5 to 50% B; 1.0-2.0 min, 50 to 95% B; 2.0-3.0 min, 95% B; and back to 5% B in 1.0 min; flow rate was 0.4 mL min^{-1}; injection volume 5 µL (in partial loop mode); column temperature at 25 °C; sample temperature at 8 °C; total run time of 4 min; weak solvent was a mixture of 427.5 mL of water and 72.5 mL of MeCN; and strong solvent was pure MeCN. In routine, a calibration curve was injected at the beginning and after every 30 injections, and three quality control standards (low, middle and concentrated) were injected after every 15 injections to assure the validity of the calibration curve. The lack-of-fit test was applied in the validation of the method to two replicates and three injections of each standard according to recommendations of Analytical Methods Committee [31].

For MS analysis, electrospray ionization (ESI) was performed in positive ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1-1.0 mg L^{-1}). Electrospray ionization spray voltage was 3000 V. Nitrogen was used as desolvation gas at 800 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 350 °C. Argon (99.999% purity) was used as collision gas at an approximate rate of 0.13 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the two functions are Inter Channel Delay: 5 ms and InterScan Time: 5 ms.

2.6. Protein determination

Samples for the determination of proteins were measured directly in Nanodrop®, except for the body of adult fish that was diluted 1:20 (v/v) with 0.1 N Tris-HCl at pH 7.4. Protein determination was carried out using the "Protein A280" method, previously programmed into the instrument by the manufacturer. A volume of 2 µL was analyzed. For proteins analysis, a method with general reference setting based on the absorbance produced by a solution of 0.1% protein (1 mg mL^{-1}) which produces at 280 nm 1.0 Abs, being 1 Abs = 1 mg mL^{-1}, was selected. Additionally, the predetermined wavelength for bichromatic normalization was 340 nm.

3. Results and discussion

3.1. Liquid chromatographic-mass spectrometric analysis

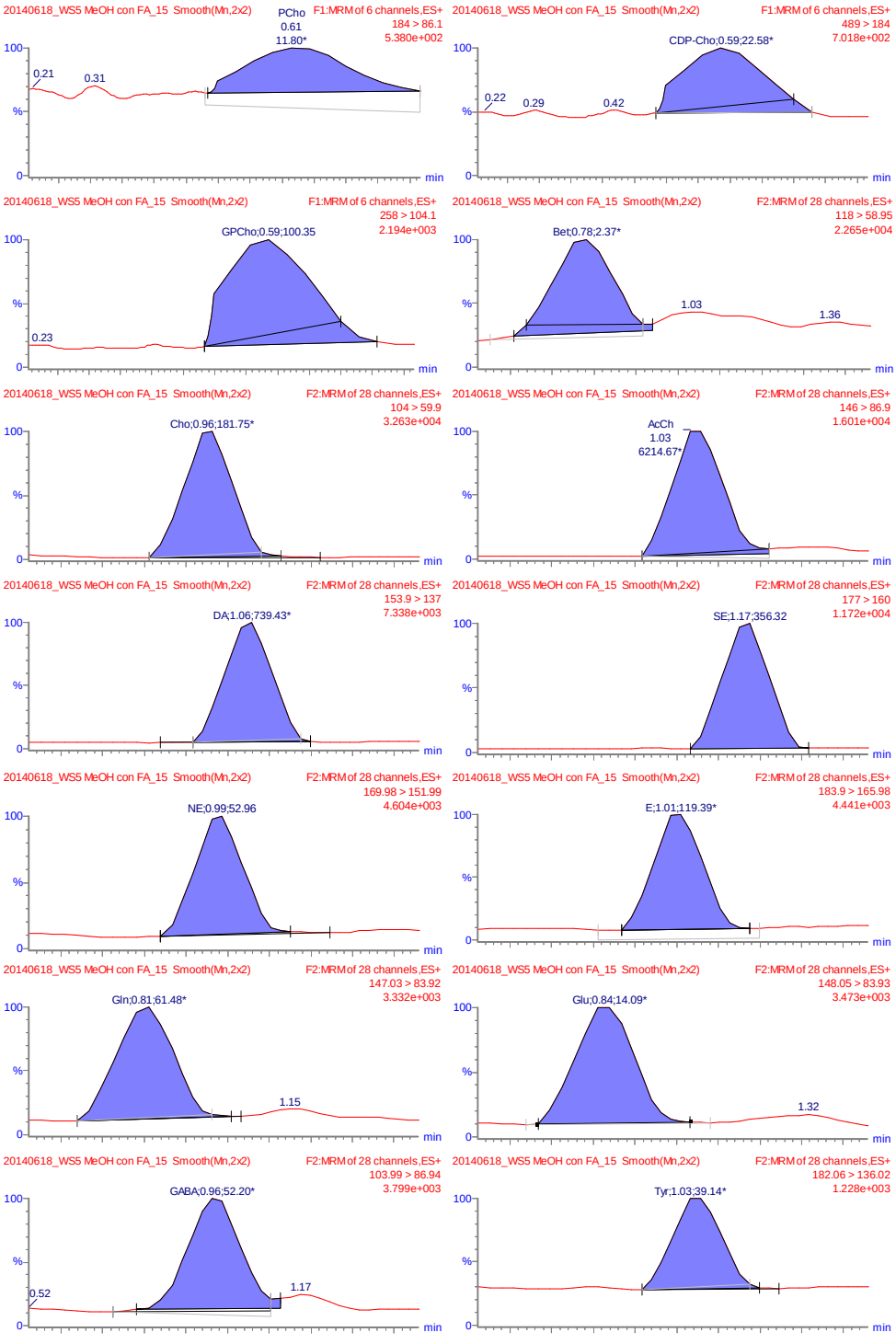
Three liquid chromatography columns were tested: an Acquity UPLC BEH C18, an Acquity UPLC BEH HILIC, and an Acquity UPLC HSS T3. All three columns provided good resolution for the analytes; however, the best separation was obtained using a BEH C18 column. Different mobile phases were also studied in order to optimize the separation and peak shapes. Two organic solvents (methanol and acetonitrile) commonly used in reversed-phase liquid chromatography were evaluated. Acetonitrile gave better results than methanol in terms of resolution and peak shape. Moreover, based on previously published methods, several additives were evaluated (ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid, formic acid, HFBA) in order to improve the analytical signal and the resolution of the chromatographic peaks. The best separation was obtained using a mobile phase composed of water, acetonitrile, HFBA and concentrated formic acid in a proportion of 900:100:1:1 (v/v/v/v) as solvent A and 0.1% (v/v) of formic acid in acetonitrile as solvent B. The linear gradient, injection mode and volumes and other parameters are described in Experimental section.

The MS/MS detection was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and combined electrospray and atmospheric pressure ionization source (ESCI) interfaces in positive and negative modes were evaluated. ESI interface in positive mode was selected because it showed higher sensitivity for all compounds of interest. The response of two product ions (two transitions), one for quantification and the other for identification or confirmation, were monitored. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Figure 1 shows chromatograms of a standard mixture of compounds.

Table 1. MS parameters for ionisation with ESI + in MRM mode.

*Function 1 (0.0 to 1.0 min)				
Compound	Precursor Ion(Da)	Product Ion (Da)	Cone (V)	Collision (eV)
Bet	118.0	57.9	35	22
		58.9	35	18
Gln	147.0	83.9	18	15
		130.0	18	10
Glu	148.1	83.9	18	15
		130.1	18	10
GABA	104.0	86.9	18	10
		68.9	18	15
PCho	184.0	86.1	30	20
		124.8	30	20
GPCho	258.0	104.1	30	17
		125.0	30	30
CDP-Cho	489.0	184.0	35	35
		264.0	35	25
*Function 2 (0.8 to 3.0 min)				
Compound	Precursor Ion (Da)	Product Ion (Da)	Cone (V)	Collision (eV)
Cho	104.0	44.9	35	15
		59.9	35	15
AcCh	146.0	60.0	23	10
		86.9	23	15
DA	153.9	90.9	20	20
		137.0	20	10
NE	170.0	106.9	12	20
		152.0	12	8
SE	177.0	115.0	15	25
		160.0	15	10
Tyr	182.1	136.0	18	14
		165.0	18	10
E	183.9	107.0	15	22
		166.0	15	10
5-HIAA	192.0	146.1	25	17
		91.1	25	37
Agm	131.2	71.9	25	15
		114.0	25	12
Trp	205.1	188.1	18	10
		146.0	18	18

* Dwell time (ms): 20; Delay (ms): 5


20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F1:MRM of 6 channels,ES+
184 > 86.1
5.380e+002
PCho
0.61
11.80*
0.21
0.31
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F1:MRM of 6 channels,ES+
489 > 184
7.018e+002
CDP-Cho;0.59;22.58*
0.22
0.29
0.42
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F1:MRM of 6 channels,ES+
258 > 104.1
2.194e+003
GPCho;0.59;100.35
0.23
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
118 > 58.95
2.265e+004
Bet;0.78;2.37*
1.03
1.36
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
104 > 59.9
3.263e+004
Cho;0.96;181.75*
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
146 > 86.9
1.601e+004
AcCh
1.03
6214.67*
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
153.9 > 137
7.338e+003
DA;1.06;739.43*
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
177 > 160
1.172e+004
SE;1.17;356.32
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
169.98 > 151.99
4.604e+003
NE;0.99;52.96
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
183.9 > 165.98
4.441e+003
E;1.01;119.39*
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
147.03 > 83.92
3.332e+003
Gln;0.81;61.48*
1.15
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
148.05 > 83.93
3.473e+003
Glu;0.84;14.09*
1.32
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
103.99 > 86.94
3.799e+003
GABA;0.96;52.20*
0.52
1.17
20140618_WS5 MeOH con FA_15 Smooth(Mn,2x2)
F2:MRM of 28 channels,ES+
182.06 > 136.02
1.228e+003
Tyr;1.03;39.14*
100
%
0
min

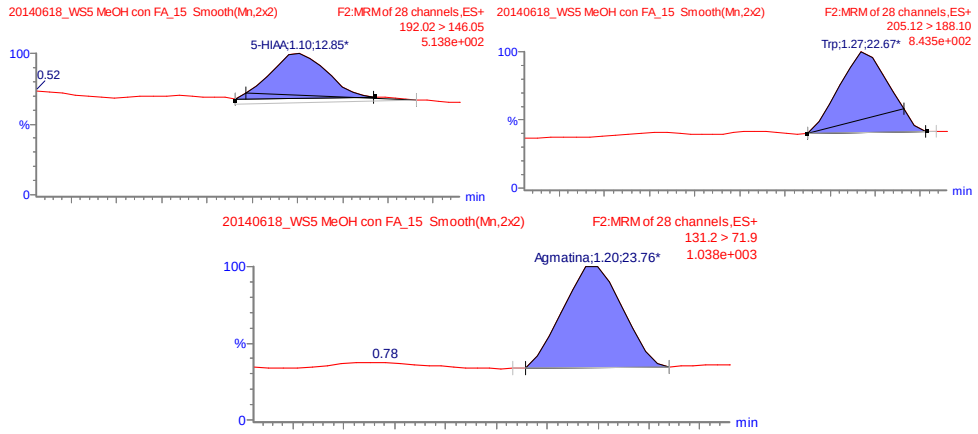


Fig. 1 Chromatograms of each analyte in an intermediate standard. Quantifier transitions are shown.

3.2. *Analytical performance*

Analytical performance was evaluated according to the recommendations of Analytical Methods Committee [31]. The calibration curve was obtained for each compound at concentrations ranging from 9-2000 ng mL^{-1} for GPCho, 18-1500 ng mL^{-1} for CDP-Cho, 8-1500 ng mL^{-1} for PCho, 10-2000 ng mL^{-1} for Bet, 0.4-1500 ng mL^{-1} for Cho, 0.1-1500 ng mL^{-1} for AcCh, 2-1500 ng mL^{-1} for DA and SE, 4-1500 ng mL^{-1} for NE, 3-1500 ng mL^{-1} for E, 38-2000 ng mL^{-1} for Gln, 25-1500 ng mL^{-1} for Glu, 2-1500 ng mL^{-1} for GABA, 7-1500 ng mL^{-1} for Tyr, 16-1500 ng mL^{-1} for 5-HIAA, 3-1500 ng mL^{-1} for Trp and 7-1500 ng mL^{-1} for Agm. The results of the lack-of-fit test are summarized in Table 2. The predicted values in quality control standards and calibration expected to not exceed ± 15% of the theoretical values.

Table 2. Analytical and statistical parameters.

	n	a	b (mL ng^{-1})	R^2 (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})	$\%P_{lof}$
GPCho	5	9.4	2.556	99.9	3	9	9-2000	10.8
CDP-Cho	5	-33.4	1.197	99.0	8	18	18-1500	35.8
PCho	5	-16.2	2.300	99.1	2	8	8-1500	95.6
Bet	5	-1165.5	47.568	99.1	3	10	10-2000	47.2
Cho	5	494.8	46.660	99.1	0.1	0.4	0.4-1500	63.9
AcCh	5	1527.3	183.657	99.4	0.02	0.1	0.1-1500	57.7
DA	5	154.9	6.743	99.1	0.4	2	2-1500	25.5
SE	5	18.2	5.839	99.2	0.8	3	2-1500	93.4
NE	5	21.1	3.039	99.4	1	4	4-1500	81.4
E	5	19.6	3.642	99.0	0.9	3	3-1500	88.7
Gln	5	116.6	4.998	99.4	11	38	38-2000	34.1
Glu	5	-8.1	5.967	99.3	7	25	25-1500	97.9
GABA	5	94.2	5.803	99.0	0.6	2	2-1500	78.9
Tyr	5	85.5	3.441	99.2	2	7	7-1500	43.1
5-HIAA	5	5.8	0.753	99.0	5	16	16-1500	95.8
Trp	5	72.8	2.373	99.5	0.8	3	3-1500	69.6
Agm	5	13.7	2.014	99.1	2	7	7-1500	89.0

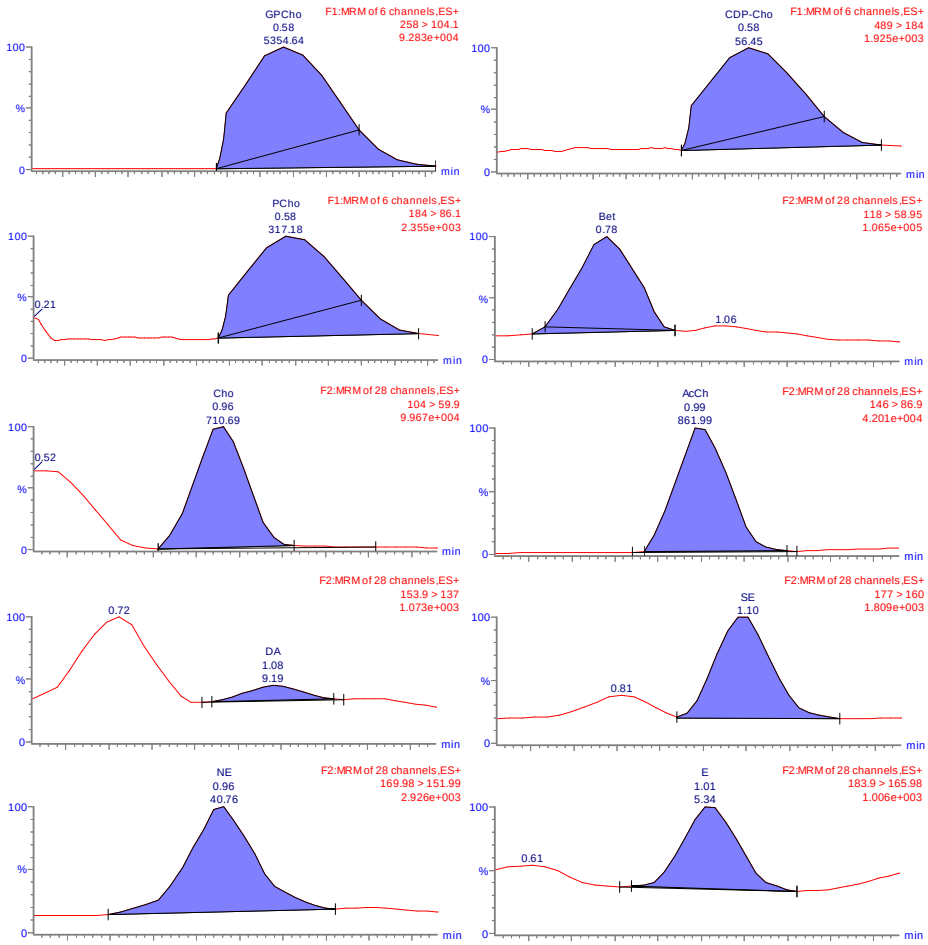
n, calibration levels; a, intercept; b, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; $\%P_{lof}$, P-value for lack-of-fit test.

3.3. Method validation

Validation of linearity, selectivity, accuracy (precision and trueness) and sensitivity was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [32].

Linearity was estimated with the determination coefficient (R^2) of the calibration curve in addition to P-value for lack-of-fit test ($\%P_{lof}$). The values obtained for determination coefficient (R^2) ranged from 99.0% for CDP-Cho, E, GABA and 5-HIAA to 99.9 % for GPCho, and P_{lof} values were higher than 5% in all cases. This indicated a good linearity within the stated ranges. Data are also shown in table 2.

Selectivity was determined by comparing the blank chromatograms with those corresponding to the samples, blank samples were Tris-HCl 0.1 M containing 10 µM of BW284c5 without adding any component to the matrix. No interferences from endogenous substances were observed at the retention times of each respective analyte (Figure 2), which eluted at 0.58 min GPCho, CDP-Cho and PCho, at 0.78 min Bet, at 0.81 min Gln, at 0.84 min Glu, at 0.96 min Cho and NE, at 0.99 min AcCh and GABA, at 1.01 min E, at 1.06 min Tyr, at 1.08 min DA, at 1.10 min SE and Agm, at 1.22 min 5-HIAA, at 1.27 min Trp. This finding suggested that the LC-MS/MS conditions provided sufficient selectivity.



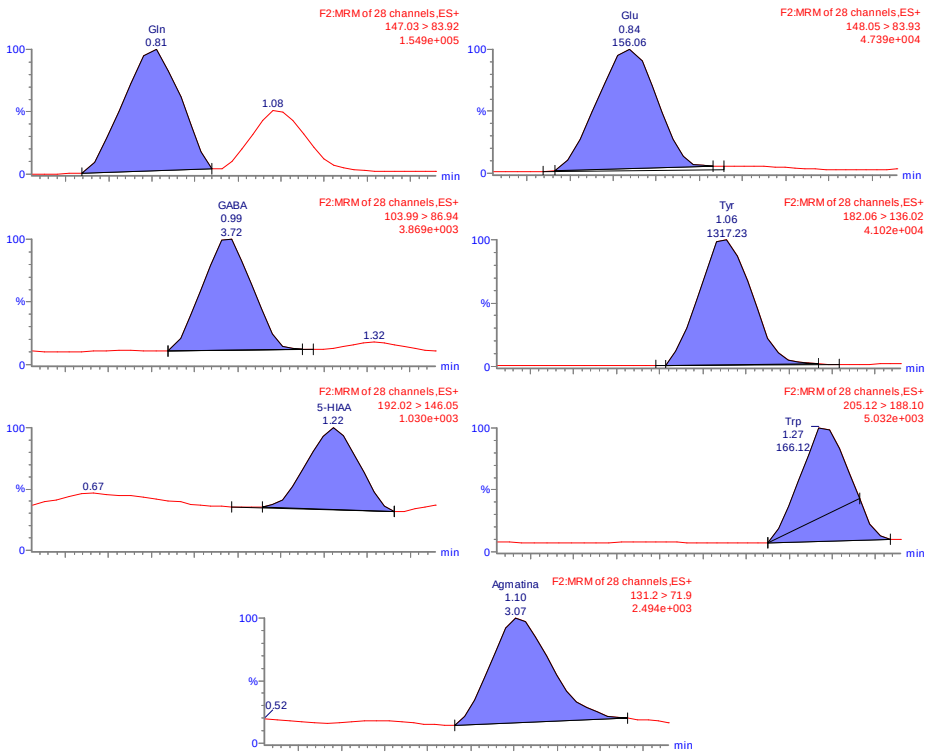


Fig. 2 Chromatograms of each analyte in body of adult zebrafish. Quantifier transitions are shown.

Accuracy (precision and trueness). Due to the lack of certified reference materials, a recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolating from the standard calibration curve. Recoveries (% R) were calculated by comparing the interpolated amounts to the theoretical spiked amounts. To evaluate the precision of the method, the intra- and inter-day precision (as % RSD) were assessed in larva matrix at three concentration levels for each compound (see Table 3). Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and were repeated for five days to determine inter-day variability. Additional assays were carried out in the other stages of zebrafish to confirm the criteria previously tested.

Recoveries and repeatability and within-laboratory reproducibility in larvae are summarized in Table 3. Additional trials in the rest of matrices are summarized in Table 4.

Table 3. Recovery, precision and accuracy of target compounds in zebrafish (embryo-larvae)

		Intra-day					Inter-day				
	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n
GPCho	13.1	13.7	1.3	104.3	9.6	3	12.6	1.8	95.7	14.4	6
	327.9	302.1	31.0	92.1	10.3	3	337.1	43.1	102.8	12.8	6
	1311.7	1499.2	41.8	114.3	2.8	3	1465.0	81.3	111.7	5.5	6
CDPCho	11.9	11.3	1.2	95.1	10.5	3	11.3	1.0	94.8	8.4	6
	298.6	319.7	38.7	107.1	12.1	3	289.0	47.9	98.5	14.1	6
	1194.3	1111.7	149.8	93.1	13.5	3	1069.2	141.5	89.5	13.2	6
PCho	6.8	5.8	0.4	85.5	6.7	3	6.4	0.8	94.8	13.0	6
	168.9	147.5	10.4	87.3	7.1	3	144.9	8.4	85.8	5.8	6
	675.7	675.3	48.1	99.9	7.1	3	682.4	34.8	101.0	5.1	6
Bet	16.6	14.2	1.2	85.8	8.6	3	15.5	1.9	93.9	12.5	6
	414.1	465.8	19.9	112.5	4.3	3	469.8	25.1	113.5	5.3	6
	1656.3	1748.4	154.9	105.6	8.9	3	1812.5	120.8	109.4	6.7	6
Cho	9.6	9.2	0.1	95.2	1.4	3	9.9	1.0	103.1	10.2	6
	240.4	269.7	7.8	112.2	2.9	3	257.4	17.0	107.1	6.6	6
	961.5	824.4	9.8	85.7	1.2	3	939.3	134.7	97.7	14.3	6
AcCh	9.9	10.0	0.3	101.2	2.9	3	10.2	0.6	102.6	5.5	6
	247.9	284.5	5.0	114.8	1.8	3	281.5	11.2	113.6	4.0	6
	991.5	868.6	121.1	87.6	13.9	3	865.9	78.0	87.3	9.0	6
DA	11.8	12.9	1.3	109.8	10.0	3	12.1	1.3	102.4	10.7	6
	294.9	296.9	22.1	100.7	7.4	3	283.6	22.9	96.2	8.1	6
	1179.7	1054.9	154.3	89.4	14.6	3	1138.5	139.3	96.5	12.2	6
SE	11.0	12.3	0.5	111.9	4.3	3	10.9	1.7	99.8	14.8	6
	274.1	305.1	14.8	111.3	4.9	3	291.2	28.4	105.9	9.8	6
	1096.2	1126.9	75.5	102.8	6.7	3	1162.2	102.4	106.0	8.8	6

Table 3 (cont). Recovery, precision and accuracy of target compounds in zebrafish (embryo-larvae)

		Intra-day					Inter-day				
	Spiked (ng mL^{-1})	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n	Observed[a] (ng mL^{-1})	SD (ng mL^{-1})	R (%)	RSD (%)	n
NE	12.7	13.4	0.7	105.7	5.4	3	12.6	1.0	99.3	8.2	6
	316.6	276.0	28.0	87.2	10.1	3	276.7	22.2	87.4	8.0	6
	1266.5	1111.4	159.8	87.8	14.4	3	1170.1	145.9	92.4	12.5	6
E	12.2	13.6	1.1	111.9	8.3	3	12.1	1.9	99.6	14.9	6
	305.0	338.8	50.4	111.1	14.9	3	322.9	36.6	105.9	11.3	6
	1220.0	1287.4	30.3	105.5	2.4	3	1315.7	102.0	107.9	7.7	6
Gln	14.4	13.6	1.2	94.2	8.6	3	11.4	1.2	97.8	9.8	6
	359.8	410.4	20.5	114.1	5.0	3	321.6	35.0	112.2	8.9	6
	1439.3	1401.5	17.8	97.4	1.3	3	1065.4	81.8	91.3	7.5	6
Glu	12.3	11.6	1.0	94.5	8.6	3	11.4	1.2	93.3	10.1	6
	307.1	310.9	17.7	101.2	5.7	3	321.6	35.0	104.7	10.9	6
	1228.4	1061.6	103.7	86.4	9.8	3	1065.4	81.8	86.7	7.7	6
GABA	12.7	12.1	1.1	95.8	9.3	3	12.0	1.3	94.8	11.1	6
	317.0	283.9	36.0	89.5	12.7	3	278.9	26.0	88.0	9.3	6
	1268.0	1413.3	73.0	111.5	5.2	3	1355.0	101.9	106.9	7.5	6
Tyr	12.3	10.6	0.5	85.6	4.6	3	11.1	0.9	89.7	7.7	6
	308.4	353.8	4.3	114.7	1.2	3	332.2	24.4	107.7	7.4	6
	1233.7	1076.9	51.7	87.3	4.8	3	1093.8	105.4	88.7	9.6	6
5-HIAA	12.7	12.9	0.6	101.4	4.5	3	12.8	0.4	101.0	2.9	6
	317.9	364.7	5.4	114.7	1.5	3	336.5	34.0	105.8	10.1	6
	1271.8	1227.8	140.4	96.5	11.4	3	1171.0	146.5	92.1	12.5	6
Agm	12.6	14.4	2.1	114.9	14.3	3	12.9	2.2	104.9	14.7	6
	314.1	308.7	45.3	98.3	14.7	3	303.7	39.6	102.4	12.0	6
	1256.5	1382.7	193.3	110.0	14.0	3	1405.7	165.7	101.2	12.3	6
Trp	12.2	11.8	1.4	96.6	11.6	3	12.6	1.4	102.7	11.2	6
	305.6	331.8	36.6	108.6	11.0	3	312.9	37.7	96.7	13.0	6
	1222.3	1266.1	188.1	103.6	14.9	3	1236.9	151.6	111.9	11.8	6

[a] Mean value; SD, standard deviation; R, % recovery; RSD, relative standard deviation; n, number of determinations

Table 4. Recovery, precision and accuracy of target compounds in zebrafish (alevin, adult body and adult brain), n=3

	Alevin					Adult body				Adult Brain			
	Spiked	Obs[a]	SD	R (%)	RSD (%)	Obs[a]	SD	R (%)	RSD (%)	Obs[a]	SD	R (%)	RSD (%)
	13.1	12.9	1.2	98.0	9.6	11.3	1.3	86.3	11.5	12.4	0.7	94.9	5.7
GPCho	327.9	297.9	20.8	90.9	7.0	309.3	37.1	94.3	12.0	363.0	52.9	110.7	14.6
	1311.7	1392.4	144.0	106.2	10.3	1296.0	47.1	98.8	3.6	1462.2	51.3	111.5	3.5
	11.9	12.2	1.4	85.6	13.4	10.4	0.9	87.0	8.5	12.3	0.1	102.7	0.7
CDPCho	298.6	324.5	48.1	107.8	14.8	295.9	33.1	99.1	11.2	303.0	36.9	101.5	12.2
	1194.3	1283.8	184.8	107.5	14.4	1068.0	104.8	89.4	9.8	1193.2	146.0	99.9	12.2
	6.8	6.1	0.4	89.8	6.1	7.2	0.8	106.5	11.3	7.5	1.0	111.2	12.7
PCho	168.9	149.8	14.5	88.7	9.7	171.2	22.0	101.3	12.9	158.9	17.6	94.1	11.1
	675.7	630.9	91.8	93.4	14.6	627.8	45.5	92.9	7.2	769.8	35.1	113.9	4.6
	16.6	17.9	2.2	107.8	12.4	14.2	0.6	85.6	4.0	18.7	1.2	113.1	6.6
Bet	414.1	474.9	4.8	114.7	1.0	444.5	41.1	107.4	9.2	474.5	1.6	114.6	0.3
	1656.3	1769.2	88.8	106.8	5.0	1540.2	47.7	93.0	3.1	1806.9	32.0	109.1	1.8
	9.6	12.2	1.1	106.7	10.3	9.6	1.1	99.8	11.5	9.8	1.4	102.2	14.4
Cho	240.4	276.1	37.8	114.9	13.7	204.6	22.3	85.1	10.9	241.4	28.5	100.4	11.8
	961.5	1067.4	81.0	111.0	7.6	892.7	128.9	92.8	14.4	978.3	93.5	101.8	9.6
	9.9	9.5	1.4	95.8	14.9	9.6	1.3	96.9	13.1	11.2	1.5	112.8	13.6
AcCh	247.9	282.9	3.0	114.1	1.1	267.1	23.4	107.8	8.8	284.3	5.3	114.7	1.9
	991.5	1107.9	25.5	111.7	2.3	861.6	53.2	86.9	6.2	1126.0	113.2	113.6	10.0
	11.8	11.8	1.7	99.6	14.6	11.3	1.7	95.7	14.8	10.3	0.5	87.0	5.2
DA	294.9	254.8	19.9	86.4	7.8	259.4	15.2	88.0	5.8	254.6	2.5	86.3	1.0
	1179.7	1019.3	132.4	86.4	13.0	1162.5	13.4	98.5	1.2	1027.5	69.7	87.1	6.8
	11.0	10.4	1.4	94.8	13.5	12.2	1.2	111.1	9.6	11.8	1.4	107.7	11.5
SE	274.1	236.9	5.3	86.4	2.2	254.5	29.7	92.8	11.7	303.3	14.6	110.7	4.8
	1096.2	1103.9	38.0	100.7	3.4	940.1	34.1	85.8	3.6	1257.8	17.4	114.7	1.4
	12.7	12.9	1.9	101.8	14.9	13.7	1.3	108.1	9.4	11.5	1.0	90.9	9.0
NE	316.6	273.7	5.4	86.5	2.0	303.2	20.0	95.8	6.6	363.6	36.2	114.8	10.0
	1266.5	1144.0	40.5	90.3	3.5	1080.2	55.5	85.3	5.1	1091.4	94.5	86.2	8.7
	12.2	11.6	1.4	95.2	12.1	12.2	1.7	99.9	13.8	10.8	0.4	88.5	3.3
E	305.0	304.6	40.5	99.9	13.3	320.1	45.3	105.0	14.1	267.7	25.7	87.8	9.6
	1220.0	1134.7	28.8	93.0	2.5	1338.5	91.1	109.7	6.8	1148.7	28.4	94.2	2.5
	14.4	14.1	1.8	97.9	12.9	16.5	2.0	114.5	12.3	12.7	0.7	88.2	5.8
Gln	359.8	319.7	22.6	88.9	7.1	328.8	5.1	91.4	1.5	367.0	19.2	102.0	5.2
	1439.3	1240.1	175.4	86.2	14.1	1226.6	5.5	85.2	0.4	1378.4	88.4	95.8	6.4
	12.3	10.9	1.2	88.9	10.7	11.1	1.7	90.7	14.9	10.8	1.3	87.5	11.9
Glu	307.1	270.6	36.9	88.1	13.6	298.1	25.4	97.1	8.5	335.8	27.6	109.4	8.2
	1228.4	1252.4	73.3	101.9	5.9	1230.4	164.1	100.2	13.3	1257.7	118.0	102.4	9.4
	12.7	11.0	0.5	88.6	4.3	11.4	0.5	89.9	4.6	13.1	1.6	103.0	12.3
GABA	317.0	272.0	22.6	85.8	8.3	301.1	45.2	95.0	15.0	272.2	5.2	85.9	1.9
	1268.0	1117.6	38.2	88.1	3.43	1270.2	14.2	100.2	1.1	1097.0	7.6	86.5	0.7
	12.3	11.7	0.4	87.1	3.4	12.5	0.7	101.1	5.4	12.9	1.9	104.2	14.8
Tyr	308.4	348.2	22.6	112.9	6.5	285.0	28.7	92.4	10.1	268.0	38.3	86.9	14.3
	1233.7	1228.0	171.2	99.5	13.9	1418.8	28.4	115.0	2.0	1396.4	72.7	113.2	5.2
	12.7	13.1	1.9	103.3	14.3	12.0	1.3	94.4	10.9	11.9	1.3	93.9	10.6
5-HIAA	317.9	352.4	36.6	110.8	10.4	326.3	16.0	102.6	4.9	315.1	41.7	99.1	13.2
	1271.8	1435.5	157.2	112.9	11.0	1330.4	160.9	104.6	12.1	1310.5	160.4	103.0	12.2
	12.6	11.5	1.2	91.8	10.4	11.5	1.5	91.8	13.2	12.8	1.6	101.5	12.8
Agm	314.1	268.0	11.0	87.4	13.2	295.9	32.3	94.2	10.9	269.6	7.4	85.8	2.8
	1256.5	1168.6	155.5	108.5	11.2	1087.5	49.6	86.5	4.6	1154.6	76.1	91.9	6.6
	12.2	10.9	1.3	89.0	12.3	13.3	1.2	108.8	9.3	11.7	1.3	95.9	11.4
Trp	305.6	267.1	35.3	85.3	4.1	271.5	28.7	88.9	10.6	298.0	12.1	97.5	4.1
	1222.3	1326.2	148.2	93.0	13.3	1321.3	66.7	108.1	5.0	1172.4	72.6	95.9	6.2

[a] Obs, Observed, mean value (ng mL^{-1}); SD, standard deviation (ng mL^{-1}); R, % recovery; RSD, relative standard deviation

The recoveries were between 85% and 115% in all cases. Relative standard deviation values (RSD) were between 0.3% for Bet and 14.9% for Trp, E, Glu and AcCh at some stages of development. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value. Precision and trueness data indicated that the methodology to determine the target compounds in zebrafish samples is accurate and precise, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Sensitivity. LOD and LOQ were calculated from the signal-to-noise ratio (LODs signal-to-noise ratio = 3, LOQ signal-to-noise ratio = 10) injecting several solutions of decreasing amounts of the compounds of interest spiked into blank samples. The calculated limits of detection (LODs, signal-to-noise ratio = 3) are in the range from 0.02 ng mL^{-1} for AcCh to 11 ng mL^{-1} for Gln, and the corresponding limits of quantification (LOQs, signal-to-noise ratio = 10) ranged from 0.1 ng mL^{-1} to 38 ng mL^{-1}, respectively. The results are shown in Table 2.

3.4. Application of the method in zebrafish and results discussion

With regards to sample extraction, several trials were done to optimize the method. The protocols that were studied were 1) liquid-liquid separation using chloroform ($CHCl_3$), MeOH, isopropanol (IPA) and water [33-35]; 2) protein precipitation with organic solvents such as MeOH or etanol (EtOH) [36], 3) acid hydrolysis by FA at different concentrations 4) combination of protein precipitation and acid hydrolysis. None of them had acceptable results: poor recoveries lower than 80% in some cases and higher than 120% in others, power matrix effects and high variability between replicates were obtained by liquid-liquid extractions; acid hydrolysis showed better recovery than liquid-liquid extraction but high variability; and protein precipitation with MeOH instead of EtOH showed better results in terms of recovery and repeatability in the preliminary tests, perhaps for being less laborious methods. Finally, with the objective in mind for accomplishing the international guidelines in

bioanalytical methods, a mix of procedures was probed between organic and acid deproteinization, obtaining acceptable results. The procedure consisted to precipitate proteins avoiding interferences by 1% FA in MeOH, following the protocols indicated below in Method Section.

Figure 2 shows chromatograms obtained for adult body sample. Under the experimental conditions, all the studied compounds were detected in the body of the adult zebrafish. However in earlier stages, not all compounds were detected. Additionally, the amount of protein per animal, expressed in mg, at different stages through the zebrafish life is shown in Figure 3A.

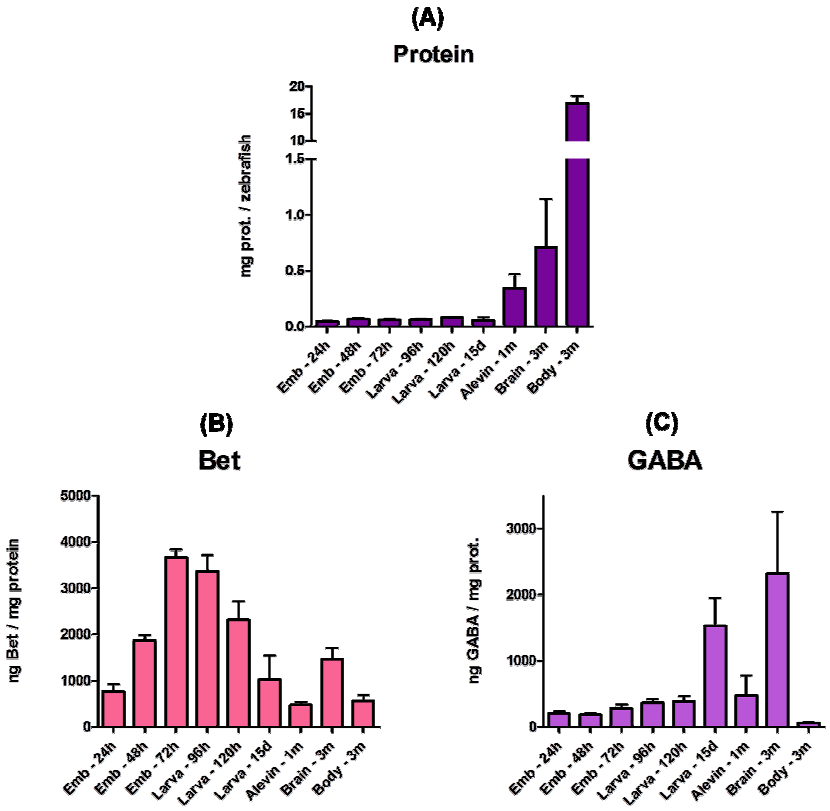


Fig. 3. Protein amount in zebrafish through the life cycle (A). Betaine (B) and GABA (C) profile normalized by mg protein (B).

Regarding the total protein content, data showed that during the embryo phases as well as in early larva there was no relevant change in the total protein

amounts. Starting from 15 day larva, total protein showed a constant increase up to reach a dramatic peak in adults. In fact, this profile in the protein amount across the development may reflect that during the early stages, there are intense differentiation processes rather than real growth, which really take off as from mature larva (15 day). Changes in protein concentration with age run in parallel to changes in neurotransmitters. It is quite typical to normalize data by protein content. However, the expression of our results by protein content instead than absolute values increased the variability and did not reflect the overall trend (data not shown). Only in some cases normalization by protein can provide additional information such as with Bet (Figure 3B) or GABA (Figure 3C).

Our method is sensitive for measuring most of the main neurotransmitters, although some molecules were not detected during the early stages, probably because the total neurotransmitter amount was under the LOQ. This is the case for zebra fish aminergic neurotransmitter systems. Thus, SE was not detectable from embryos up to 120 h larva, showing low levels in mature larva, juvenile and brain from adult fishes (Figure 4A). Interestingly, SE is much more abundant in the body of adult animals, which is in agreement with the fact that near 90 % of SE in the organism is located in the intestine [37]. However, although the detection of SE in our study is limited to the later stages, some molecules related to the SE metabolism, such as the precursor Trp (Figure 4B) or, especially the metabolite 5HIAA (Figure 4C), were accurately measured in all the developmental stages analyzed. Likewise, the other aminergic neurotransmitter, DA, was also detected only in mature animals but close to LOQ. However, its metabolic precursor Tyr was effectively detected across all the developmental period (Figure 4D).

Likewise, E, another neurotransmitter related to tyrosine metabolism, was only accurately measured in the body of adult fishes. In younger subjects the E levels were close or under the LOQ (Figure 4E). However, its precursor NE was properly measured even from the pharyngula period on (48 hpf), but not in embryos during the segmentation period (24 hpf). The levels of NE during embryonic and larval stages remain reasonably constant, increasing the

amount of NE in mature larva, juvenile and adults, both in brain and especially in adult body (Figure 4F).

AcCh was detected at very low levels in embryos and larvae, showing an important increase from mature larva to adults, especially in the body (Figure 4G). Since AcCh is a signaling molecule related to neuromuscular junctions it is easy to correlate the increase in the AcCh amount with the development of the muscular tissues and the animal movement capability during growth [38]. Interestingly, others molecules related to AcCh metabolism, such as Cho (Figure 4H) and Bet (Figure 4I) showed a similar profile particularly at later stages of life. At early stages, Bet showed an increase between the pharyngula period at 48 hpf and early larval stages at 120 hpf. This was also observed in AcCh and Cho although it was less evident and the scale of the graph did not allow seeing this profile. This rise in the amount of Bet during early stages is even more remarkable when the level of analyte is corrected by the total protein amount (Figure 3B).

In zebrafish, the GABAergic inhibitory control of motor neurons and the balance between excitatory and inhibitory synapses on interneurons and motor neurons underlies the normal functioning of locomotor circuits that produce rhythmic motor output [39]. In this method three metabolic steps related to the GABA system are analyzed: GABA (Figure 4J), its precursor Glu (Figure 4K) as well as Gln (Figure 4L), which is the Glu precursor. These three analytes were accurately measured from the segmentation period at 24h embryos, showing a progressive increase concomitantly with animal development and growth. It is worthy to point out that the total amounts of these three molecules are inversely proportional to their relative order in the anabolism GABA pathway ([Gln] > [Glu] > [GABA]).

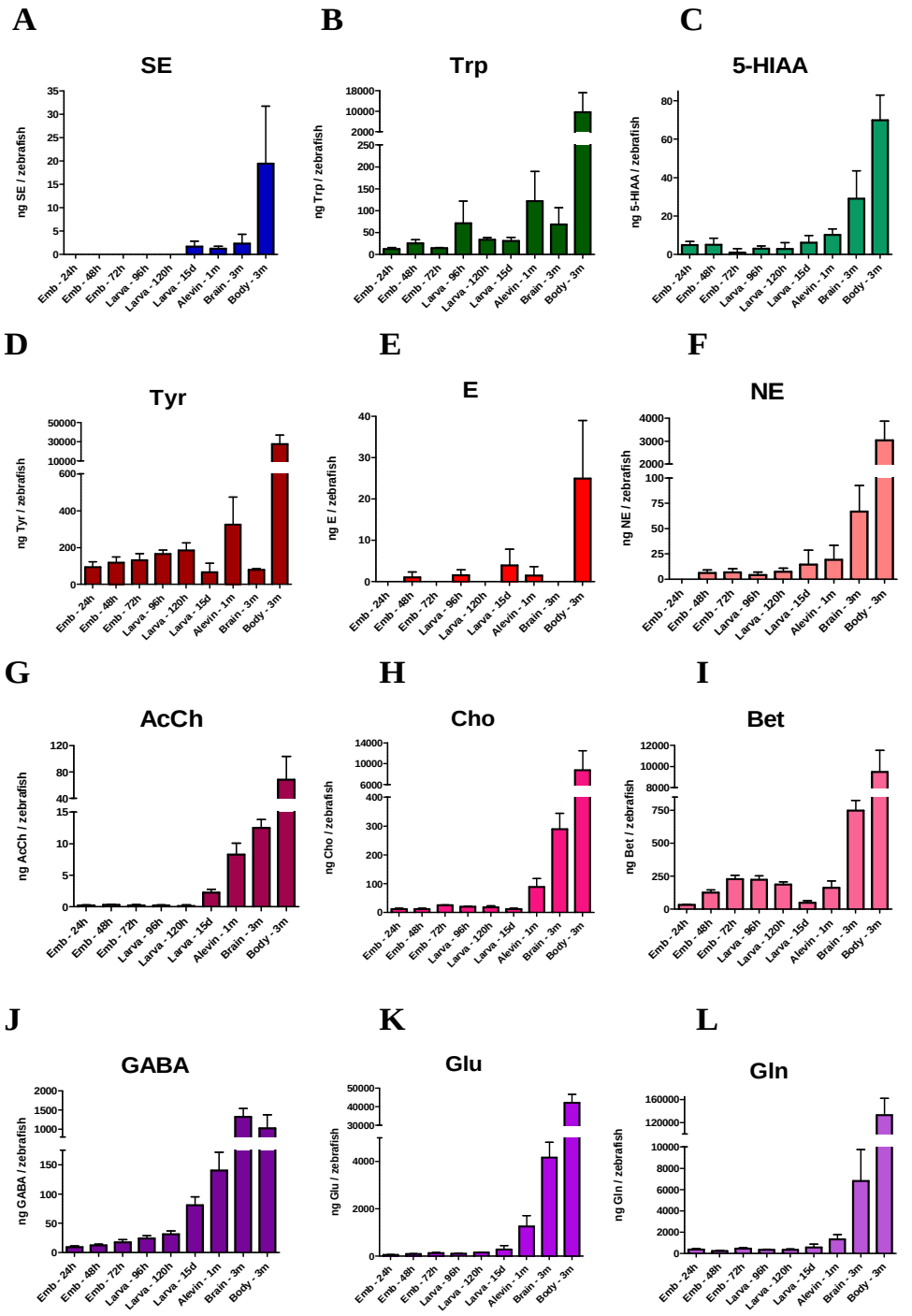


Fig. 4. Highlighted neurocompounds graphed in ng compound per animal. Serotonin (A); Triptophan (B); 5-Hydroxyindolacetic acid (C); Tyrosine (D); Epinephrine (E); Norepinephrine (F); Acetylcholine (G); Choline (H); Betaine (I); GABA (J); Glutamic acid (K); Glutamine (L).

As far as we know this is the first time a method is described for measuring Agm, and Trp in zebrafish samples. In addition, although there is a lot of information about the cholinergic system in this model, particularly about AcCh receptors and related enzymes, this is the first time that AcCh and CDP-Cho levels are measured too. Agm showed the same profile of evolution with the development as AcCh. This method opens the doors to future research on the role of Agm in zebrafish and therefore to the study in this model of conditions in which Agm has been involved, such as ischemic injury, convulsive seizures, and opiate analgesia [40]. Thus, taking into account that the zebrafish model is a widely recognized screening tool for drug discovery [41,42], the induction of neurological disorders [43,44] as well as for neurotoxicological assessment [2, 45], the possibility of measuring up to 17 compounds related with the main neurotransmitter systems in a simple analytical method will completement and reinforce the use of this organism in multiple applications in the field of neurosciences.

4. Conclusions

UHPLC-MS/MS is used for the first time to analyze a large number of neurotransmitters, metabolites and precursors in zebrafish. This methodology has acceptable quality parameters in terms of linear dynamic range, selectivity, accuracy and sensitivity, and provides a number of advantages such as minimal run times, low solvent consumption and waste production, thereby reducing the final cost. Moreover, it can provide a relevant tool for the neuroscience research performed in zebrafish. The use of zebrafish model shows important advantages, such as easy management, cost reduction, similarity to the human genome or similarity of the neurotransmitters system. Thus, taking into account that the zebrafish model is a widely recognized screening tool for drug discovery, the induction of neurological disorders as well as for neurotoxicological assessment, the possibility of measuring up to 17 compounds related with the main neurotransmitter systems in a simple analytical method will complement and reinforce the use of this organism in multiple applications in the field of neurosciences. The proposed method will facilitate future studies related with brain development.

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CAPÍTULO II

Oligosacáridos presentes en leche materna (HMOs)

II.1 OBJETIVOS

De la misma manera el objetivo principal era dar apoyo analítico al proyecto focalizado en determinar este segundo tipo de biomoléculas pertenecientes a los carbohidratos complejos presentes en la leche materna. Los objetivos específicos son:

- ✓ Proponer metodología de buenas características para la determinación de los oligosacáridos complejos conocidos como HMOs y metabolitos o precursores de los mismos, en suero y orina de rata mediante cromatografía de líquidos-espectrometría de masas en tándem (LC-MS/MS).

- ✓ Validar el método analítico propuesto en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación específicas para métodos bioanalíticos.

- ✓ Aplicar la metodología validada a muestras reales obtenidas en experimentos específicos tanto en condiciones naturales como con intervención nutricional, para observar los niveles basales en distintas etapas de la vida de un roedor y los mecanismos de absorción en los mismos.

II.2 ESTRATEGIAS DE TRABAJO

La estrategia seguida para alcanzar los objetivos fijados se basó en las siguientes etapas.

A. **Proceso de muestreo.** Obtención de suero y orina de rata a través de protocolos específicos para las medidas basales y para el estudio de curvas de absorción después de la administración oral de diferentes ingredientes a estudiar.

B. **Análisis mediante cromatografía de líquidos-espectrometría de masas.** Se desarrolla esta etapa, al igual que en el resto de trabajos con procedimientos similares especialmente dirigidos a cada biomolécula en particular. Se siguieron, en la medida de lo posible, las recomendaciones indicadas en las Guías de Validación para métodos analíticos biológicos mencionadas anteriormente.

C. **Aplicación del método a la determinación de los compuestos de interés en muestras reales**. Una vez obtenidos todos los parámetros y condiciones ideales de trabajo, se llevó a cabo el diseño experimental con animales vivos, administrando ingredientes específicos por vía oral de forma aguda para hacerles el seguimiento a los analitos de interés tanto a niveles basales como en curvas cinéticas para estudiar la biodisponibilidad y su destino.

II.3 ESTADO ACTUAL DE LA INVESTIGACIÓN. ANTECEDENTES BIBLIOGRÁFICOS

Para tener una medida cuantificable del interés que despiertan estas biomoléculas en el mundo científico y más específicamente desde el punto de vista analítico, en las siguentes figuras se refleja el crecimiento de investigaciones publicadas a lo largo de los años. En la Figura 49 se muestra una búsqueda en la base de datos científica Scopus usando como entrada el término "HMOs". En este caso son casi 4000 documentos encontrados, pero se observa un descenso en los últimos años que podría hacernos pensar que ha disminuido el interés científico por estas biomoléculas. Si hacemos una nueva búsqueda usando los vocablos completos "human milk oligosaccharides" y evitamos la abreviatura, podemos ver el ascenso en documentos publicados (Figura II.1).

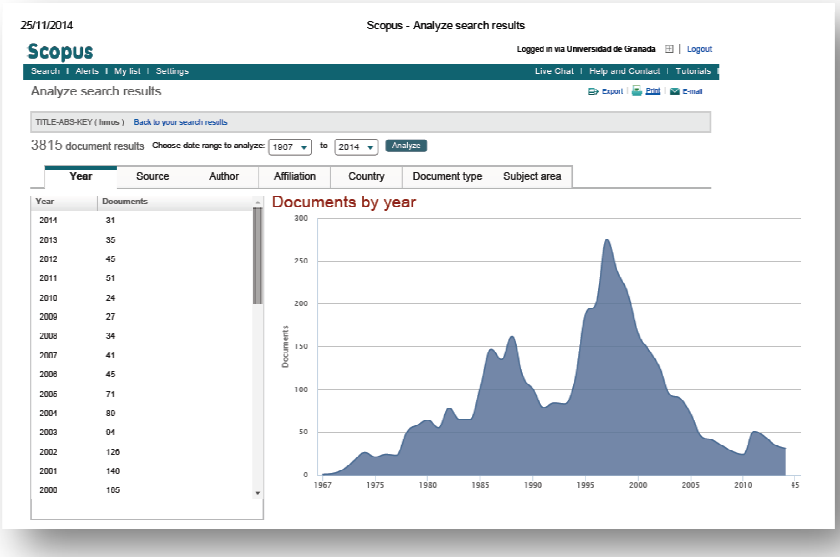


Figura II.1. *Búsqueda bibliográfica en Scopus de todas las publicaciones relacionadas con el término "HMOs"*

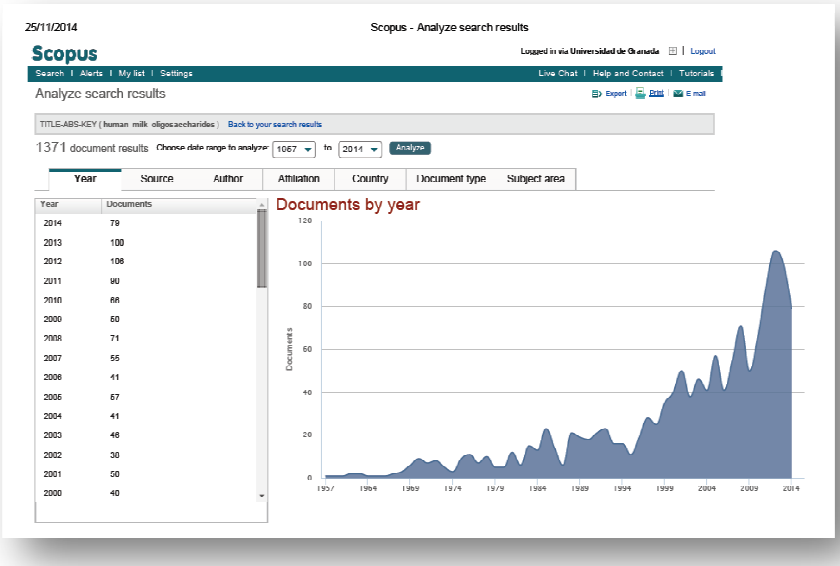


Figura II.2. *Búsqueda bibliográfica en Scopus de todas las publicaciones relacionadas con el término "human milk oligosaccharides"*

En esta segunda búsqueda encontramos más de 1000 documentos. Estos documentos están repartidos entre artículos y revisiones mayoritariamente en áreas como la medicina y la bioquímica (Figura II.3).

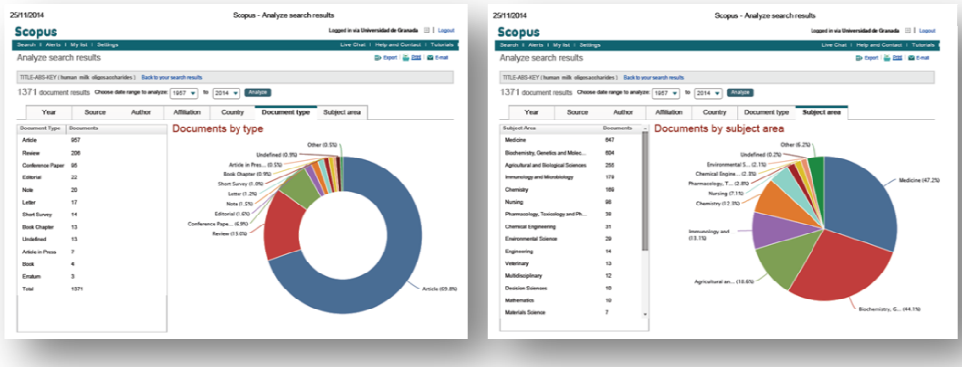


Figura II.3. *Tipos de documentos publicados con el término de "human milk oligosaccharides" y áreas de interés*

Entre los resultados obtenidos se encuentra el artículo presentado en la presente Tesis Doctoral. En la Figura II.4 se muestra el interés mostrado de esta publicación durante un año según un informe facilitado por Scopus. El número de descargas son 1230, principalmente en Estados Unidos (73%) y China (7%).

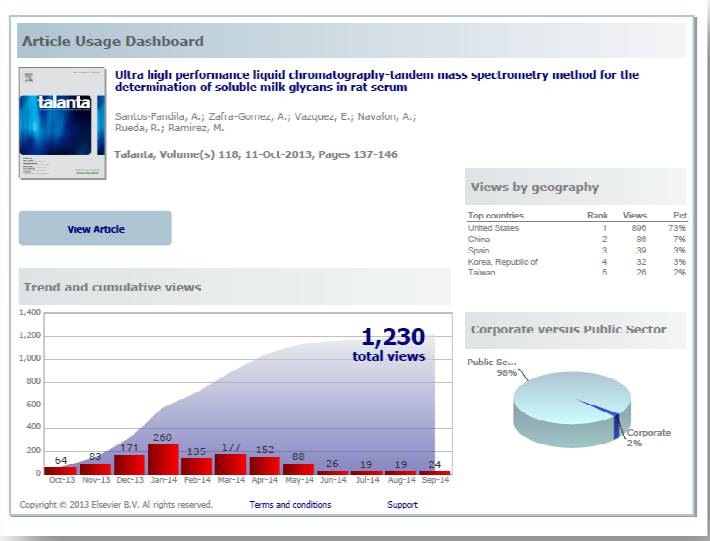
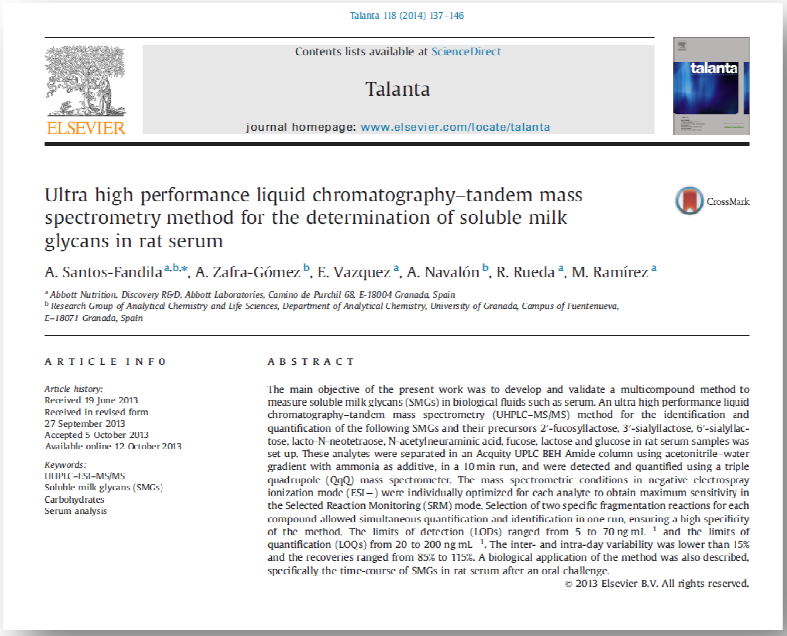


Figura II.430. *Informe de ScienceDirect y Scopus con más de 1000 descargas en un año, principalmente en Estados Unidos y China*

II.4 DETERMINACIÓN DE SGMs EN SUERO DE RATA MEDIANTE UHPLC-MS/MS (Publicación III)

En este apartado se presenta el artículo publicado en la revista **Talanta** de la editorial Elsevier (ver Figura II.5).



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Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum

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ABSTRACT

The main objective of the present work was to develop and validate a multicompound method to measure soluble milk glycans (SMGs) in biological fluids such as serum. An ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the identification and quantification of the following SMGs and their precursors 2′-fucosyllactose, 3′-sialyllactose, 6′-sialyllactose, lacto N neotetraose, N acetylneuraminic acid, fucose, lactose and glucose in rat serum samples was set up. These analytes were separated in an Acquity UPLC BEH Amide column using acetonitrile–water gradient with ammonia as additive, in a 10 min run, and were detected and quantified using a triple quadropole (QqQ) mass spectrometer. The mass spectrometric conditions in negative electrospray ionization mode (ESI−) were individually optimized for each analyte to obtain maximum sensitivity in the Selected Reaction Monitoring (SRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter- and intra-day variability was lower than 15% and the recoveries ranged from 85% to 115%. A biological application of the method was also described, specifically the time-course of SMGs in rat serum after an oral challenge.

Figura II.5. *Ultra high performance liquid chromatography-tandem mass spectrometry method for the determination of soluble milk glicans in rat serum. Talanta 118 (2014) 137-146 (factor de Impacto de 3.511)*

Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum

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ABSTRACT

The main objective of the present work was to develop and validate a multicompound method to measure soluble milk glycans (SMGs) in biological fluids such as serum. An ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for the identification and quantification of the following SMGs and their precursors 2'–fucosyllactose, 3'–sialyllactose, 6'–sialyllactose, lacto–N–neotetraose, N-acetylneuraminic acid, fucose, lactose and glucose in rat serum samples was set up. These analytes were separated in an Acquity UPLC BEH Amide column using acetonitrile–water gradient with ammonia as additive, in a 10 min run, and were detected and quantified using a triple quadrupole (QqQ) mass spectrometer. The mass spectrometric conditions in negative electrospray ionization mode (ESI–) were individually optimized for each analyte to obtain maximum sensitivity in the Selected Reaction Monitoring (SRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter– and intra–day variability was lower than 15% and the recoveries ranged from 85–115%. A biological application of the method was also described, specifically the time–course of SMGs in rat serum after an oral challenge.

Keywords: UHPLC–ESI–MS/MS; Soluble Milk Glycans (SMGs); Carbohydrates, Serum analysis

Highlights:

- Multi-analyte method for the determination of soluble milk glycans in rat serum.
- Validation of the method: quality parameters, accuracy (trueness and precision).
- Evaluation of the time–course of SMGs in rat serum after an oral challenge.
- *In vivo* absorption of SMGs from the intestine to the systemic circulation is demonstrated.

1. Introduction

Human milk is a complex biological fluid composed mainly of lipids, proteins, and lactose. Oligosaccharides are the third most abundant component in human milk, after lactose and lipids. They are present in large amounts, ranging from 5 to 23 g L^{-1}. The monosaccharides used for the biosynthesis of human milk glycans are glucose, galactose, N–acetylglucosamine, fucose and N-acetylneuraminic acid (SA, acronym of sialic acid). Despite these basic structures, the possible combinations of monosaccharides and plausible linkages contribute to the overall diversity and complexity of SMG structures to the point that more than 150 structurally distinct SMGs have been identified so far [1].

SMGs exert important biological effects. Namely, they have a prebiotic effect, selectively serving as a source of energy and nutrients for bacteria to colonize the infant intestine [2]. Beyond the prebiotic effects, a large body of evidence suggests that SMGs may protect against infections by mimicking the attachment sites for certain pathogens [2], influence various stages of gut maturation in vitro [3], and prevent necrotising enterocolitis in neonatal rats [4]. SMGs were previously considered indigestible. However, it is currently

known that their structure changes before reaching the colon and that they can be absorbed as they appeared in plasma of neonatal rats [5] and in urine of infants [6]. SMGs can also exert systemic effects. For instance, it has been shown that: 1) fucosylated and sialylated SMGs reduce selectin–mediated leukocyte rolling, adhesion, and activation [7, 8]; 2) oligosaccharides are often found as a component of glycoproteins or glycolipids and there is evidence of a role of glycoproteins in adaptive functions of neuronal membrane components determining the efficiency of interneuronal connections [8]; and 3) sialoconjugates have been also shown to participate in the establishment of synaptic pathways, calcium transportation, binding of neurotransmitters, cell–to–cell interactions and axon regeneration [9, 10].

An important tool for initiating any study on SMGs is a quantitative method to measure different SMGs in biological matrices. The separation of SMGs has been traditionally a challenge; oligosaccharides are polar compounds that are not well resolved by traditional reversed–phase chromatography. In addition, oligosaccharides do not generally contain strong intrinsic chromophores, resulting in low specificity and sensitivity in optical absorbance detection. Nonetheless, some studies have been published in the scientific literature for the determination of these compounds such as gel permeation chromatography [11], reverse–phase high performance liquid chromatography [12], high performance anion–exchange chromatography with pulsed amperometric detection (HPAEC–PAD) [13, 14], capillary electrophoresis [15], precolumn derivation HPLC with different detectors (ultraviolet, photodiode array ultraviolet–visible, differential refractive index detector) [16, 17], and also Nuclear Magnetic Resonance Spectroscopy [18].

All of the previously published methods based on LC are limited by cumbersome protocols requiring derivatization precolumns and long–run times, which do not fully resolve the major oligosaccharides to baseline, and have low sensitivities. HPAEC–ECD improves detection limits, but requires a long analysis time. Currently, mass spectrometry offers several advantages such as greater selectivity, specificity and sensitivity. Some of these techniques involve matrix–assisted laser desorption (MALDI) or time–of–flight mass

spectrometry (TOF/MS) [19–26], microfluidic chips and mass spectrometry technology (HPLC–Chip/TOF–MS) [27], and negative ion mode electrospray mass spectrometry (ESI–MS) [28, 29].

Herein, we report an UHPLC–MS/MS method with negative electrospray ionization using ammonium hydroxide as additive for the separation and determination of specific carbohydrates and metabolites in serum of rats to support pharmacokinetic studies of SMGs carried out to confirm the appearance of these metabolites in serum of rats when they were administered orally. The method focused on four main SMGs: 2'–fucosyllactose (2'–FL), 3'–sialyllactose (3'–SL), 6'–sialyllactose (6'–SL), lacto–N–neo–tetraose (LNnT) and their parental mono– and disaccharides: sialic acid (SA), fucose (Fuc), lactose (Lact) and glucose (Glu). The chemical structures are shown in Figure 1. To our knowledge, no multicomponent methods for the quantitative determination in serum samples of all the proposed compounds in a single analysis have been previously described in the literature.

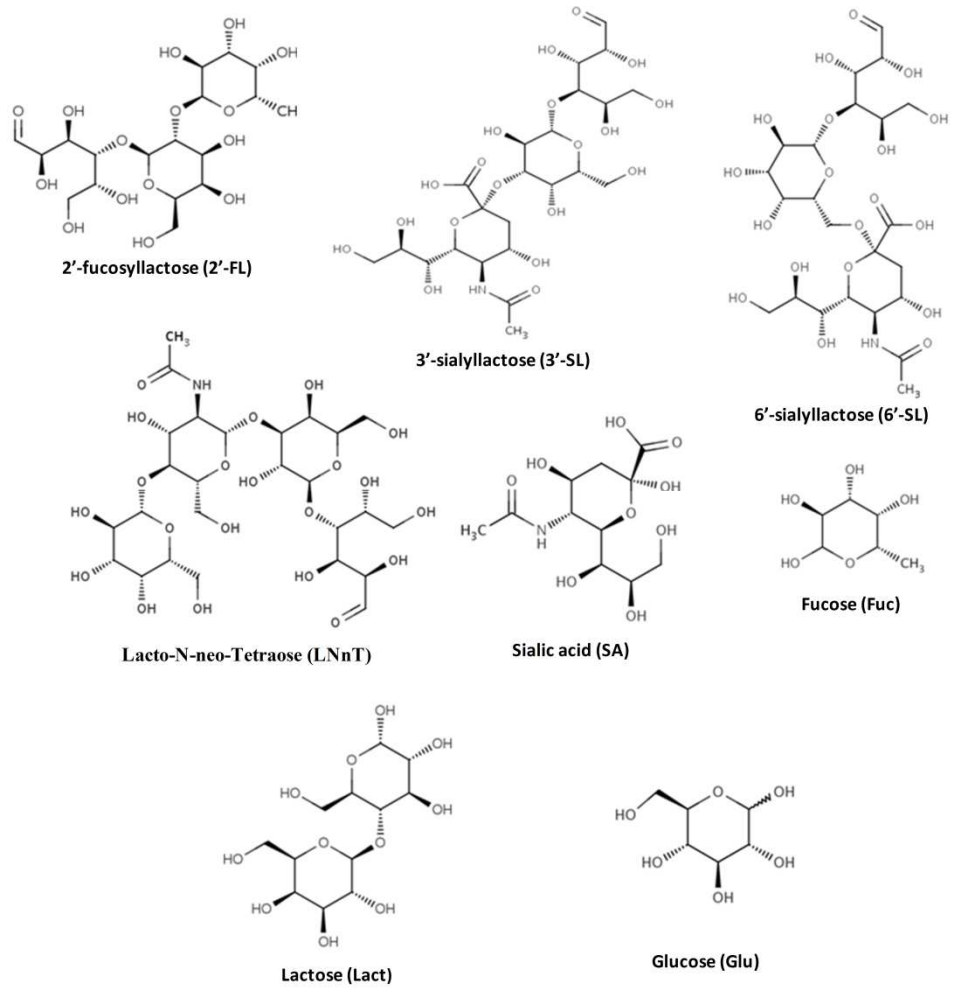

Fig. 1. Chemical structures of the human milk oligosaccharides and related compounds of interest.

2. Materials and methods

2.1. *Chemicals and reagents*

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli–Q system from Millipore (Bedford, MA, USA). SA, Fuc, Lact and Glu were supplied by Sigma–Aldrich (Madrid, Spain); 2'–FL, 3'–SL and 6'–SL were all derived from

bacterial synthesis; and LNnT was synthesized from a yeast fermentation system and purified by crystallization [30]. The purity and content of each SMG was measured by high performance anion–exchange chromatography with pulsed amperometric detection (HPAEC/PAD). Acetonitrile (MeCN) LC–MS grade, ethanol (EtOH) HPLC grade and ammonia solution 25% (v/v) eluent additive for LC–MS were from Scharlab (Barcelona, Spain).

A stock solution was prepared by weighing 0.002 g of each compound into a 10 mL flask, except for Lact and Glu, for which 0.005 g were weighed. Then, water was added up to the final volume. The solution remained stable for at least one month at 4 °C. After that, six work standard solutions were prepared for calibration purposes. The first one (WS6) was obtained by dilution to 1 mL of 100 µL of the stock solution. Then, 500 µL, 100 µL, 25 µL and 5 µL of WS6 solution were diluted to a final volume of 1 mL to obtain standards WS5, WS4, WS3 and WS2, respectively. Finally 150 µL of WS2 were diluted to 1 mL to prepare WS1. In all cases, an aqueous solution of ammonia 0.1% (v/v) was used for standard preparation. The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS4) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment. The stock solution was stable for 1 month at 4 °C. The working standard solutions were prepared from the stock solution for each experiment.

2.2. *Apparatus and software*

Detection and quantification of the studied compounds were performed using an UPLC Acquity® system from Waters (Milford, MA, USA) equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector. The chromatograph was connected on–line to a triple quadrupole mass spectrometer detector (TQD) with an electrospray ionization (ESI) interface. Three different polarity chromatographic columns were tested in order to achieve a good resolution: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 µm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 µm particle size), and Acquity UPLC BEH

Amide (2.1 mm × 100 mm i.d., 1.7 µm particle size) from Waters. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis.

Analytical balance with a precision of 0.1 mg, vortex–mixer, maximum recovery LC vials and screw caps from Waters, eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom), and speed vac evaporator from Heraeus Instrument Thermo Scientific (Madrid, Spain) were also used.

2.3. *Animal manipulation*

Thirty Sprague Dawley female rats (~300 g body weight) (Charles River Laboratories, France) were used. The animals were kept in pairs in standard cages at constant room temperature (22 ± 2 °C) and 45–55% humidity under a regular 12 h light/dark schedule. Food and water were freely available. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101–2005, 86/609/CEE).

Animals were assigned to five experimental groups (n = 6). Group 1 received a single dose of 2´–FL (1.75×10^{-3} mole kg^{-1}); group 2, 6´–SL (1.75×10^{-3} mole kg^{-1}); group 3, 3´–SL (1.75×10^{-3} mole kg^{-1}); group 4, LNnT (1.75×10^{-3} mole kg^{-1}) and group 5, a mix of 2´–FL, 6´–SL, 3´–SL, and LNnT (4.37×10^{-4} mole of each SMGs kg^{-1}, representing 1.75×10^{-3} mole kg^{-1} in total).

All the solutions were prepared in water and were administered by intragastric gavage. The animals were fasted for 12 h and a blood sample was taken from the caudal veins (time 0). After the gavage, serial blood samples were collected at 30, 60, 90, 120, 150, 210 and 240 min. Blood samples were allowed to clot and then, centrifuged at 1800 × g for 10 min. Serum was stored at –20 °C.

2.4. *Sample Preparation*

The initial extraction protocol was based on the procedures previously proposed in the scientific literature [18, 27]. The protocol was then modified and optimized in order to improve the extraction of the analytes from serum samples. An aliquot (80 µL) of serum sample was placed into a 10 mL glass tube and extracted with a solution containing 2.0 mL of a 2:1 (v/v) mixture of chloroform and methanol, and 0.4 mL of deionized water. After shaking for 2 min in a vortex–mixer, samples were centrifuged at 2400 × g at 4 °C for 30 min. The lower chloroform layer was discarded and the upper layer was collected. For protein precipitation, 1 mL of cold ethanol was added and the mixture was left at 4 °C overnight. Then, the solution was centrifuged at 2400 × g at 4 °C for 20 min and the supernatant was separated and dried in a rotary vacuum pump evaporator at 30 °C. The dried samples were re–dissolved in a total volume of 80 µL of a 0.1% (v/v) aqueous solution of ammonia and the final extract was centrifuged in a microcentrifuge at 14,600 × g for 10 min. The supernatant was placed in a chromatographic vial for injection into the UPLC–MS/MS system (1 µL injection volume).

2.5. *Liquid chromatographic conditions*

Chromatographic analysis was performed using a UPLC BEH Amide column (2.1 × 100 mm, 1.7 µm particle size) from Waters. The flow rate was 300 µL min^{-1}, the column was maintained at 25 °C, the sample at 20 °C and the injection volume was 1 µL in partial loop mode. A gradient mobile phase consisting of 0.1% (v/v) ammoniacal aqueous solution (solvent A) and 0.1% (v/v) ammonia in acetonitrile (solvent B) was used. Gradient conditions were: 0.0–3.0 min, 10 to 25% A; 3.0–8.0 min, 25 to 40% A; 8.0–8.1 min, 40 to 10% A; and 8.1–10.0 min 10% A to stabilize the initial conditions. The total run time was 8.1 min, and the post–delay time for reconditioning the column with 10% A was 1.9 min. Weak solvent was a mixture of 25 mL of water and 75 mL of acetonitrile, and strong solvent was a mixture of 80 mL of water and 20 mL of acetonitrile. These solutions were stable for at least one week at room temperature.

2.6. *Mass spectrometric conditions*

ESI was performed in negative ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1–1.0 mg L^{-1}). Electrospray ionization spray voltage was 3500 V. Nitrogen was used as desolvation gas at 800 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 400 °C. Argon (99.999% purity) was used as collision gas at an approximate rate of 0.13 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the single function are Inter Channel Delay, 5 ms and InterScan Time, 5 ms.

Table 1
MS/MS parameters for ionisation with ESI- in MRM mode.

Compound	Parent Ion (Da)	Daughter (Da)	Dwell time (ms)	Cone (V)	Collision (eV)	Delay (ms)
Fuc	163.0	58.9	20	15	15	5
		88.9	20	15	5	5
Glu	179.0	58.8	20	13	15	5
		89.0	20	13	8	5
SA	308.1	87.0	20	25	17	5
		170.1	20	25	10	5
Lact	341.1	100.9	20	15	15	5
		161.1	20	15	8	5
2'-FL	7.2	161.0	20	20	12	5
		205.0	20	20	17	5
6'-SL	632.2	87.1	20	60	35	5
		290.1	20	60	28	5
3'-SL	632.2	87.1	20	62	30	5
		264.0	20	62	28	5
LNnT	706.2	179.0	20	28	25	5
		263.0	20	28	20	5

3. Results and discussion

3.1. *Liquid chromatographic analysis*

Based on the recommendations obtained from the scientific literature [19–22], some preliminary studies were carried out to optimize chromatographic separation and signal intensity using a standard mixture of compounds. First, an Acquity UPLC BEH C18, an Acquity UPLC BEH HILIC and an Acquity UPLC BEH Amide columns were tested in order to achieve a good separation of peaks. The amide column provided the best resolution for the studied analytes in the shortest time. Consequently, the Acquity UPLC BEH Amide column was the one we selected for further experiments.

Because our aim was to obtain higher sensitivity and selectivity in a short time, the effect of mobile phase was also studied. In order to optimize the separation and peak shapes, different solvents and additives were checked. Two organic solvents (methanol and acetonitrile) commonly used in liquid chromatography were evaluated. Acetonitrile gave better results than methanol, so we selected acetonitrile as optimum for mobile phase. Moreover, several additives (ammonia, ammonium acetate, triethanolamine, ammonium formate, acetic acid and formic acid) were also assayed. The best separation, peak shapes and ionization of the compounds were obtained with a mobile phase composed of 0.1% (v/v) of ammonia in acetonitrile as solvent A and 0.1% (v/v) of ammonia in water as solvent B. The linear gradient described previously was used.

On the other hand, in order to improve the sensitivity of the method, a study to evaluate the possibility of increasing the injection volume and to evaluate different modes of injection was also performed. Injection volumes ranging from 1 to 10 µL were studied and an extra broadening of the peaks was observed at injection volumes higher than 2 µL; therefore, a volume of 1 µL was selected. Related to injection mode, full loop required a greater amount of sample compared to partial loop, and due to the sample amount limitations, partial loop was selected.

Finally, the stability of standards and samples was also studied. The stability was studied in different days for four weeks. It was demonstrated that refrigerated standards after a week were acceptable, but refrigerated serum extracts were not. Therefore, it is advisable to prepare work solutions, standards and processed samples freshly for each experiment.

3.2. *Mass spectrometric analysis*

The MS/MS detection method was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and ESCI interfaces in positive and negative modes were evaluated. ESI interface in negative mode was selected because it showed higher sensitivity for all compounds of interest. For each compound two product ions (two reactions) were monitored: one for quantification and the other for confirmation. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperature, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Figure 2 shows chromatograms of a standard mixture of compounds.

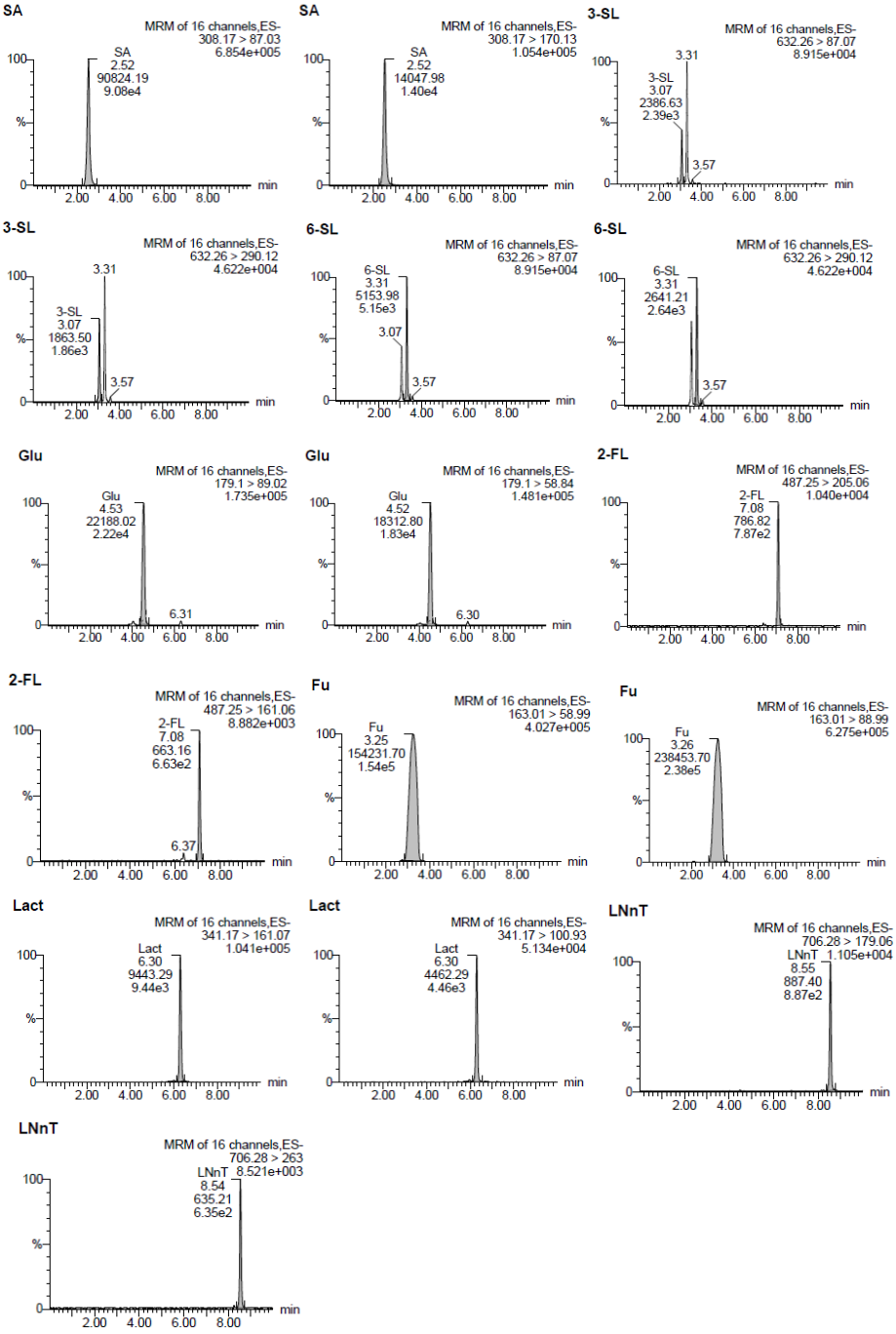

Fig. 2. Chromatogram of each analyte standard in an aqueous solution of ammonia 0.1% (v/v) (two transitions). The first transition was used for quantification and the second for confirmation at concentrations of 20 µg mL^{-1} for SA, 3'–SL, 6'–SL, 2'–FL, Fuc, LNnT and 50 µg mL^{-1} for Glu and Lact.

3.3. *Analytical performance*

Analytical performance was evaluated according to the recommendations of Analytical Methods Committee [31]. First, calibration curves were established using serum, plasma and solvent to check the absence of matrix effect. A Student's t-test was applied in order to compare the calibration curves. First, we compared the variances estimated as $S_{y/x}^2$ by means of an *F*–Snedecor test. The *t*–Student test showed no statistical differences among slope values for the calibration curves in all cases and, consequently, the calibration was carried out in solvent. On the other hand, although the results were very similar, serum had minor variability in replicates, for this reason serum instead of plasma was chosen as sample.

The calibration curve for each compound, was obtained in MRM mode by injecting 1 µL of different standard solutions (prepared in 0.1%, v/v, ammonia aqueous solution) in the concentration range from 0.02–20 µg mL^{-1} for SA, 3'–SL, 6'–SL, 2'–FL, Fuc and LNnT and 0.04–50 µg mL^{-1} for Glu and Lact. Each calibration level was made in triplicate, and analyzed twice. Table 2 shows the analytical parameters obtained.

Table 2
Analytical and statistical parameters.

	n	a	b (mL ng^{-1})	R^2 (%)	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	LDR (µg mL^{-1})	P_{lof} (%)
SA	6	15	7680	99.9	0.005	0.020	0.02 – 1000	95.2
Fuc	6	674	185	99.8	0.030	0.100	0.10 – 1000	70.7
3'-SL	6	-14	243	99.0	0.020	0.060	0.06 – 60	94.2
6'-SL	6	-32	237	99.4	0.020	0.060	0.06 – 60	17.7
Glu	6	154	150	99.5	0.030	0.100	0.10 – 500	91.9
Lact	6	-33	80	99.4	0.070	0.200	0.20 – 100	65.1
2'-FL	6	12	243	99.9	0.005	0.020	0.02 – 100	81.3
LNnT	6	-36	50	99.3	0.020	0.060	0.06 – 100	66.8

n, calibration levels; a, intercept; b, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; P_{lof} (%), P-value for lack-of-fit test.

Quality controls were injected after every 20 injections to assure the validity of the calibration curve. The predicted value expected was not to exceed ± 15% of the theoretical value.

3.4. *Method validation*

Validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food and Drug Administration (FDA) guideline for bioanalytical assay validation [32].

Linearity. Linearity of the calibration curves was quantified by both linear coefficient of determination (%R^2) and *P*–values of the *lack–of–fit* test (%P_{lof}). The linearity for all compounds within this wide concentration range was achieved with R^2 ranging from 99.0% for 3'–SL to 99.9% for SA and 2'–FL, and P_{lof} were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

Selectivity. The specificity of the method was determined by comparing the chromatograms of blanks with those corresponding to the samples. No interferences from endogenous substances were observed at the retention times of each respective analyte (Fig. 2), which eluted at 2.97 min, 3.25 min, 3.51 min, 3.73 min, 4.43 min, 6.11 min, 6.87 min and 8.31 min. for SA, Fuc, 3'–SL, 6'–SL, Glu, Lact, 2'–FL and LNnT respectively. This finding suggested that the LC–MS/MS conditions provided sufficient selectivity.

Accuracy: precision and trueness. Due to the lack of certified reference materials, a recovery assay with spiked samples was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation from the standard calibration curve. Recoveries were calculated by comparing the obtained amounts with the theoretical spiked amounts. As shown in Table 3, the recoveries fell between 85% and 115% in all cases.

Table 3
Recovery, precision and accuracy of target compounds in serum samples.

	Intra-day					Inter-day			
	Spiked (μg mL^{-1})	Found[a] (μg mL^{-1})	R (%)	RSD (%)	n	Found[a] (μg mL^{-1})	R (%)	RSD (%)	n
SA	0.91	0.84	92.3	12.1	3	0.85	93.4	13.0	15
	9.05	7.73	85.4	2.4	3	7.98	88.2	5.2	15
	27.2	25.7	94.8	5.3	3	26.3	96.7	5.1	15
Fuc	11.2	9.53	85.2	3.1	3	9.57	85.6	3.8	15
	112	95.6	85.5	3.5	3	96.5	86.4	3.7	15
	335	311	92.8	4.3	3	309	92.1	4.8	15
3'-SL	0.19	0.16	88.1	7.3	3	0.18	96.2	9.1	15
	1.86	1.72	92.3	5.5	3	1.75	94.0	6.3	15
	5.59	4.76	85.0	4.9	3	5.06	90.5	8.3	15
6'-SL	0.60	0.62	96.8	7.9	3	0.59	98.1	8.8	15
	6.03	5.25	87.2	4.6	3	5.37	89.1	6.6	15
	18.1	15.5	85.8	2.1	3	15.8	87.5	1.7	15
Glu	1.10	1.17	106.3	8.0	3	1.16	105.4	9.6	15
	11.0	11.2	101.5	9.9	3	11.3	102.7	9.9	15
	33.0	34.7	103.7	4.9	3	34.3	103.7	5.1	15
Lact	1.02	0.77	85.7	9.9	3	0.90	90.0	13.6	15
	10.2	9.50	94.3	6.4	3	9.58	94.3	6.2	15
	30.5	25.9	85.2	2.6	3	24.9	81.5	2.6	15
2'-FL	0.20	0.19	95.0	14.0	3	0.19	98.8	14.9	15
	1.95	2.00	102.6	10.7	3	2.11	108.2	11.8	15
	5.86	6.61	115.0	11.1	3	6.73	114.8	12.0	15
LNnT	0.15	0.13	87.1	12.1	3	0.16	107.9	13.2	15
	1.46	1.36	93.1	4.9	3	1.49	101.6	8.6	15
	4.39	3.93	90.3	2.0	3	3.96	90.3	3.6	15

[a] *Mean value; R, % recovery; RSD, relative standard deviation; n, number of determinations*

To assure precise quantifications of the global method the intra– and inter–day precisions (as relative standard deviation, RSD) were assessed at

three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate the intra–day variability. The procedure was repeated on five consecutive days to determine inter–day variability. The results of repeatability and within–laboratory reproducibility are summarized in Table 3. RSD values fell between 1.7% for 6'–SL at a concentration of 18.1 μg mL^{-1}, and 14.9% for 2'–FL at the lower concentration assayed. This last value was probably due to the fact that the concentration was close to the limit of quantification (LOQ). Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value, except at the LOQ, which it should not deviate by more than 20%. Precision and trueness data indicated that the methodology to determine the target compounds in serum from rat is highly accurate, precise, and robust, and that the presence of co–extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Sensitivity. Limit of detection (LOD) and limit of quantification (LOQ) are two fundamental aspects that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives. In this work, these parameters were calculated from the signal–to–noise ratio. The calculated LODs (signal–to–noise ratio = 3) are in the range from 0.005 μg mL^{-1} for SA and 2'–FL to 0.07 μg mL^{-1} for Lact, and the corresponding LOQs (signal–to–noise ratio = 10) ranged from 0.02 to 0.20 μg mL^{-1}, respectively. The values obtained are summarized in Table 2.

3.5. *Application of the method*

Samples of serum from rats were collected following the protocol previously described in section 2.3. The objective of this experiment was to determine the time–course of each analyte when different ingredients were administered to the animals orally. Figure 3 shows chromatograms obtained for a serum sample.

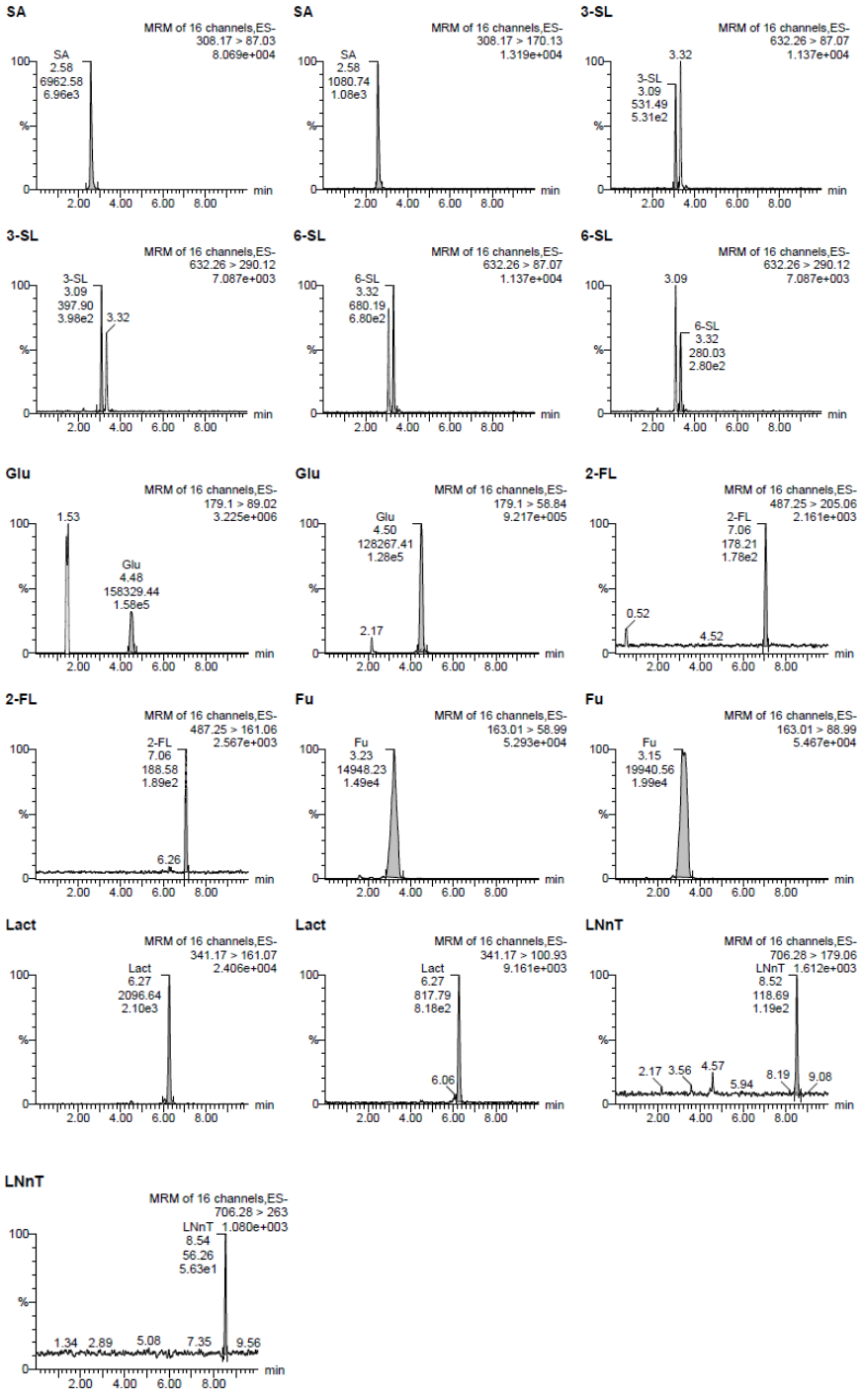

Fig. 3. Chromatograms obtained for a serum sample (two transitions). The first transition of each analyte was used for quantification and the second for confirmation.

Figure 4 shows the time–course of the four SMGs tested in this study: 2´–FL, 3´–SL, 6´–SL and LNnT in rat serum. For the purpose of simplicity this section was focused on the compounds that were administered although the mono– and disaccharides: SA, Fuc, Lact and Glu were also detected.

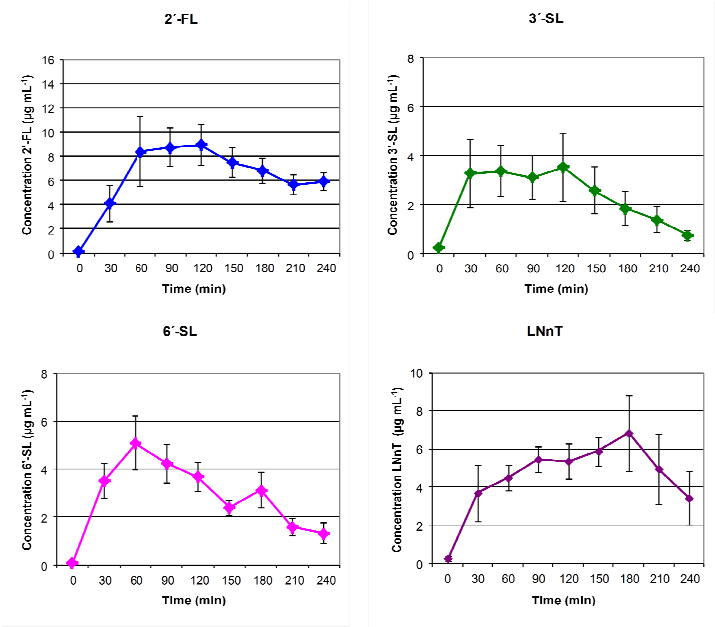

Fig. 4. Time–course for the four SMGs tested in the study: 2´–FL, 3´–SL, 6´–SL and LNnT. On the "y" axe is the concentration of each analyte in µg mL^{-1}, on "x" axe is the time expressed in minutes.

Both sialylated SMGs, 3´–SL and 6´–SL, in addition to 2´–FL, exhibited a similar absorptive profile: their levels peaked in the bloodstream between 30 to 60 min after the gavage. However, LNnT exhibited a significantly slower absorption rate, reaching the maximum level in plasma at 180 min after feeding. On the other hand, not all the SMGs were cleared from the blood stream with the same efficacy. Figure 4 shows how both sialylated SMGs are almost completely removed from plasma 240 min after the challenge, whereas the plasma levels of LNnT and, specially, 2´–FL remain significantly higher at the end of the study (240 min). This fact clearly points out that each SMGs may have different mechanisms of absorption in the intestine and/or may be taken up differently by peripheral tissues or excreted by the urinary system. In that

sense, it has been previously reported that there might be different mechanisms underlying the absorption of structurally different SMGs. Thus, Gnoth and collaborators reported in 2001 that while non–sialylated SMGs cross monolayers of cultured intestinal epithelial cells by receptor–mediated transcytosis and paracytosis, sialylated SMGs only use paracytosis [33]. However, it remains unknown which receptors facilitate absorption, as well as their absorption rate, appearance in the circulation and clearance from the system [34].

While it is becoming evident that SMG structure determines their specific function, understanding the metabolic fate of ingested SMGs is key in assessing their biological roles. There is little knowledge about how, when and where they are metabolized. SMGs have long been regarded as metabolically "inert" to the host, because significant amounts are excreted in feces. However, it is well known that a small percentage of SMGs is believed to be absorbed intact in the small intestine and later excreted in urine, which opens speculation on possible systemic effects, e.g. in the immune system or in the context of neuronal development [35]. These potential beneficial systemic effects of SMGs on neonatal health come from indirect evidence, namely in vitro data and the presence of SMGs excreted in urine of breast–fed, but not formula–fed infant [35].

The development of a method such as the one shown in this article, and its application to the reported experiment in rats allowed us to show that SMGs are absorbed and detected in the blood stream. Up to now, only a recent article described the appearance of 3´SL in plasma of neonatal rats [5]. No other SMGs were detected, so that the authors concluded that selective absorption occurs. There were many differences between this study and ours, with regard to the analytical method, the age of the animals, and the dosing. Despite this, our research does not support the hypothesis of a selective absorption, as the four SMGs: 2'–FL, '3–SL, 6'–SL and LNnT, given alone or in combination, were detected in serum showing that all of these compounds can reach the systemic circulation as intact SMGs.

4. Conclusions

The determination and quantification of SMGs using UHPLC–MS/MS in serum samples was successfully performed on an Acquity UPLC BEH Amide column, using 0.1% (v/v) ammonia in acetonitrile and 0.1% (v/v) ammonia in water as mobile phase and triple quadrupole mass spectrometry detection in negative electrospray ionization mode. The analytical performance of the method was validated providing a powerful tool for the simultaneous determination of eight compounds that include the most abundant SMGs and related metabolites. The simplicity and sensitivity of the new method made it possible to conduct an *in vivo* study in rats to demonstrate the absorption of these compounds from the intestine to the systemic circulation, which constitutes one of the first studies showing this aspect *in vivo*. The proposed method could also provide opportunities to study all of these components in a wide variety of situations.

Acknowledgments

The authors are indebted to all the participants, without whom this work would not have been possible. We are grateful to the team of technicians of Abbott which helped in the tasks of care and maintenance of animals, and in the sampling protocol. We also thank Rachael Buck, PhD, and Pedro Prieto, PhD, for their contribution to the revision of this manuscript.

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II.5 STUDY OF ABSORPTION OF 2'-FL FROM INTESTINE TO PLASMA COMPARTMENT IN RAT PUPS (Publicación IV. Libro de Actas de Congreso)

En esta sección se presenta el resumen presentado en el 48th Annual Meeting of The European Society for Paediatric Gastroenterology, Hepatology and Nutrition, May 2015 Amsterdam, Netherlands: ESPGHAN2015.

ESPGHAN
48th ANNUAL MEETING
of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition

6 - 9 May 2015
Amsterdam RAI Exhibition and Convention Centre
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Dear Dr Enrique Vazquez,

Thank you for your abstract submission for the 48th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition to be held in Amsterdam, The Netherlands, 6 – 9 May 2015.

On behalf of the Scientific Programme Committee, we have great pleasure to inform you that your abstract

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ABSORPTION OF THE HUMAN MILK OLIGOSACCHARIDE 2'-FUCOSYLLACTOSE IN RAT PUPS.

has been accepted as a Poster for display.

Your abstract has been renumbered and your final poster number is:

PO-N-0364

Please note information below for your poster display:

Display Date:	**From 07:30 Thursday 7 May 2015 until 12:00 on Saturday 9 May 2015**
Poster Display Location:	**Hall 3**
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Mandatory information:	**The poster number has to be indicated in the top right hand corner The dimensions of your poster should be a maximum: Width: 93cm x Height: 223cm (Portrait format).**
Presenting Author:	**Maria Ramirez**
Poster guidelines:	**Specific guidelines are available on http://www.espghan2015.org/for - delegates/abstracts on the abstract page of the website**

Figura 31. *Abstract Congreso internacional de ESPGHAN*

Nutrition

Basic Science

ESPGHAN2015-1293

ABSORPTION OF THE HUMAN MILK OLIGOSACCHARIDE 2'-FUCOSYLLACTOSE IN RAT PUPS

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Presentation Preference: Poster

Objectives and Study: Human milk contains all the nutrients necessary to support infant growth and development, including a rich repertoire of human milk oligosaccharides (HMOs). The HMO fraction is unique regarding its diversity, quantity and complexity. 2'-fucosyllactose (2'-FL) is the most abundant HMO in the majority of human milk. In contrast, trace amounts of oligosaccharides are present in mature bovine milk and in milk-based infant formula. Although the prebiotic effects of HMOs have been established *in vitro*, HMOs may have systemic effects. Recent findings described the presence of HMOs in the plasma and urine of breast-fed infants providing evidence that HMOs reach the circulation. Furthermore, systemic levels of 2'-FL correlate with levels present in human milk. Consequently, HMOs have the potential to impact health beyond the intestine.

Aim: To investigate the time-course of 2'-FL absorption from the intestine to the blood stream as well as its excretion in the urine compartment after an oral gavage at increasing doses in rat pups.

Methods: Four hour-fasted Sprague Dawley rat pups (1-12 day old) were given a unique oral gavage of 2'-FL at several doses (1, 2.5, 5 and 10 g/L). Pups were sacrificed (n=8) at different times after the gavage (0, 30, 60, 90, 120, 180 and 240 min) and serum and urine samples were collected. Samples were analyzed by UHPLC-MS/MS for the quantification of HMOs.

Results: After the oral bolus, 2'-FL was quickly taken up from the intestine to the plasma compartment. The appearance of circulating 2'-FL as well as the

excretion of 2'-FL in urine after an oral bolus was dose-dependent. At the doses tested, no saturation effect was noticed regarding the absorption of 2'-FL from the intestine to the blood stream.

Conclusion: In rat pups, orally-given 2'-FL is effectively absorbed from the intestine to the circulation in a dose-dependent manner. This result confirms observations in breast fed infants, and suggests systemic effects of HMOs in support of health and development.

Disclosure of Interest: E. Vazquez Conflict with: staff of Abbott Nutrition, A. Santos Conflict with: staff of Abbott Nutrition, M. Ramirez Conflict with: staff of Abbott Nutrition, P. Prieto Conflict with: staff of Abbott Nutrition, R. Buck Conflict with: staff of Abbott Nutrition, R. Rueda Conflict with: staff of Abbott Nutrition.

Keywords: None.

Animal Manipulation

Rats were supplied by Charles River laboratories (France). Sprague Dawley rats (SD) were allowed to acclimate to Abbott animal facility before running experimental approaches. Animal were kept under a 12 h light/dark cycle at 21-24°C, 40-70% humidity, with free access to food and water unless otherwise specified. animal procedures were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 53/2013).

Analytical Method

An UHPLC–MS/MS method for the identification and quantification of the following glycans and their precursors: 2'-FL, 3'-SL, 6'-SL, LNnT, N-acetylneuraminic acid, fucose, lactose and glucose in serum and urine from rat was previously developed by the Granada team (Santos-Fandila et al. 2014.

Talanta 118: 137–146). These analytes were separated in an Acquity UPLC BEH Amide column using acetonitrile–water gradient with ammonia as additive, in a 10 min run, and were detected and quantified using a triple quadrupole (QqQ) mass spectrometer. The mass spectrometric conditions in negative electrospray ionization mode (ESI-) were individually optimized for each analyte to obtain maximum sensitivity in the Selected Reaction Monitoring (SRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter- and intra-day variability was lower than 15% and the recoveries ranged from 85% to 115%.

The sample preparation was based on the procedure mentioned above. The protocol in serum was the same; it consisted basically in a liquid-liquid extraction with a mixture of chloroform and methanol (2:1, v/v), and deionized water, and incubation overnight with cold ethanol to precipitate proteins. After centrifugation, the samples were dried, re–dissolved and placed in a chromatographic vial for injection into the UPLC®–MS/MS system. Urine did not need extraction; the urine samples were diluted with water, and filtrated and centrifuged in an Amicon filter (pore size: 10000k, 14000 rpm, 30 min) before injection.

Method Application

Objective: To run an experiment in healthy normal rat pups to evaluate the absorption of 2'-FL given by oral gavage.

Pilot experiment. We did not have previous data about how 2'-FL is absorbed in rat pups, and therefore we run a pilot trial in order to confirm if the initially designed dosages were higher enough to show a good analytical signal in the serum of rat pups. We evaluated 5 doses of 2'-FL administered orally in pups, both males and females (n=2 per dose). The doses were 50, 5, 1, 0.1 and 0.03 g/L encoded as doses 1, 2, 3, 4 and 5. The 2'-FL solutions were given by a

300 µL gavage to 10 day-old pups fasted during 4 hours. The serum was collected 1 hour after the 2'-FL administration. Table 1 describes the circulating 2'-FL concentration 1 hour after the gavage in males as well as in females for all the doses. Figure 1 shows a graphic representation of the same data.

Table 1. *2'-FL concentration in serum from pups after 1h of gavage.*

	Male	Female
Doses	**2'-FL (ug/ml)**	**2'-FL (ug/ml)**
Time 0	0,0000	0,0000
1	13,9576	15,0572
2	1,7197	2,1370
3	0,5486	0,4557
4	0,1061	0,0530
5	< 0,02	< 0,02

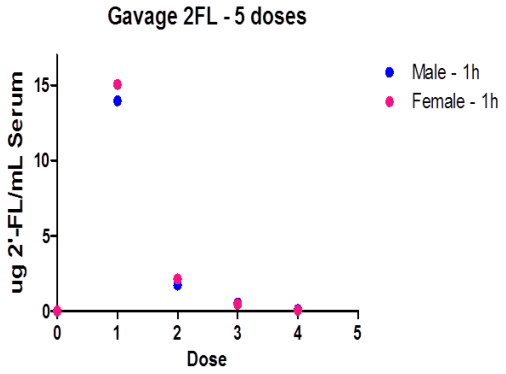


Figure 1. *2'-FL concentration in serum from pups after 1h of gavage at increasing doses of 2'-FL.*

We concluded that dose 5 (0.03 g/L) was too low to generate measurable levels of 2'-FL in the plasma compartment and that dose 1 (50g/L) seemed to be too high. In conclusions, we decided to conduct the experiment with four doses of 2'-FL: 1, 2.5, 5 and 10 g/L encoded as doses A, B, C and D.

Experimental design and methods. Two days after delivery, all the litters were mixed and readjusted in order to keep the same number of pups for each dam (n=10 pups for dam, five males and five females). The day of the experiment, the pups were 9-11 days old. The pups were separated from the dams and fasted during 4 hours while were kept warm in 28-30°C-thermostated cages. According to the pilot experiment described above, 2'-FL was given at four different doses: 1, 2.5, 5 and 10 g/L (encoded as doses A, B, C and D, respectively). The gavages were given using a soy formula as a free-HMO vehicle (0.3 mL per gavage). At different times after the gavage (30, 60, 90, 120, 180 and 240 min) pups were sacrificed (n=8 each sampling time, 4 males and 4 females) and blood and urine were collected and frozen for HMOs

determination by UHPLC-MS/MS. An additional group of 12 pups (6 males and 6 females) were also sacrificed without any gavage in order to get basal levels.

Results. Basal levels. Figure 2 displays the amounts of sialic acid, 3'-SL, 6'-SL, 2'-FL as well as the carbohydrates fucose, lactose and glucose of pups after 4 hours of fasting. We have also included in this figure serum basal data in adult rats in order to make easier the comparisons between the HMOs level in pups and adults.

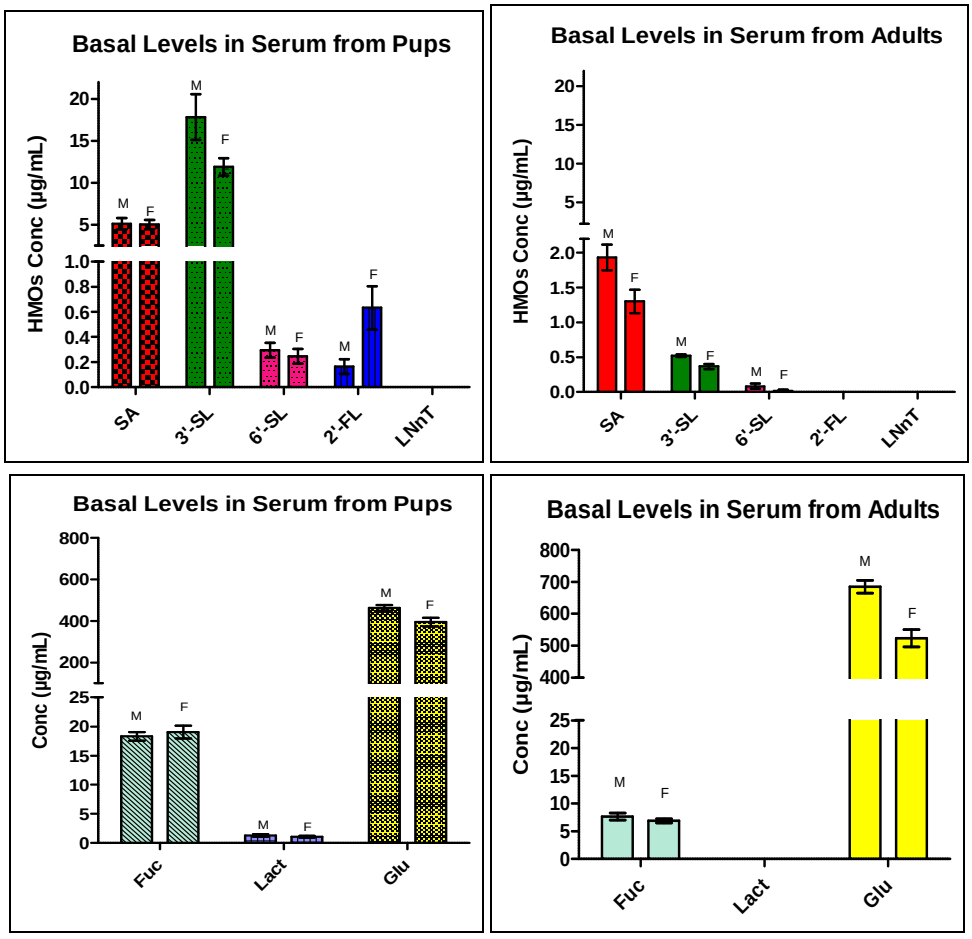


Figure 2. *Basal levels of HMOs and related compound in pups and adult rats. M: male; F: female.*

Results. Serum data in pups after gavage with 2'-FL. Figure 3 shows the absorption of 2'-FL in pups from intestine to plasma compartment during 3 h after the gavages (3A, male and female pups together; 3B, split by gender).

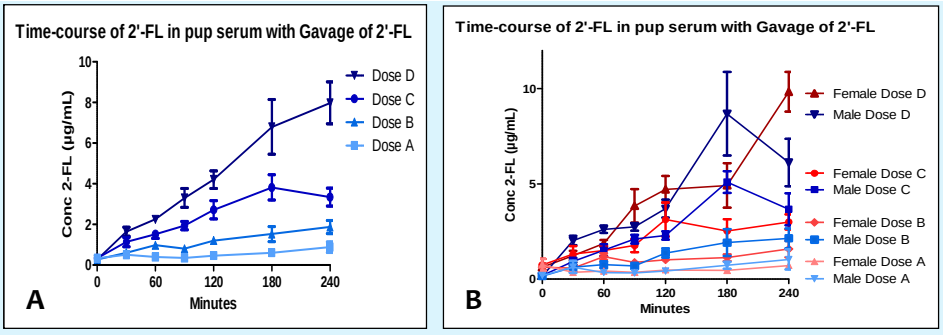


Figure 3. *Time-course of 2'-FL appearance in serum from pups given with different doses of 2'-FL. Graph A: average of the concentration of serum 2'-FL with data from both male and female compiled; Graph B: average of the serum concentration of 2'-FL in males and females separately.*

The absorption curves were absolutely dose-dependent, although the lower doses (A and B) induced an absorption rate much slower than in higher doses, where the slopes of the curves (doses C and, especially dose D) were more remarkable (interaction dose x time $p<0.000$, multifactorial ANOVA) . Overall, there were no effect of sex ($p>0.7$, multifactorial ANOVA) but there was a significant interaction of time x sex which was due to a higher content of 2'-FL in males than in females only at 180 min and that did not have any biological relevance.

Regarding the other HMOs and associated compounds, there were no significant changes (data not shown) during the time-course in any of them respect to those levels in the baseline excepting for fucose. Interestingly, when a gavage of 2´-FL was administrated to the pups, fucose increased proportionally to the concentration of 2´-FL with the same type of profile than in 2´-FL, reaching also maximum absorption at 180 minutes (Figure 4). This finding was not observed in adult rats, and so, it seems that this could be a phenomenon linked to early infancy and that may be related to the higher

intestinal permeability of developing pups. Likewise, these data may support the concept of fucose absorption as a marker of intestinal maturity.

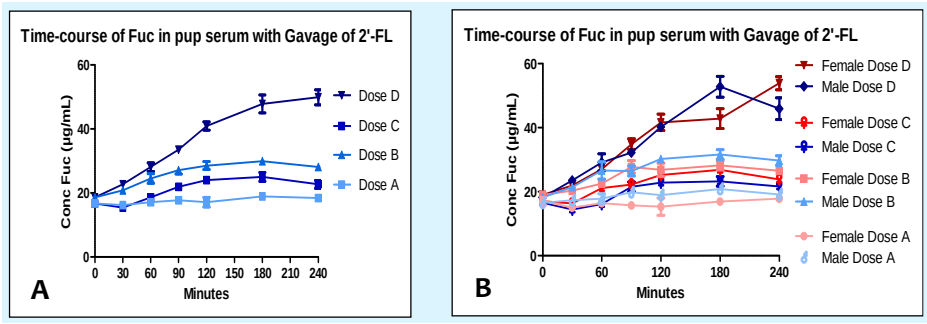


Figure 4. *Time-course of fucose appearance in plasma from pups given with different doses (A and C) of 2'-FL. Graph A concentration of serum fucose with data from both male and female compiled; Graph B: concentration of serum fucose with data from males and females separately.*

Lactose serum concentrations were 10 times higher in pups than in adult rats (see Figure 5). Moreover, no significant changes were noted in serum lactose concentration after the gavages with 2'-FL unlike adult rats, in which serum lactose level was progressively increased after the gavage with 2'-FL.

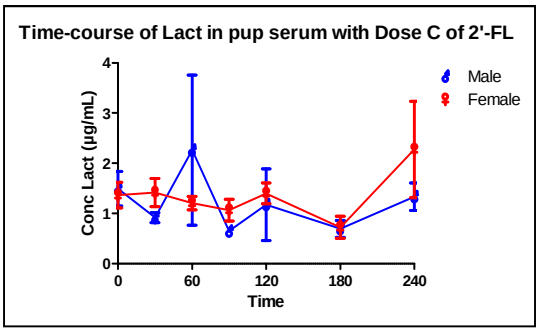


Figure 5. *Time-course of lactose appearance in serum in pups given 5g/L 2'-FL (Dose C).*

Results. Urine data in pups after gavage with 2'-FL. The time courses of 2'-FL and related compounds in urine were quite similar to those described for serum samples. The excretion of 2'-FL was dose-dependent and exhibited a progressive and constant increase over time (Figure 6A). There were no

significant differences between the concentration of 2'-FL in urine of males and females (figure 6B).

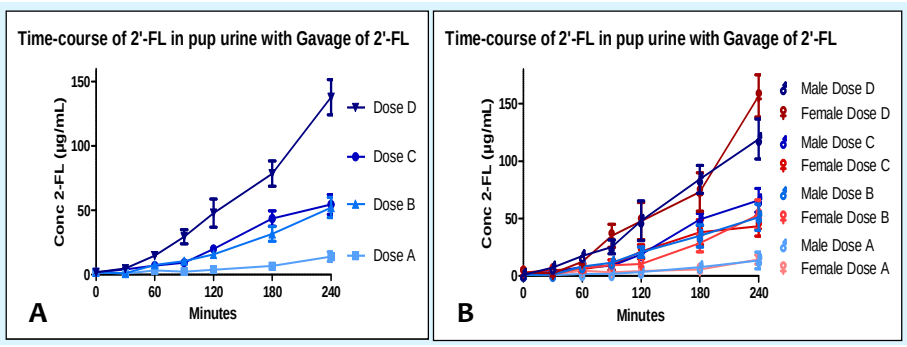


Figure 6. *Time-course of 2'-FL appearance in urine from pups gavaged with different doses of 2'-FL. Graph A: average of the concentration of serum 2'-FL with data from both male and female compiled; Graph B: average of the serum concentration of 2'-FL in males and females separately.*

Sialic acid, 3'-SL and 6'-SL were detected in urine of pups, at baseline and after the gavages with 2'-FL. Like in blood, these HMOs and related analytes, excepting fucose, did not change over the 3 hours after the gavage of 2'-FL, independently of doses and gender (data not shown). Interestingly, LNnT, which was not detected in the plasma compartment, was measured in urine at low levels (0.5-2.5 µg/ml). LNnT was detected in urine samples at the baseline as well as in all the other sampling times, not showing a remarkable variation in its urine concentration over time (Figure 7). This fact supports the presence of LNnT in blood although it could not be detected in serum.

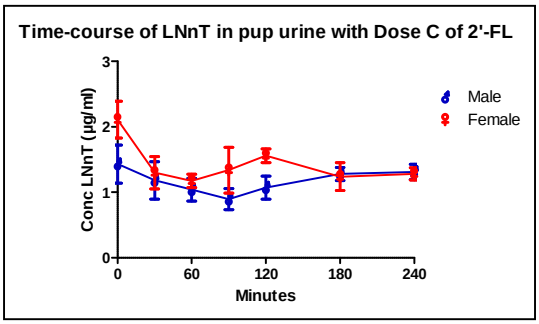


Figure 7. *Levels of LNnT in pup urine during three hours after the gavages with 5g/L 2'-FL (dose C).*

Fucose increased in urine over time in a dose-dependent manner as it did in serum (figure 8).

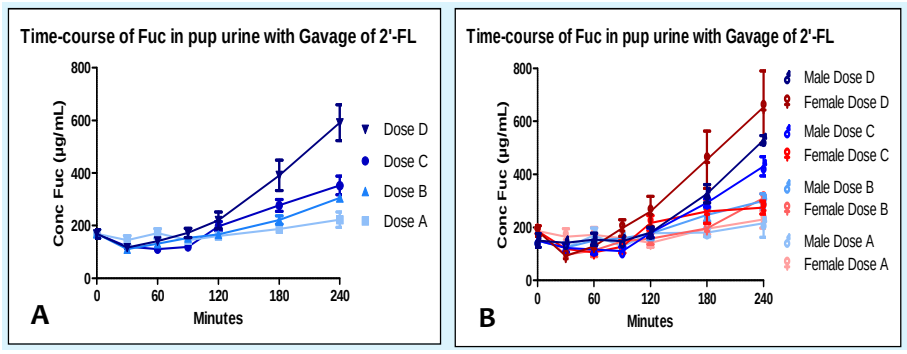


Figure 8. *Time-course of Fuc in pup urine when different doses of 2'-FL were given. Graph A: average of the concentration of urine 2'-FL with data from both male and female compiled; Graph B: average of the urine concentration of 2'-FL in males and females separately.*

Biochemical Findings

Comparing both experiments, some findings could be highlighted:

- When 2'-FL, 6'-SL and LNnT were given by gavage in adult rats, there was a quick absorption from the intestine to the plasma compartment. The time courses of absorption were dose-dependent, and generally, the circulating levels of these HMOs remains elevated respect to baseline even at 300 minutes after the gavage.

- The oral administration of 2'-FL affected the concentrations of other molecules such as Fuc. These alterations in constitutive levels were noted in serum and, especially in urine. The gradual increase of Fuc in serum may be due to possible hydrolysis of these molecules at the gastrointestinal tract, mainly through the commensal bacteria metabolism, with the concomitant release of Fuc.

- Rat pups showed higher circulating level of SA, 6'-SL, 2'-FL and specially, 3'-SL as well as Fuc and Lact than adult animals.

- The appearance of 2'-FL in plasma compartment as well as in urine of pups after 2'-FL gavage was dose-dependent. Unlike data obtained in adults (where 2'-FL reached a peak and then progressively decreased), levels of 2'-FL were increasing constantly over time, not reaching a defined peak or plateau phase at least for the 4 hours of experiment.

CAPÍTULO III

HMB y Leucina

III.1 OBJETIVOS

El primer objetivo general de este tercer capítulo fue demostrar el paso del HMB a través de la barrera hematoencefálica. Como segundo objetivo se planteó conocer mejor los el estado natural del HMB y la Leu a distintas edades de un roedor a lo largo de su vida, determinando estos analitos en diversas muestras biológicas. Los objetivos específicos son:

- ✓ Proponer metodología analítica de buenas características para la determinación de Leucina y su metabolito HMB mediante cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS) en distintas matrices como cultivos celulares, suero, orina, leche, microdializados de cerebro de rata y otras matrices biológicas.

- ✓ Validar los métodos analíticos propuestos en términos de exactitud (veracidad y precisión), linealidad, selectividad y sensibilidad de acuerdo a los requerimientos de las guías de validación específicas para métodos bioanalíticos.

- ✓ Aplicar la metodología desarrollada en distintos experimentos para aportar información sobre el mecanismo del HMB y la Leucina en un animal en distintos fluidos biológicos y en distintas etapas de la vida, tanto en condiciones normales como bajo intervención nutricional.

III.2 ESTRATEGIAS DE TRABAJO SEGUIDAS

La estrategia seguida para alcanzar los objetivos fijados en este tercer capítulo fue similar a las dos anteriores.

A. **Proceso de microdiálisis y protocolo en cultivos celulares.** Fue el mismo ya implantado en el primer trabajo de esta Tesis sobre neurotransmisores, con ligeras modificaciones. Las muestras de cultivo celular fueron obtenidas a través de un servicio externo contratado.

B. **Análisis mediante cromatografía de líquidos-espectrometría de masas.** Este segundo punto es común en todos los trabajos expuestos en esta Tesis Doctoral. Los protocolos de trabajo han sido desarrollados en cada capítulo con las modificaciones y especificaciones adecuadas en cada caso.

C. **Aplicación del método a la determinación de los compuestos de interés en muestras de microdializados y cultivos celulares.** Una vez obtenidos todos los parámetros y condiciones ideales de trabajo, se realizaron experimentos con animales vivos, administrando vía oral el propio HMB de forma aguda, para seguir la evolución de los dos compuestos de interés (HMB y Leu) en el fluido intersticial del cerebro, obtenido a través de la técnica de microdiálisis. Los resultados obtenidos en cultivo celular son complementarios, ya que la información de los experimentos *in vitro* permite predecir qué se encontrará en los experimentos *in vivo*.

D. **Aplicación del método cromatográfico de determinación de HMB y Leu en otras muestras biológicas.** Usando como base el método cromatográfico previamente desarrollado, se validó un nuevo método con distintos tratamientos de muestra en suero, orina, leche materna y otra matriz biológica (no detallada por motivos de confidencialidad) para determinar niveles basales de HMB y Leu a lo largo de la vida de un roedor. Se compararon además niveles basales en distintas situaciones fisiológicas y etapas de la vida del animal.

III.3 ESTADO ACTUAL DE LA INVESTIGACIÓN. ANTECEDENTES BIBLIOGRÁFICOS

Según la base de datos Scopus el interés en todos estos años en el HMB ha ido en aumento (Ver Figura III.1). Mayoritariamente con artículos en el área de Medicina y Bioquímica.

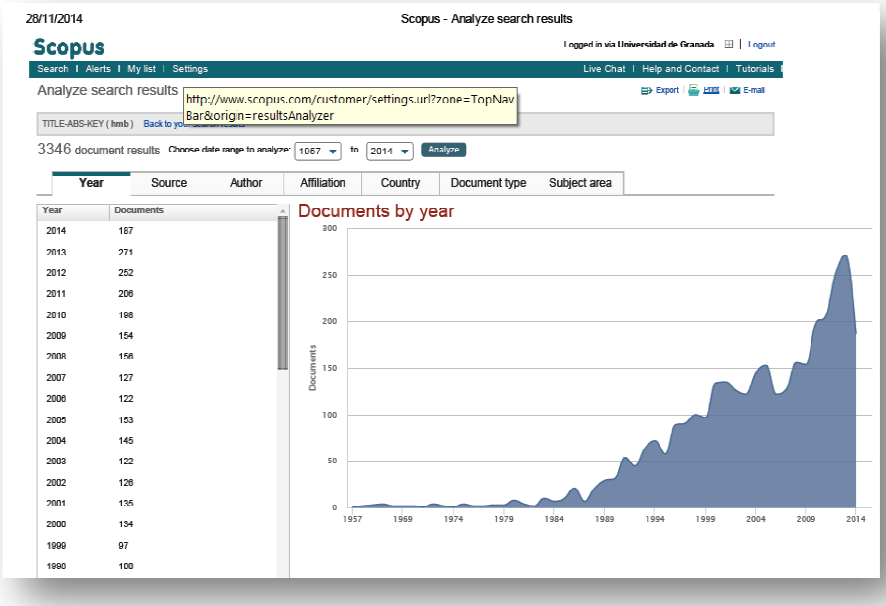
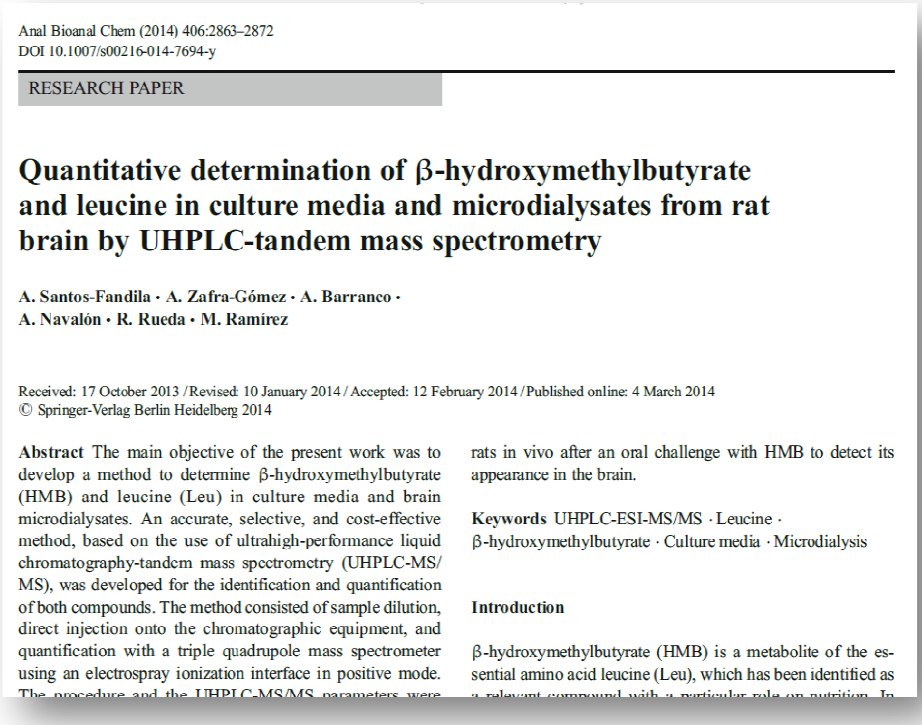


Figura III.1. *Informe de Scopus de publicaciones sobre HMB*

III.4 DETERMINACIÓN DE HMB Y LEU EN CULTIVOS CELULARES Y MICRODIALIZADOS DE CEREBRO DE RATA MEDIANTE UHPLC-MS/MS (Publicación V)

En este apartado se presenta el artículo publicado en la revista Analytical **Bioanalytical Chemistry (ABC)** de la editorial Springer (ver Figura III.2).



Anal Bioanal Chem (2014) 406:2863–2872
DOI 10.1007/s00216-014-7694-y

RESEARCH PAPER

Quantitative determination of β-hydroxymethylbutyrate and leucine in culture media and microdialysates from rat brain by UHPLC-tandem mass spectrometry

A. Santos-Fandila · A. Zafra-Gómez · A. Barranco · A. Navalón · R. Rueda · M. Ramírez

Received: 17 October 2013 / Revised: 10 January 2014 / Accepted: 12 February 2014 / Published online: 4 March 2014

Abstract The main objective of the present work was to develop a method to determine β-hydroxymethylbutyrate (HMB) and leucine (Leu) in culture media and brain microdialysates. An accurate, selective, and cost-effective method, based on the use of ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), was developed for the identification and quantification of both compounds. The method consisted of sample dilution, direct injection onto the chromatographic equipment, and quantification with a triple quadrupole mass spectrometer using an electrospray ionization interface in positive mode. The procedure and the UHPLC-MS/MS parameters were

rats in vivo after an oral challenge with HMB to detect its appearance in the brain.

Keywords UHPLC-ESI-MS/MS · Leucine · β-hydroxymethylbutyrate · Culture media · Microdialysis

Introduction

β-hydroxymethylbutyrate (HMB) is a metabolite of the essential amino acid leucine (Leu), which has been identified as

Figura III.2. *Quantitative determination of β-hydroxymethylbutirate and leucine in culture media and microdialistes from rat brain by UHPLC-tandem mass spectrometry. ABC, 406 (2014) 2863-2872 (Factor de Impacto: 3.578)*

Quantitative determination of β-hydroxymethylbutyrate and leucine in culture media and microdialysates from rat brain by UHPLC–tandem mass spectrometry

A. Santos-Fandila · A. Zafra-Gómez · A. Barranco · A. Navalón · R. Rueda · M. Ramírez

Abstract The main objective of the present work was to develop a method to determine β-hydroxymethylbutyrate (HMB) and leucine (Leu) in culture media and brain microdialysates. An accurate, selective and cost-effective method, based on the use of ultrahigh-performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS), was developed for the identification and quantification of both compounds. The method consisted of sample dilution, direct injection onto the chromatographic equipment and quantification with a triple quadrupole mass spectrometer using an electrospray ionization interface in positive mode. The procedure and the UHPLC–MS/MS parameters were accurately optimized to achieve the highest recoveries and to enhance the analytical characteristics of the method. For chromatographic separation, an Acquity UPLC BEH Hilic column using acetonitrile-water gradient with formic acid as additive was employed. The total run time was 4 min. The limits of detection (LODs) obtained ranged from 0.01 to 0.04 μg mL-1 and the limits of quantification (LOQs) from 0.04 to 0.12 μg mL-1. Precision (expressed as relative standard deviation) was lower than 15%, and the determination coefficient (R^2) was higher than 99.0% with a residual deviation for each calibration point lower than ± 25%. Mean recoveries were between 85 and 115%. The method was successfully applied to the analysis of both compounds, HMB and Leu, in samples obtained from an experiment of blood brain barrier (BBB) passage in vitro and to an experiment of brain microdialysis in rats in vivo after an oral challenge with HMB to detect its appearance in the brain.

Keywords UHPLC–ESI–MS/MS · Leucine · β-Hydroxymethylbutyrate · Culture media · Microdialysis

Introduction

β-Hydroxymethylbutyrate (HMB) is a metabolite of the essential amino acid leucine (Leu), which has been identified as a relevant compound with a particular role on nutrition. In fact, some of the effects exerted by Leu are now being attributed to this metabolite. Namely, the following functions have been reported for HMB. This compound is considered as an anti-catabolic agent associated with protein synthesis and attenuation of degradation [1-3], and therefore, it exerts a potentiating effect on strength and muscle mass in sport [4-9] as well as in clinical situations of increased protein degradation (cachexia), decreased rate of muscle protein synthesis (inactivity), or alteration of both (sarcopenia) [10-13]. Furthermore, the reduction of muscle atrophy and increase of muscle hypertrophy have been shown to be mediated by Akt phosphorylation and improved anabolic signaling via the m-TOR pathway [14,15]. HMB is also a readily available substrate for the synthesis of cholesterol needed to form and stabilize sarcolemmas in muscle [4]. Beyond its effects on muscle, HMB exhibits immune modulator properties *in vitro* on cell-mediated immunity, proliferative response of lymphocytes and peripheral mononuclear cells and cytokine production [16,17] and finally, it induces positive changes in oxidative metabolism, mitochondrial biogenesis and fatty acid oxidation [18-20].

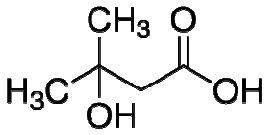
Due to the increasing reported effects of this compound, a number of studies are being carried out to investigate HMB bioavailability and mechanism of actions both *in vitro* and *in vivo*. These studies require accurate analytical methods to measure low levels of HMB in different biological fluids.

Our research group is interested in studying the role of HMB on brain functionality due to the important implications in this tissue of some of the molecular mechanisms reported for HMB. The capability of HMB to cross the blood brain barrier and its appearance on brain fluids should be proved before studying any effect in experimental animals or humans. As far as we know, there are no analytical methods to determine HMB and its precursor Leucine in

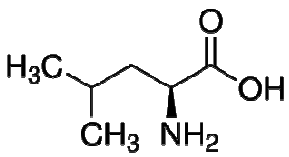
brain fluids or neural cell cultures. For this reason it was necessary to develop a new method that allows us to determine them in these specific matrices.

The classical method of bromide/bromate titration, microbiological assays, or colorimetric methods such as the McCarthy & Sullivan test [21] were developed for high concentrations of amino acids and were not specific; therefore, they have been substituted by more powerful chromatographic methods. In this sense, gas chromatography [22-25] and liquid chromatography with ultraviolet, fluorescence or mass spectrometry detection have been used [26-32]. However, these methods usually require larger sample volumes and are laborious processes lasting for several hours or even days. HMB cannot be measured by the amino acid chromatographic methods because it lacks the α-amino group and therefore commonly employed the derivatization techniques are not applicable.

In the present work, an ultra high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) method for the quantification of HMB and its precursor Leu was developed and validated. The method was applied to an *in vitro* study of blood brain barrier (BBB) pass and to microdialysis experiments to prove if orally administered HMB is able to reach the brain in rats. Fig. 1 shows the chemical structures of compounds.



β-Hydroxymethylbutyrate (HMB)



Leucine (Leu)

Fig. 1 Chemical structures of the studied compounds.

Materials and methods

Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). HMB and Leu were supplied by Sigma-Aldrich (Madrid, Spain). Methanol, acetonitrile and formic acid (LC-MS grade) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, Massachusetts, USA). A stock solution was prepared by weighing 0.1 g HMB and 0.02 g Leu into a 10 mL flask with water. The solution remained stable for at least one month at 4 °C. After that, six work standard solutions for calibration purposes were prepared to make calibration curve specifically depending on the matrix.

Ringer-HEPES buffer solution (NaCl, 150 mM; KCl, 5.2 mM; $CaCl_2$, 2.2 mM; $MgCl_2$ $6H_2O$, 0.2 mM; $NaHCO_3$, 6 mM; HEPES, 5 mM; glucose 2.8 mM) was used for the preparation of intermediate solutions and standard solutions for calibration purposes.

In culture media an intermediate solution (N° 1) was prepared by diluting 25 µL of the stock solution to 1 mL with Ringer-HEPES buffer solution. A second intermediate solution (N° 2) was prepared by diluting 25 µL of solution N° 1 to 1 mL with Ringer-HEPES buffer solution. Work standards for calibration purposes, were prepared by taking aliquots of the intermediate solutions, and diluting to 1 mL with Ringer-HEPES buffer solution into maximum recovery vials. The calibration standards were injected at the beginning and end of each sample series. A quality control standard was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution for each experiment.

In microdialysates, the work standard solutions were prepared similarly with the exception of the diluting media that was water-aCSF (1:1; *v*/*v*).

Apparatus and software

Detection and quantification of the analytes were performed using an UPLC® Acquity system from Waters (Milford, MA, USA) equipped with a binary pump, vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector. The chromatograph was connected on-line to a triple quadrupole mass spectrometer detector (QqQ) with an electrospray ionization (ESI) interface from Waters (Milford, MA, USA). An Acquity UPLC BEH C18 (100 mm × 2.1 mm I.D., 1.7 µm particle size), Acquity UPLC BEH HILIC (150 mm × 2.1 mm I.D., 1.7 µm particle size), and Acquity UPLC BEH Amide (100 mm × 2.1 mm I.D., 1.7 µm particle size) from Waters were tested as chromatographic columns. MassLynx software version 4.1 was used for instrument control and for data acquisition and analysis. Auxiliary apparatuses were: analytical balance with a precision of 0.1 mg, vortex-mixer, maximum recovery LC vials and screw caps from Waters, 0.2 µm nylon filters and eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, UK).

Blood brain barrier experiment

A co-culture of bovine brain capillary endothelial cells on one side of a filter (luminal compartment, representative of the blood side) and rat glial cells on the other (abluminal compartment, representative of the brain side) was used as an *in vitro* model of the BBB (Advancell, Barcelona Spain) [33]. Briefly, experiments were performed in triplicate with filters containing a confluent monolayer of endothelial cells (for evaluation of the compound passage), or in triplicates with empty filters coated with only collagen (filter test). Ringer-HEPES was added to the lower compartment (abluminal) in the first well of the plate. A volume of 1 mL Ringer-HEPES containing HMB was placed in the upper compartment (luminal side). Every 15 min after the addition of HMB, the insert was transferred to another well of the six-well plate. At the end of the each incubation periods, aliquots of abluminal liquid were collected and frozen. At the end of the experiment, 60 min incubation time, an aliquot of the luminal compartment was also taken. The samples were lyophilized, diluted in water,

and filtered through a 0.2 μm nylon filter. The volume filtered was introduced into a vial and injected into the UPLC®–MS/MS system (2 μL injection volume).

Three parameters were calculated taking into account the measurements performed with this chromatographic method, and according to Cecchelli et al [33, 34]:

- Mass balance. The percentage of compound recovered at the end of the experiment:

$$Mass\,Balance\,(\%) = \frac{[HMB]\,Lumin al,t\ 60\min + [HMB]\,Ablumin al,t\ 60\min}{[HMB]\,Lumin al,t\ 0\min}$$

- Clearance volume: It is an independent-concentration transport parameter that increases linearity with time. It is plotted versus time and the linear regression analysis calculated.

$$Clearance\,(\mu L) = \frac{[HMB]\,Ablumin al x VA}{[HMB]\,Lumin al,t\ 0\min}$$

where VA is the volume of the abluminal chamber.

- The slope of the clearance curves for the co-culture is denoted PSt, where PS is the permeability x surface area product (in μL min^{-1}). The slope of the clearance curve for the filter only covered with collagen is denoted PSf. The PS value for the endothelial monolayer (PSe) is calculated from:

$$\frac{1}{PSe} = \frac{1}{PSt} + \frac{1}{PSf}$$

The PSe values are divided by the surface area of the filter (4.7 cm^2 for transwell inserts) to generate the endothelial permeability coefficient (Pe, in centimetres per minute).

Microdialysis experiment

The microdialysis technique was previously described in a recent publication [35]. The technique was done in Sprague-Dawley rats and animal procedures were conducted in conformity with the institutional guidelines that are in compliance with national laws and EC policies for the Care and Use of Laboratory Animals (RD 2101-2005, 86/609/CEE). Briefly, a probe was implanted in the left lateral hippocampus area of the animal brain under general anaesthesia. The animal was allowed to recover from surgery and the day of the experiment, a microdialysis probe was inserted into the guide cannula of the probe. The probe was perfused with aCSF at a constant rate of 2 µL min^{-1}. A bolus dose of HMB (250 mg/kg body weight) was given by gavage 100 min after starting the experiment and sampling was continued for additional 220 min. Microdialysate samples were automatically collected every 20 min directly into vials, and frozen at -80 °C until analysis. All studies included a 20 min washout period prior to collecting the dialysates. Six dialysate samples were then collected and defined as basal samples.

Artificial cerebrospinal fluid (aCSF) is commonly used when sampling brain interstitial fluid. This solution closely matches the electrolyte concentrations of cerebrospinal fluid. It is commercially available in high purity water and analytical grade reagents, micro-filtered and sterile. Final ion concentrations in this solution were (in mM): Na 150; K 3.0; Ca 1.4; Mg 0.8; P 1.0; Cl 155. Samples (typically 40 µL) of microdialysate obtained from *in vivo* studies were placed in maximum recovery vials. Samples were diluted with equal amount of water and they were ready to direct injection into the UPLC®–MS/MS system (2 µL injection volume).

Liquid chromatographic conditions

Chromatographic separation was performed using an Acquity UPLC BEH HILIC (150 mm × 2.1 mm I.D., 1.7 µm particle size) from Waters. Samples and standards separation was achieved using a gradient mobile phase consisting of pure water as solvent A, and 0.1 % (*v*/*v*) of formic acid in acetonitrile as solvent

B. Gradient conditions at a 0.3 mL min^{-1} flow rate were: 0.0-1.0 min, 90 to 20 % B; 1.0-3.0 min, 20 to 10% B; 3.0-3.1 min, back to 90 % B; and maintaining 90 % B until 4 min. An injection volume 2 µL (in partial loop mode) was used, the column temperature was maintained at 30 °C, the sample temperature at 20 °C. Weak solvent was a mixture of 450 mL of acetonitrile and 50 mL of water, and strong solvent was pure water. These solutions were stable for at least one week at room temperature.

Mass spectrometric conditions

ESI was performed in positive ion mode. The tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. The mass spectrometric conditions were optimized for each compound by continuously infusing standard solutions (0.1-1.0 mg L^{-1}). Electrospray ionization spray voltage was 4.5 kV. Nitrogen was used as desolvation gas at 850 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The temperature of the source was 120 °C and the desolvation temperature was 400 °C. Argon (99.999 % purity) was used as collision gas at an approximate rate of 0.10 mL min^{-1}. Optimized parameters for each compound are listed together with the mass transitions in Table 1. Common parameters in the single function are Inter Channel Delay of 5 ms and InterScan Time of 5 ms.

Table 1 MS parameters for ionization with ESI+ in MRM mode

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Dwell time (ms)	Cone (V)	Collision (eV)	Delay (ms)
HMB	119.0	59.0	100	17	12	5
		101.0	100	17	5	5
Leucine	132.1	43.9	100	23	20	5
		85.9	100	23	10	5

Results and discussion

Liquid chromatographic analysis

Initial experiments were designed to evaluate chromatographic conditions based on the recommendations obtained from the scientific literature [25, 26, 28-31, 36-38]. No method for simultaneous determination of HMB plus Leucine was found. To determine Leu there was an extensive bibliography to take into account. However, less information was available for HMB.

Three liquid chromatography columns were tested: an Acquity UPLC BEH C18, an Acquity UPLC BEH HILIC, and an Acquity UPLC BEH Amide. The BEH HILIC column provided the best resolution in the shortest time. Different mobile phases were also studied in order to optimize separation and peak shapes. With that objective in mind, two organic solvents commonly used in reversed-phase liquid chromatography (methanol and acetonitrile) were evaluated which acetonitrile gave better results than methanol. Moreover, several additives were evaluated (ammonium hydroxide, ammonium acetate, ammonium formate, acetic acid and formic acid) to improve the analytical signal and the resolution of the chromatographic peaks. The best separation was obtained using a mobile phase composed of 100 % water as solvent A and 0.1 % (*v*/*v*) formic acid in acetonitrile as solvent B. The gradient described previously was used.

To improve the sensitivity of the method, a study was performed to evaluate the possibility of increasing the injection volume and to evaluate different modes of injection. Injection volumes ranging from 1 to 10 µL were studied. An extra broadening of the peaks was observed at volumes higher than 2 µL; therefore, this volume was selected. An investigation of injection mode showed that full loop required a greater amount of sample compared to partial loop. Therefore, partial loop was chosen.

The stability of the standards was also studied, which included an investigation of different additives to extend the expiration date. Acetic acid,

formic acid and several mixtures of acetonitrile, methanol, and water were tested. The results showed that the addition of additives did not increase the stability of the standard nor the signal response. Therefore, the decision was to prepare the working standards daily in the solution used to collect samples, Ringer-HEPES buffer or aCSF for cell culture or microdialysates respectively.

Mass spectrometric analysis

The MS/MS detection method was set up by direct infusion of each individual compound to optimize the response of the precursor ion. ESI and ESCI® (electrospray and atmospheric pressure combined ionization) interfaces in positive and negative modes were evaluated. ESI interface in positive mode was selected because it showed higher sensitivity. The response of two daughter ions (two reactions), one for quantification and the other for identification or confirmation, were monitored. The most abundant transition ion was selected to obtain maximum sensitivity for quantification. The parameters optimized for the precursor ions were capillary and cone voltages, source and desolvation temperatures, and desolvation gas flow. For product ions, the optimized parameters were collision energy (CE) and dwell times. The parameters selected to obtain optimum responses are presented in Table 1. Additionally, Fig. 2 shows chromatograms of a standard mixture of HMB and Leucine.

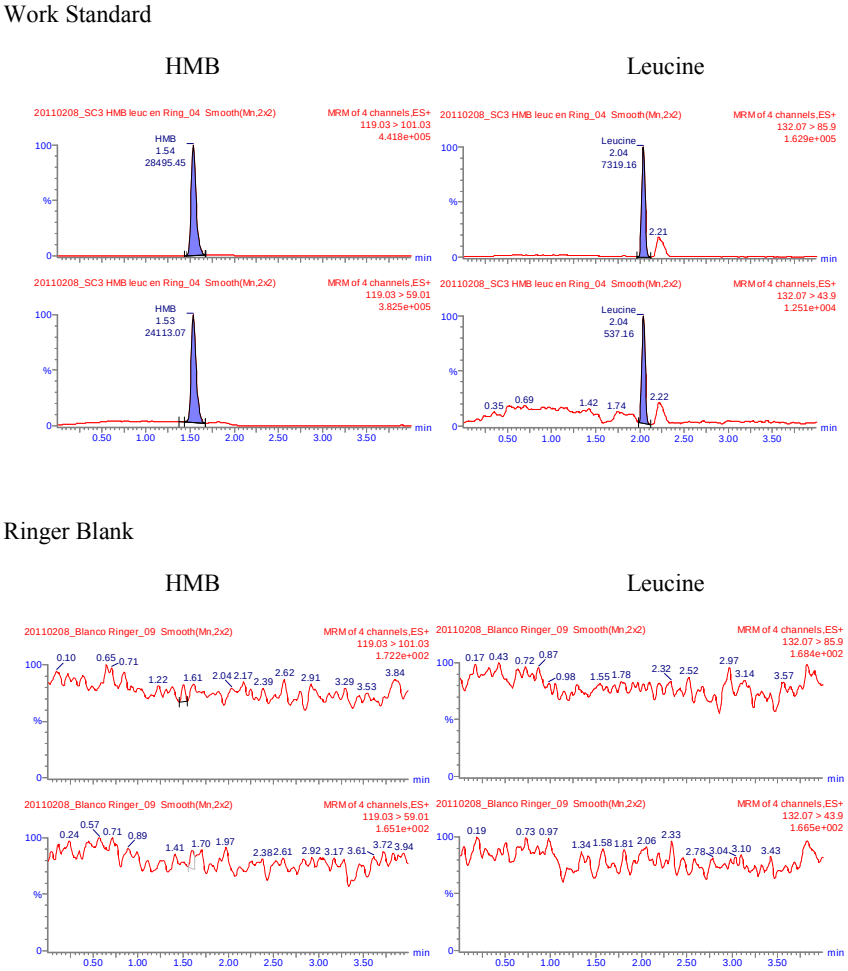


Fig. 2 Chromatograms of each analyte in a blank and in an intermediate standard (two transitions). The first transition of each analyte was used for quantification and the second for confirmation.

Analytical performance

A calibration curve was obtained for each compound by injecting 2 µL of different standard solutions at concentrations ranging from 0.1–25 µg mL^{-1} for HMB and 0.04–5 µg mL^{-1} for Leu, respectively. Quality control samples were injected every 20 injections to assure the validity of the calibration curve. The predicted value was expected to not exceed ± 15% of the theoretical value. Analytical performance was evaluated according to the recommendations of

Analytical Methods Committee [39]. The *lack-of-fit* test was applied to two replicates and three injections of each standard (six concentration levels). The results are summarized in Table 2.

Table 2 Analytical and statistical parameters

	n	b (mL μg^{-1})	R^2 (%)	LOD ($\mu g\ mL^{-1}$)	LOQ ($\mu g\ mL^{-1}$)	LDR ($\mu g\ mL^{-1}$)	P_{lof} (%)
HMB	6	0.150	99.9	0.04	0.12	0.1 - 25	10.7
Leucine	6	4.059	99.0	0.01	0.04	0.04 - 5	79.4

n, number of calibration levels; b, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; $\%P_{lof}$, P-value for *lack-of-fit* test

Method validation

Validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation [40].

Linearity. Linearity of the calibration curves was evaluated using coefficients of determination (R^2, %) and *P*-values of the *lack-of-fit* test (%, P_{lof}). The values obtained for R^2 ranged from 99.0 % for Leu to 99.9 % for HMB, and P_{lof} values were higher than 5 % in both cases. This indicated a good linearity within the stated ranges.

Selectivity. The specificity of the method was determined by comparing the chromatograms of blanks with those corresponding to the samples. No interferences from endogenous substances were observed at the retention times of each respective analyte (Fig. 2), which eluted at 1.54 min and 2.04 min for HMB and Leu respectively. This finding suggested that the UPLC®–MS/MS conditions provided sufficient selectivity.

Accuracy (precision and trueness). Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples

were analyzed using the proposed method and the concentration of each compound was determined by interpolating from the standard calibration curve. Recoveries were calculated by comparing the interpolated amounts to the theoretical amounts, spiked amounts. As shown in Table 3, the recoveries were between 85 and 115 % in all cases.

To evaluate the precision of the method, the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and were repeated for five days to determine inter-day variability. The results of repeatability and within-laboratory reproducibility are summarized in Table 3. RSD values were between 1.3 % and 13.6 %. The highest RSD was for Leu on the lower level of recovery. This would be due to the fact that the concentration was close to the limit of quantification (LOQ). Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15 % of the actual value, except at the LOQ, where it should not deviate by more than 20 %. In this specific case also meets with the stipulated limits. Precision and trueness data indicated that the methodology to determine the target compounds in culture media and microdialysates from rat brain is highly accurate, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Table 3 Recovery, precision and accuracy of target compounds in Ringer solution samples

		Intra-day				Inter-day			
	Spiked (μg mL^{-1})	Found[a] (μg mL^{-1})	R (%)	RSD (%)	n	Found[a] (μg mL^{-1})	R (%)	RSD (%)	n
HMB	1.01	1.09	108.4	7.3	3	1.14	113.4	7.7	15
	5.04	5.74	113.9	2.9	3	5.62	111.5	7.1	15
	20.2	21.0	103.9	3.7	3	22.9	113.4	6.2	15
Leucine	0.11	0.12	106.8	9.9	3	0.11	100.9	13.6	15
	1.10	1.12	101.8	1.3	3	1.16	105.4	2.6	15
	4.38	4.72	107.8	1.9	3	4.88	111.4	10.7	15

[a] Mean value; R, % recovery; RSD, relative standard deviation; n, number of determinations

Sensitivity. Limit of detection and LOQ are two fundamental parameters that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. The LOD is the minimum amount of analyte detectable in the sample while the LOQ is the minimum amount that can be quantified. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives. In this work, these parameters were calculated from the signal-to-noise ratio. The found LODs (signal-to-noise ratio = 3) were 0.04 μg mL^{-1} for HMB and 0.01 μg mL^{-1} for Leu, and the corresponding LOQs (signal-to-noise ratio = 10) were 0.1 μg mL^{-1} for HMB and 0.04 μg mL^{-1} for Leu, respectively. The values obtained are shown in Table 2.

Application of the method

The method was applied to the study of brain permeability to HMB. Leu was also determined because it is the internal precursor of HMB, as it was explained before. Two lines of research were covered to carry out this objective. The first one was the *in vitro* study of the transfer of this compound in a model of cell culture mimicking the BBB. The second one was a microdialysis experiment. Microdialysis in the brain is a well-established technique for the measurement of the time-course of different compounds in the brain that can be produced

internally in response to an activity or an insult, such as neurotransmitters, or that can reach the brain and appear in the interstitial brain fluid after a systemic administration; the latest approach is the second example provide in this article.

Samples of cell cultures and microdialysates from rat brain were collected following the protocols previously described. Fig. 3 shows chromatograms obtained for a cell culture and microdialysate samples.

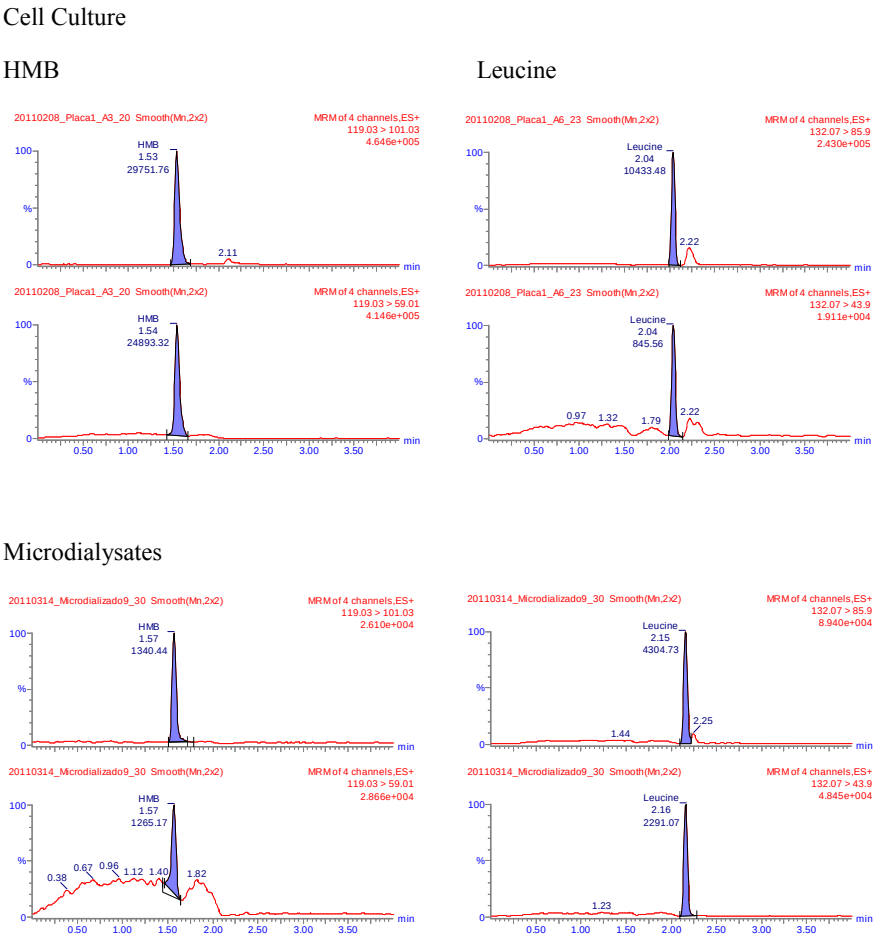


Fig. 3 Chromatograms of each analyte in cell culture and microdialysate samples are shown with two transitions. The first transition of each analyte was used for quantification and the second for confirmation.

Blood brain barrier experiment

The measurements of the *in vitro* model are shown in Table 4 and Fig. 4. The mass balance values with the filter without cells and with the filter and the cells after 60 min experiment were acceptable (81.5 and 86.7 %, respectively). The rate of solute diffusion across the endothelial cell monolayer was less than the diffusion across the filters only covered with collagen (PSf = 2.18 versus PSf = 16.42 µL min^{-1}). The passage of HMB is consequently not restricted by the permeability of the filter and the endothelial cell monolayer is really a physical barrier. The Pe determined across the BBB in this *in vitro* model indicated that HMB would penetrate the brain at a slow rate (Pe = $0.54 \cdot 10^{-3}$ cm min^{-1}). Leucine was also detected likely due to the presence of this compound in residual culture media and/or the release of Leu from the collagen or cell layers. Leu was not added to the initial solution (data not shown).

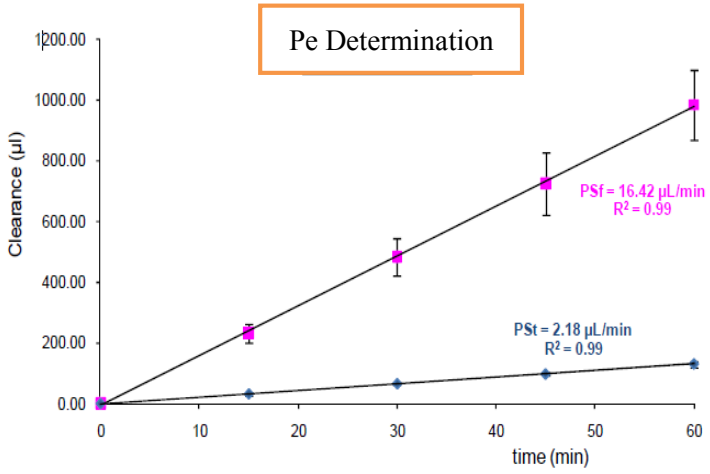


Fig. 4 Clearance curves for HMB in the blood brain barrier in vitro experiment.

Microdialysis experiment

Fig. 5 shows an example of the time-course of HMB in the brain interstitial fluid when was administered in an animal *in vivo* by a gavage of HMB (250 mg kg^{-1} body weight).

Table 4 Blood Brain Barrier *in vitro* experiment

Concentration of HMB in plate 1 without cells (µg mL^{-1})					
Sample	0 min	15 min	30 min	45 min	60 min
Filter A	12.2	2.68	4.49	5.72	6.51
Filter B	12.4	2.01	3.54	4.68	5.62
Filter C	12.1	1.96	3.48	4.44	5.42
AVERAGE	12.2	2.22	3.84	4.95	5.85
SD	0.2	0.40	0.57	0.68	0.58
Concentration of HMB in plate 2 with cells (µg mL^{-1})					
Sample	0 min	15 min	30 min	45 min	60 min
Filter A	17.4	0.66	1.24	1.77	2.33
Filter B	17.9	0.51	1.00	1.49	1.90
Filter C	18.0	0.46	1.02	1.46	1.91
AVERAGE	17.8	0.54	1.09	1.57	2.05
SD	0.3	0.10	0.13	0.17	0.25

SD: Standard deviation

During the basal period we did not detect any level of HMB in the microdialysates, although a stable basal level of Leu was detected. HMB appeared in hippocampus 40 min after administration (sample 7), and increased sharply for 2 h and 20 min (sample 12-13) where the maximum concentration was reached (more than 4 µg mL^{-1}). HMB level decreased thereafter although at the end of the experiment (more than 3.5 h after the gavage) it had not reached basal level yet. Despite the fact that the *in vitro* BBB model showed a slow rate of penetration, the *in vivo* approach clearly showed that HMB peaks on the brain.

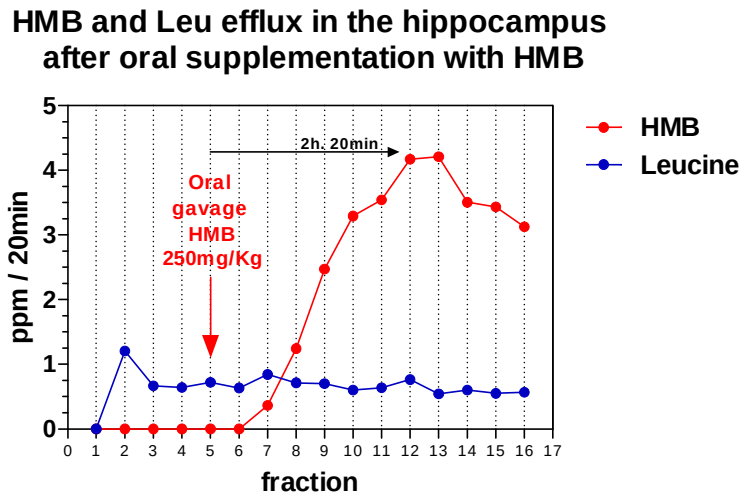


Fig. 5 Time-course of the HMB and Leu overflow from the hippocampus over the 300 min. Values are expressed as parts per million, ppm (μg mL^{-1}), in fractions of 20 min (ppm/20 min). Rat received a gavage of HMB (250 mg/kg b.w.) at the end of fraction 5 (the time of administration was highlighted in red)

Leu concentration remained unchanged during the time course of the experiment. To have a better understanding of the Leu evolution in figure 5 is necessary to explain in more detail: the experiment started when the microdialysis probe was inserted into the cannula previously implanted in the brain of the animal. That is, the Fraction 1 was collected just after the insertion of the microdialysis probe in the cannula into the brain of the animal. Previously there was a washout period, and obviously the first fraction collected was part of the washing liquid, being a remnant in the pipes. On the other hand, Leu in the first collected fraction after the insertion of microdialysis probe (Fraction 2) increases slightly, due to the damage produced in the tissue which releases amino acids. Thereafter, the concentration of Leu returned to baseline level.

As far as we know this is the first study showing that HMB given orally appeared in brain interstitial fluids, which means that despite its relatively hydrophilic properties was able to cross the blood brain barrier in an experimental model of brain microdialysis in rats. Studies in animals have reported that HMB peaked in plasma within 2 h following consumption [41]. Our results are in agreement with these results and point out that the peak of

HMB concentration in hippocampus is subsequent to the peak reported in plasma. HMB is the active metabolite of the amino acid Leu, an essential amino acid [42]. Leu levels remained constant regardless of changes of HMB concentration confirming the irreversibility of the conversion of HMB to Leu [43]. However an individual would have to consume larger quantities of all amino acids to achieve the effective levels of HMB [4]. This could justify the need for HMB supplementation.

HMB supplementation enhances the gain of muscle mass. This effect could be mediated through a direct action on the mTOR pathway [18]. mTOR is also involved in the molecular mechanisms of learning and memory that are impaired in Alzheimer's Disease as well as in the regulation of neuronal reactions [44]. In fact, the brain structure where the microdialysis probe was inserted, the hippocampus, is closely related to learning and memory formation [45,46]. The understanding of the kinetics and biodistribution of HMB in the brain could open new avenues of nutritional research for brain function.

Conclusions

The proposed method is a powerful tool for the simultaneous determination of HMB and Leu in microdialysates samples and culture cells. This novel method minimizes the time and total cost of the analysis reducing sample volumes in comparison with other previous published methods. Its simplicity and sensitivity makes possible the identification and quantification of both compounds in culture media and brain microdialysates, two applications related to the study of the biological fate of HMB.

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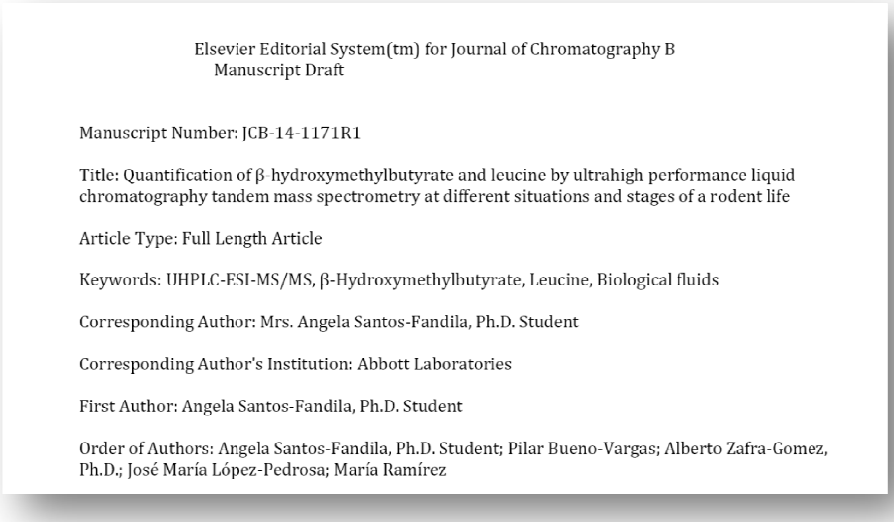
III.5 DETERMINACIÓN DE HMB Y LEU EN DIFERENTES SITUACIONES Y ETAPAS DE LA VIDA DE UN ROEDOR MEDIANTE UHPLC-MS/MS (Publicación VI)

En este apartado se presenta el trabajo enviado para su publicación a la revista internacional **Journal of Chromatography B** de la editorial Elsevier (Figura III.3). Primera revisión 15/04/2014



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Article Type: Full Length Article

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Figura III.3. *Quantification of β-hydroxymethylbutyrate and leucine by ultrahigh performance liquid chromatography tandem mass spectrometry at different situations and stages of a rodent life. Revision Draft of Journal of Chromatography B (Factor de Impacto: 2.694)*

Quantification of β-hydroxymethylbutyrate and leucine by ultrahigh performance liquid chromatography tandem mass spectrometry at different situations and stages of a rodent life

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ABSTRACT

The main objective of this work was to develop a method to measure Leucine (Leu) and β-hydroxymethylbutyrate (HMB) at basal levels in serum, urine, milk and brain microdialysates in rats. Ultrahigh performance liquid chromatography-electrospray-tandem mass spectrometry (UHPLC-ESI-MS/MS) was used as analytical technique. The sample treatment was simple and consisted of dilution with methanol and centrifugation for serum and urine, dilution with water and filtration with an Amicon filter for milk, and treatment with formic acid with no further dilution for microdialyzates. The procedures for sampling and the UHPLC-MS/MS parameters were accurately optimized to achieve the highest recoveries and to enhance the analytical characteristics of the method. For chromatographic separation, an Acquity UPLC BEH Amide column using acetonitrile-water gradient with formic acid as additive was used. The total run time was 4 min. The analytical characteristics (accuracy, selectivity and sensitivity) of the proposed method were evaluated. The limits of detection (LODs) obtained ranged from 0.4 to 7 ng mL^{-1} and the limits of quantification (LOQs) from 1 to 22 ng mL^{-1}. Precision, expressed as relative standard deviation (% RSD), was lower than 15% in all cases, and the determination coefficient (R^2) was equal or higher than 99.0% with a residual deviation for each calibration point lower than ± 25%. Mean recoveries were between 85 and 115%. The method was successfully applied to these matrices

being able to detect significant differences between physiological situations, strains and stages of life.

Keywords:

UHPLC–ESI–MS/MS, β-Hydroxymethylbutyrate, Leucine, Biological fluids

Highlights:

- The natural evolution of HMB and leucine during the life of a rodent is studied
- UHPLC-MS/MS is used to measure these compounds in different biological matrices
- The method is validated in terms of selectivity, accuracy and sensibility
- Important information regarding the metabolic pathway of HMB and Leu is obtained
- Perinatal status, different ages and strains of animals are evaluated

1. Introduction

Beta-hydroxy-beta-methylbutyrate (HMB) is a metabolite of the amino acid leucine (Leu). The first step in HMB metabolism is the reversible transamination of Leu to alpha-ketoisocaproate (α-KIC) that occurs mainly extrahepatically. Following this enzymatic reaction, the majority of α-KIC is irreversibly oxidized to isovaleryl-CoA via the enzyme branched chain keto acid dehydrogenase (BCKAD). In the minority alternative pathway (approximately 5% of metabolized Leu), HMB is produced from α-KIC by the cytosolic enzyme KIC dioxygenase [1, 2] (See Figure 1).

HMB functions are well known. Namely, it is an anti-catabolic agent associated with protein synthesis and attenuation of protein degradation [3, 4], which makes it suitable for increasing strength and muscle mass in sport nutrition [5-9], as well as for clinical situations of increased protein degradation (cachexia), decreased rate of muscle protein synthesis (inactivity), or alteration

of both (sarcopenia) [10-12]. In addition, it is a substrate for the synthesis of cholesterol needed to form and stabilize sarcolemmas in muscle [5], it has immune modulator properties [13, 14], it downregulates apoptosis during immobilization and recovery [15] and also it produces improvement in oxidative metabolism [16].

Due to the catabolism of Leu, people have a daily endogenous synthesis of HMB between 0.3 to 0.4 g, but plasma HMB levels can be increased by five- to ten-fold after feeding Leu (60 g) or HMB (3 g). Kinetic studies in animals have reported that the half-life of HMB in plasma is about 2 h and that about 34% of HMB is excreted in urine. In humans, the timecourse kinetics of HMB in plasma and urine showed that the half-life of HMB is also about 2 h while only 14 to 29% of the HMB is excreted in urine, depending on the dose. Those data suggest that 70 to 85% of the ingested oral HMB is retained in the body for further metabolism [1, 2, 17]. There may be a number of factors such as gender, race, age or pathologies that could affect the basal levels and excretion of HMB and/or Leu. It is important to explore these levels of HMB and Leu in different biological fluids to have knowledge about how they vary naturally throughout the life of an animal under different conditions. This information could be relevant for the use of these compounds as dietary supplements or nutritional products with specific health outcomes.

Accurate and sensitive analytical methods are necessary for the analysis of low levels of HMB and Leu present in specific biological fluids. Classically, several methods have been published for determining amino acids such as Leu [18], with techniques such as HPLC [19-24] or gas chromatography [25-27]. So far, these methods had some shortcomings such as low specificity for HMB or the sample treatment complexity. Since HMB contains an α-hydroxy in place of the α-amino group, the techniques involving derivatization steps are not recommended. Nissen et al. published a method to determine HMB in plasma by gas chromatography coupled with mass spectrometry (GC-MS) [28] but this method requires high sample volumes and laborious protocols including derivatization steps. Recently, two analytical methods have been published to improve these inconveniences. Firstly, in 2013 Deshpande et al published a

study of the bioavailability of HMB in plasma by LC-MS [29] and secondly, our group has recently published a method to measure HMB and Leu by UHPLC-ESI-MS/MS in relatively clean biological fluids, namely cell cultures and brain microdialysates [30].

The present work aims to extend the use of this method to other biological fluids such as serum, urine and milk. Brain microdialysates were also revisited to improve sensibility. Sample treatments were optimized for the fluids mentioned above and the method was validated according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assays [31]. Subsequently, basal levels HMB in these sample matrices and on different biological conditions and stages of life were measured.

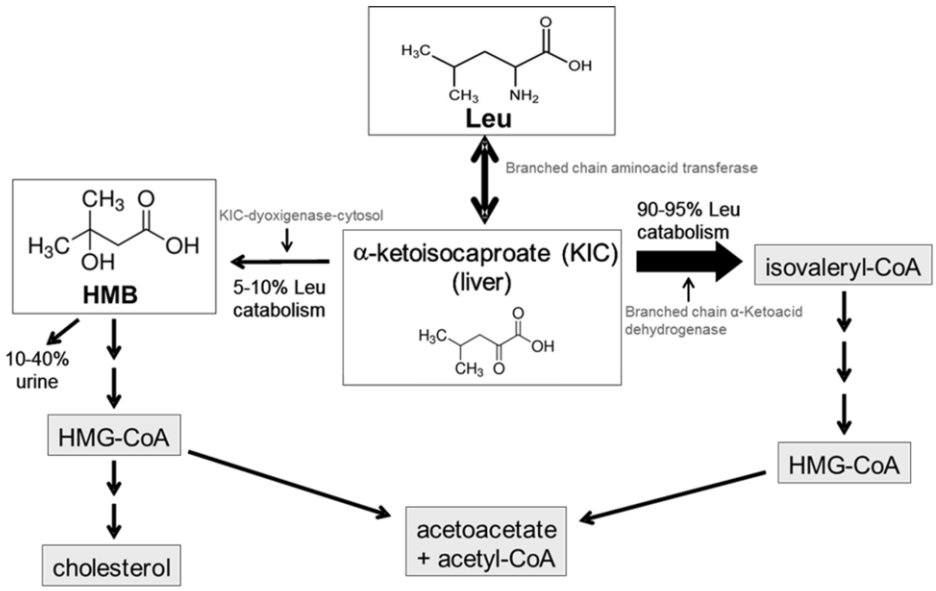

Fig. 1 Chemical structures of HMB and Leu and the metabolic route.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless specified otherwise. Water (18.2 MΩ cm) was purified and filtered by a specific LC-MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). β-Hydroxymethylbutyrate (HMB) and Leu were supplied by Sigma-Aldrich (Madrid, Spain). LCMS grade methanol (MeOH), acetonitrile (MeCN), ethanol (EtOH) and formic acid (FA) were purchased from Scharlab (Barcelona, Spain). Artificial cerebrospinal fluid (aCSF) was purchased from Harvard Apparatus (Holliston, Massachusetts, USA).

A stock solution of compounds was prepared by weighing 0.04 g HMB and 0.01 g Leu into a 10 mL flask and dilution with water. The solution remained stable for at least one month at 4 °C. Five work standard solutions for calibration purposes were prepared specifically depending on the studied matrix. For serum and urine, an intermediate solution (No. 1) was obtained by dilution of 50 µL of the stock solution to a final volume of 10 mL with MeOH. A second intermediate solution (No. 2) was prepared by diluting 200 µL of solution No. 1 to 1 mL with MeOH. Work standards for calibration purposes, named WS1, WS2, WS3 and WS4 were prepared by diluting 10, 50, 200 and 500 µL of the intermediate solution N° 2 to a final volume of 1 mL with MeOH in a maximum recovery vial. The standard WS5 was the solution No. 2. The same process was followed for other matrices, but in that case the solvent used for dilutions was different. Specifically, to measure HMB and Leu in milk the solvent was LC-MS water.

For microdialysates from rat brain a modification of the previously published article was done. The use of an improved chromatographic column by the manufacturer caused higher sensitivity than in the previous work due to narrower and higher peaks were obtained compared with those obtained in previous experiments by HILIC column. So with this improvement basal levels could be detected. Furthermore, as these levels were close to the limit of quantification, it was decided avoid dilution to improve the signal and work

within the linear dynamic range of the method. For both calibration and samples processing, and in order to concentrate the basal level to the optimal value, no dilution of samples was made.

The calibration standards were injected at the beginning and end of each sample series. A quality control standard (WS3) was injected after every twenty injections. Calibration standards were freshly prepared from the original stock solution in each experiment.

2.2. Apparatus and software

Detection and quantification of the analytes were performed using an UPLC® Acquity system from Waters (Milford, MA, USA) equipped with a binary pump, vacuum membrane degasser, a thermostated column compartment, an autosampler, and an automatic injector was used. The chromatograph was connected on-line to a triple quadrupole mass spectrometer detector (TQD) with electrospray ionization (ESI) interface. The following chromatographic columns were tested: Acquity UPLC BEH C18 (2.1 mm × 100 mm i.d., 1.7 µm particle size), Acquity UPLC BEH HILIC (2.1 mm × 150 mm i.d., 1.7 µm particle size), Acquity UPLC BEH HILIC (2.1 mm × 100 mm i.d., 1.7 µm particle size), and Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 µm particle size) from Waters. MassLynx software version 4.1 from Waters (Milford, MA, USA) was used for instrument control and for data acquisition and analysis. An analytical balance with a precision of 0.1 mg, a vortex-mixer, a speed vac evaporator from Heraeus Instrument Thermo Scientific (Madrid, Spain), maximum recovery LC vials and screw caps from Waters, 0.2 µm nylon filter, 0.2 µm polytetrafluoroethylene (PTFE) filter and 0.5 mL 10K Amicon filter from Millipore (Bedford, MA, USA) and an eVOL automated analytical syringe from SGE Analytical Science (SGE Europe, United Kingdom) and were also used.

2.3. Animal work

Sprague-Dawley (SD) rats were provided by Janvier and Charles River Laboratories (France). Zucker Lean Rats (ZLR) were obtained from Charles

River Laboratories. Adult animals were fed with a purified rodent diet according to the AIN-93 recommendations [32]. Pups were nursed by their own mothers. Animals were housed at constant room temperature (22 ± 2 °C) and 45-55 % humidity under a regular 12-hour light/dark schedule.

Serum and urine samples were measured in: a) pup rats (male and female) at postnatal day (PND) 1-3, 12-14 and 19-20, b) 13 week-old rats (male and female), c) gestating rats at gestational day (GD) 0, 5, 12 and 18 (13-16 weeks-old), d) mothers rats after delivery at PND 1-3, 12-14 and 19-20 (16-18 weeks-old), e) adult female rats which had been ovariectomized (OVX) at 24 weeks of age simulating menopause and rats of the same age which suffered the surgery but the ovaries were not removed (SHAM), and g) Zucker lean rats (ZLR), a strain that is the natural non-diabetic control for Zucker diabetic rats at several time points (10, 15 and 20 weeks of age). In addition, serum of 70 week-old males was measured, milk samples were also obtained from SD dams at PND 1-3, 12-14 and 19-20, and microdialysate samples were determined in adult male SD rats.

The following techniques were used to obtain blood samples depending on the experimental design: terminal bleeding in dead endpoint experiments and in rat pups experiments, and tail punction in longitudinal studies. Urine was obtained after involuntary urination or full day collection in metabolic cages in longitudinal studies or by punction of the bladder after dead. Milk samples were taken by manual expression in anesthetized animals after stimulation of milk secretion by intraperitoneal administration of oxytocin. The samples were taken either after 3-4 h fasting or overnight fasting (12-18 hours) depending on the experimental design. All the samples were stored at -80°C until analysis. Microdialysate samples were taken in the same way that in our previous published article mentioned above.

Protocols for all experimental procedures were carried out according to ethical guidelines for animal experimentation at the Spanish National Research Council (RD 53/2013).

2.4. Sample preparation

Serum samples were diluted in a specific volume of methanol depending on the concentration of the analyte to be within the linear working range. The addition of methanol precipitates proteins and cleans the sample before injection into the LC system. Dilutions were done always in methanol LC-MS grade and were as follows:

- Normal rats including pups, young and adult animals: 50 µL of serum + 50 µL of MeOH.
- Zucker rats (ZLR): 40 µL of serum + 260 µL of MeOH.

The dilutions were vigorously shaken for 1 min, centrifuged at 14000xg for 10 min at 8ºC, collected into maximum recovery vials and injected into the UPLC®.

Urine samples were also diluted in a specific volume of MeOH depending on the concentration of the analyte to be within the linear working range:

- Normal rats: 10 µL + 1.5 mL of MeOH LC-MS grade.
- ZLR: 50 µL + 8 mL MeOH.

The dilutions ware shaken for 1 min and the liquid layer was transferred to maximum recovery vials and injected directly into the UPLC®.

For rat milk samples, 50 µL of milk were diluted with 100 µL of purified LC-MS water. They were shaken vigorously for 1 min and centrifuged in a 10K Amicon Filter at 14600 x g for 30 min at 10ºC. The extract was collected in maximum recovery vials to inject into the UPLC®. A second dilution was made to determine Leu within the linear dynamic range (LDR) of work. In that case, 20 µL of the extract were taken in a new vial and 480 µL of LC-MS quality water were added. The mixture was shaken and then injected into the chromatographic system.

Finally, for microdialysates from rat brain, a modification of the work previously published by our research group [30] was done for the determination of the basal levels of HMB and Leu in microdialysate samples. Microdialysate samples (typically 40 µL) were automatically collected every 20 min directly into vials and frozen at −80 °C until analysis. They were placed in maximum recovery vials. Two microliters of FA were added and then mixed for 10 seconds on a vortex-mixer; it was ready to direct injection into the UPLC®.

2.5. Liquid chromatography-mass spectrometry conditions

The development of the liquid chromatographic method is based in the previously reported in a published article [30]. Two LC columns were checked again: an Acquity UPLC BEH HILIC (2.1 mm × 100 mm i.d., 1.7 µm particle size) and an Acquity UPLC BEH Amide (2.1 mm × 100 mm i.d., 1.7 µm particle size) from Waters, and in this case, since the manufacturer has recently improved the stability of the amide column, better separations, showing a higher resolution in the shortest times were obtained. Accordingly, the sensitivity was increased by 10 times. The best separation was obtained in the same way using a mobile phase composed of water as solvent A and 0.1% (v/v) of FA in acetonitrile as solvent B. A linear gradient was established as follow: 0.0-1.0 min, 90 to 20% B; 1.0-3.0 min, 20 to 10% B; 3.0-3.1 min, back to 90% B; and maintaining 90% B until 4 min. Injection or washing weak and strong solvents were a mixture of 450 mL of acetonitrile and 50 mL of water, and pure water respectively Flow rate was 0.3 mL min-1, injection volume 2 µL, the column temperature was maintained at 30 °C, the sample temperature at 20 °C and total run time was 4 min. Weak solvent was a mixture of 450 mL of acetonitrile and 50 mL of water, and strong solvent was pure water. These solutions were stable for at least one week at room temperature.

For MS/MS detection, ESI was performed in positive ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Electrospray ionization spray voltage was 4500 V. Nitrogen was used as desolvation gas at 850 L min^{-1} and as auxiliary gas in the cone at 50 L min^{-1}. The

temperature of the source was 120 °C and the desolvation temperature was 400 °C. Argon (99.999 % purity) was used as collision gas at an approximate rate of 0.1 mL min^{-1}. Precursor ion selected for HMB was 119 m/z while product ions were 101 and 59 m/z for quantification and confirmation respectively. For Leu, the precursor ion was 132 and the product ions were 86 m/z (quantification) and 44 m/z (confirmation). Regarding cone voltage, 17 V was optimized for HMB and 23 V for Leu. The collision energies were 12 / 5 eV for HMB and 20 / 10 eV for Leu. Other common parameters for both compounds were dwell time, 100 ms; Delay time, 5 ms; Inter Channel Delay, 5 ms and InterScan time, 5 ms. Figure 2 shows the chromatogram of a standard mixture of compounds in MeOH, water and aCSF solution used to test the matrix effect in different solvents.

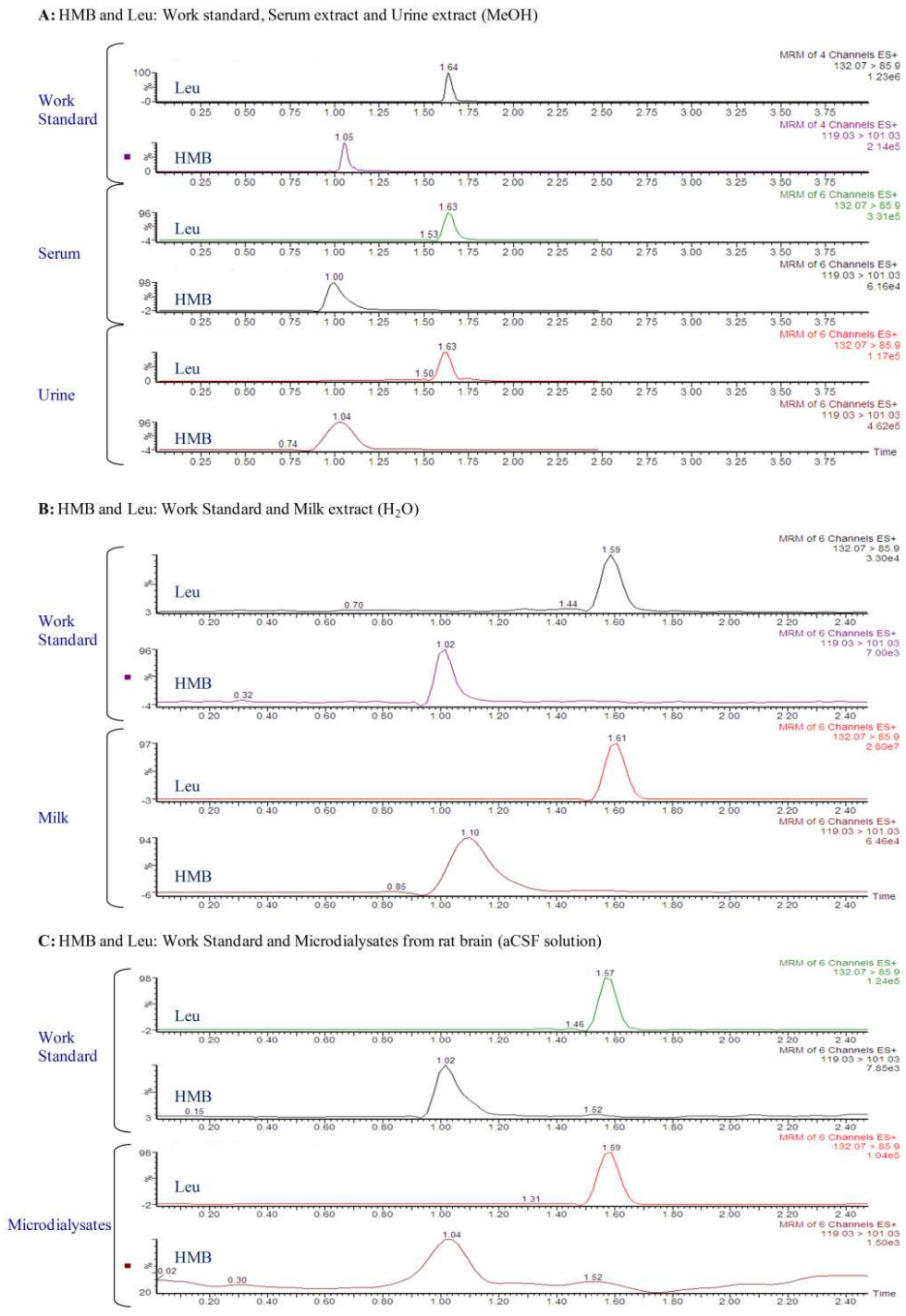

Fig. 2 Chromatograms of each analyte in calibration solution and in sample extracts. Quantification transition is shown. (A) HMB and Leu in work standard, serum extract and urine extract (MeOH); (B) HMB and Leu in work standard and milk extract (H_2O); (C) HMB and Leu in work standard and microdialysates from rat brain (aCSF solution).

2.6. Data presentation and Statistical Calculation

The results were presented as mean ± SD in Tables and Figures. Significant differences are stated in figures. One-way ANOVA and post-hoc comparison by Bonferroni's correction or Student's t-test were applied when appropriate.

3. Results and Discussion

3.1. Sample treatment for the different matrices

The optimal sample processing method for each matrix was studied. Since HMB is a molecule with amphipathic chemical character, it was reasonable to avoid protocols that include liquid-liquid partition steps, where polar and non polar solvents are used, and then HMB could be partitioned between both mediums. In fact, Deshpande et al. [29] used the liquid-liquid extraction for plasma samples, obtaining very low recovery rates. In the present work, a simple treatment of sample by dilution with a solvent was evaluated to reduce the interferences and to degrade proteins.

For serum and urine, four treatments were compared: deproteinization with EtOH; MeOH; a mixture MeOH:FA (1:1, v/v) and pure FA . The best results were obtained using MeOH, which provided recovery rates and other validation parameters within acceptance levels (recovery rates between 85-115%, relative standard deviation < 15%). For rat milk, similar experiments were done.

Different solvents (MeOH, EtOH, water), with and without additives (FA) and filtration devices (Amicon filter with centrifugation, nylon filter, PTFE filter) were tested, obtaining the best results with a simple dilution with water (without additives) and filtering with a 10K Amicon filter. Finally, for microdialysate samples a protocol similar to the one previously published [30] was applied, but in this case the new Amide column increased 10 times the sensitivity since were obtained narrower and higher peaks than in the previous Hilic column and higher ratio signal/noise (S/N) were achieved. Therefore basal levels were detected, although the values were close to quantification limits. A

slight modification in the procedure involved an acidification of the medium with 2µL FA with no further dilution. No significant matrix effects were observed after studying with different dilutions of samples where each analyte was quantified to confirm that the final concentrations, after applying the appropriate dilution factor, were the same in all cases.

3.2. Calibration curves

A calibration curve was obtained for each compound by injecting 2 µL of different standard solutions in different solvents depending on the sample, at concentration levels ranging from 0.03 to 4.00 µg mL-1 for HMB and 0.01 to 1.00 µg mL-1 for Leu. Standards for quantification of analytes in serum and urine were prepared in MeOH; water was used for milk and aCSF solution was used in the case of microdialysates. The analytical performance was evaluated according to the recommendations of Analytical Methods Committee and the Guidance for Industry, Bioanalytical Method Validation [31, 33]. The lack-of-fit test was applied to two replicates and three injections of each standard (five concentration levels). The results for every type of sample are summarized in Table 1.

Table 1. Analytical and statistical parameters.

Sample Type	Solvent		b (mL µg^{-1})	R^2 (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
Serum, Urine	Methanol	HMB	1767	99.1	6	19	19-4500
		Leu	44842	99.1	0.9	3	3-1600
Milk	Water	HMB	2091	99.6	7.0	22	22-4500
		Leu	45939	99.0	0.4	1	1-1600
Brain Microdialysates	Ringer Solution	HMB	862	99.0	4	15	15-4500
		Leu	44992	99.9	0.8	3	10-1600

b, slope; R^2, coefficient of determination; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; P-value for *lack-of-fit* test > 5% in all cases

Quality controls were injected after every 20 injections to assure the validity of the calibration curve. The predicted value was expected to not exceed ± 15% of the theoretical value.

3.3. Method validation

The analytical method was validated in terms of linearity, selectivity, sensitivity and accuracy (trueness and precision), according to the protocols described in the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [31].

Linearity of the calibration curves. It was evaluated using coefficients of determination (%R^2) and P-values for the lack-of-fit test (%P_{lof}). The values obtained for R^2 ranged from 99.0 to 99.9 % for Leu and from 99.0 to 99.6 % for HMB, and Plof values were higher than 5% in all cases. This indicated a good linearity within the stated ranges.

Selectivity. The specificity of the method was determined by comparing the chromatograms of blank solvents with those corresponding to the samples. The two compounds of interest were found in all the cases at basal levels. No interferences from endogenous substances were observed at the retention times of each respective analyte (Figure 2), which eluted at 1.0 min and 1.6 min for HMB and Leu respectively. This finding suggested that the LC-MS/MS conditions provided sufficient selectivity.

Accuracy: precision and trueness. Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation in the standard calibration curve. Recoveries were calculated by comparing the found amounts with the theoretical amounts (spiked amounts). As is shown in Table 2, the recoveries were between 85% and 115% in all cases for serum, urine and milk; plasma samples were also tested but the results obtained were not acceptable and this matrix was discharged for further experiments. Based on this fact, serum was selected to continue the experiments.

The use of a filter is generally recommended to obtain clean samples and to avoid obstruction of the UPLC® Acquity system. However, we found that if the sample was centrifuged and the supernatant collected unfiltered and directly injected, there were no obstruction issues and recovery rates were maintained. Filtration was only used for milk samples.

The precision of the method (as relative standard deviation, RSD) was assessed at three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and the procedure was repeated for five days to determine inter-day variability in order to obtain a total of 15 measurements (n=15). The results of within-laboratory reproducibility are summarized in Table 2. RSD values were between 5.7% and 14.8%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value.

Table 2. Recovery and precision of target compounds in samples.

		Spiked ($\mu g\ mL^{-1}$)	Found[a] ($\mu g\ mL^{-1}$)	Recovery (%)	RSD (%)	n
Serum	HMB	0.79	0.78	98.7	14.5	15
		1.58	1.58	100.0	11.7	15
		3.17	3.03	95.6	13.4	15
	Leu	0.09	0.09	101.1	9.9	15
		0.18	0.17	94.7	8.4	15
		0.37	0.37	101.9	13.7	15
Urine	HMB	0.24	0.24	101.6	10.0	15
		0.64	0.64	100.8	8.9	15
		1.61	1.61	101.2	7.1	15
	Leu	0.24	0.22	103.7	13.9	15
		0.63	0.59	97.5	10.8	15
		1.57	1.33	86.5	5.7	15
Milk	HMB	0.32	0.29	89.5	14.6	15
		0.59	0.53	90.2	13.6	15
		4.04	4.58	113.4	12.7	15
	Leu	0.05	0.05	99.7	12.2	15
		0.50	0.45	89.8	11.9	15
		1.00	0.97	96.5	13.9	15

[a] Mean value; RSD, relative standard deviation; n, number of determinations

Precision and trueness data indicated that the methodology to determine the target compounds in serum, urine and milk samples was highly accurate and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method. Microdialysate samples were not revaluated in terms of accuracy due to the recent publication about the validation in this matrix [30]. Only different dilutions were quantified to confirm that there was no matrix effect in mycrodialysates.

Sensitivity. The limits of detection and quantification were calculated from the signal-to-noise ratio. The calculated LODs (signal-to-noise ratio = 3) were in the range from 4-7 ng mL^{-1} for HMB and 0.4-0.9 ng mL^{-1} for Leu; and the corresponding LOQs (signal-to-noise ratio = 10) ranged from 15-22 and 1-3 ng mL^{-1}, respectively. The values obtained are shown in Table 1.

3.4. Method application. Determination of HMB and Leu contents

Serum. Table 3 gathers all the results in serum from normal animals at different ages and situations of life under overnight fasting (12-18h) and mild fasting (2-4h). The biological variability was relatively high being the RSD > 15% in most of the cases. Despite this, it is worth pointing out the following biological results that are depicted also in figures (see the reference to the figures where appropriate):

Table 3. Basal levels of HMB and Leu in serum at different conditions and stages of life.

				Sample concentration ($\mu g\ mL^{-1}$)	
Rat strain	Gender /condition	Age in weeks (stage in days)	Fasting	HMB	Leu
SD	Female /lactating	16 (PND1-3)	3-4 h	0.55 ± 0.33	9.22 ± 2.94
		17 (PND12-14)		1.08 ± 0.69	7.82 ± 5.85
		18 (PND19-20)		0.37 ± 0.12	10.8 ± 2.8
SD	Female /gestating	13 (GD-0)	3-4 h	0.90 ± 0.30	15.7 ± 5.1
		14 (GD-5)		0.77 ± 0.26	12.0 ± 3.1
		15 (GD-12)		0.67 ± 0.12	9.95 ± 3.64
		16 (GD-18)		0.68 ± 0.11	6.13 ± 2.22
SD	Male /pup	1 (PND1-3)	3-4 h	1.64 ± 0.40	14.8 ± 9.8
		2 (PND12-14)		0.42 ± 0.30	14.9 ± 3.5
		3 (PND19-20)		0.41 ± 0.02	9.91 ± 2.20
SD	Female /pup	1 (PND1-3)	3-4 h	1.29 ± 0.36	7.78 ± 2.24
		2 (PND12-14)		0.52 ± 0.07	13.5 ± 5.9
		3 (PND19-20)		0.42 ± 0.03	7.59 ± 1.71
SD	Male /virgin	13	Overnight	0.53 ± 0.03	7.49 ± 0.37
SD	Female /virgin	13	3-4 h	0.94 ± 0.33	18.7 ± 7.9
		13	Overnight	0.51 ± 0.10	9.43 ± 4.34
SD	Female /OVX	30	Overnight	0.39 ± 0.06	16.6 ± 7.6
	Female /SHAM	30		0.44 ± 0.15	15.9 ± 3.6
SD	Male	72	3-4 h	0.27 ± 0.06	12.8 ± 3.6
ZLR	Male	10	3-4 h	0.17 ± 0.12	25.8 ± 3.4
ZLR		15		0.14 ± 0.17	18.0 ± 6.2
ZLR		20		0.28 ± 0.34	23.3 ± 6.0

SD: Sprague-Dawle rats; PND: Postnatal day; GD: gestational day; OVX: Ovariectomized rats; SHAM: rats wich suffer the surgery but the ovaries are not removed; ZLR: Zucker Lean Rats. Significant differences are displayed in the figures

a) There were no differences on HMB serum levels during gestation being the levels similar to those found before gestation (V). Leu concentrations tended to decrease in gestation, being significantly different at the end of gestation with regard to the levels in non-pregnant rats (Figure 3).

b) Pup rats (both males and females) had higher levels of HMB in serum at PND1-3 (Figure 3).

c) No differences were found by sex both in pups (Figure 3) or adult animals (overnight fasted 13 weeks-old male vs females in Table 3).

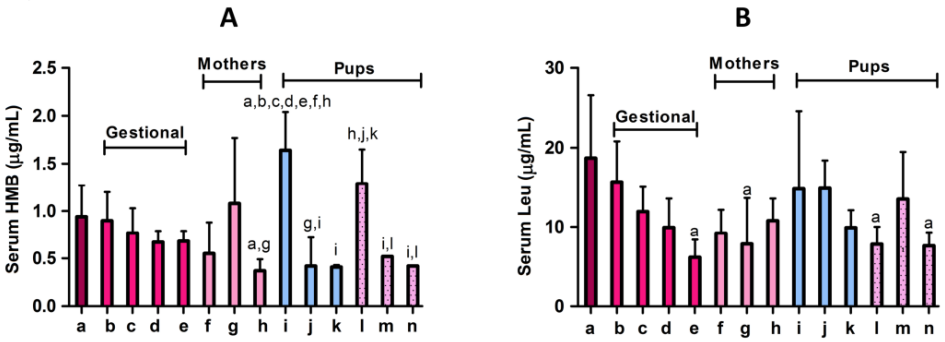

Fig. 3 (A) Concentration of HMB in serum during perinatal status; (B) Concentration of Leu in serum during perinatal status. Letters express one-way ANOVA test significant differences with refereed groups. Letters are defined as a: Virgin; b: GD0; c: GD5; d: GD12; e: GD18; f: Mum-PND1-3; g: Mum-PND12-14; h: Mum-PND19-20; i: Male PND1-3; j: Male PND12-14; k: Male PND19-20; l: Female PND1-3; m: Female PND 12-14; n: PND 19-20.

d) There was an effect of the fasting type: overnight fasting lowered the levels of HMB and Leu in serum (comparison between overnight fasted and mild fasted female rats at 13 weeks of age in Figure 4: A for HMB and B for Leu).

e) Ovariectomization by itself did not affect basal levels of HMB or Leu as the comparison with Sham operated animals did not yield significant differences. However, the levels of HMB tended to decrease and those of Leu to increase in ovariectomized adult rats to the point of being significantly different from young animals (Figure 4: C for HMB and D for Leu).

f) HMB levels decreased and Leu levels increase with age. In this regards, there were significant differences between males at 13 weeks and 70

weeks of age (data in Table 3, Figure 4: E for HMB and F for Leu). Although both groups were not in the same fasting conditions, young animals were overnight and old animals were 3-4 h fasted; this would favour even higher differences between both groups, as mild fasting was associated with higher levels particularly of HMB. This result is also in line with the finding in overiectomized rats, where the combination of ovariectomization and age reduced the content of HMB. A reasonable explanation for this effect would be that the catabolism of Leu (which is about 5% in normal conditions) could be reduced with aging.

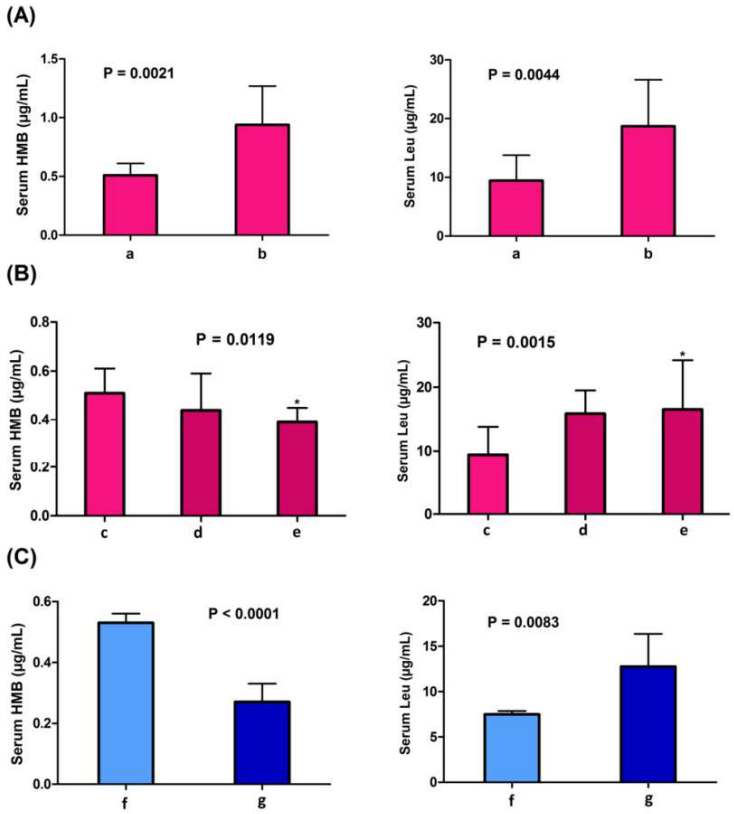

Fig. 4 HMB and Leu in serum at different stages; Student's t-test were applied and significant differences were found. Comparison between overnight and mild fasted female rats at 13 weeks of age (A for HMB and B for Leu); comparison between young female rats versus SHAM and OVX (a) rats (C for HMB and D for Leu); comparison between young male rats of 13 weeks old and male rats of 70 weeks old (E for HMB and F for Leu).

g) With regard to differences between strains, ZLR rats had less content of HMB and more of Leu in serum than SD rats (Figure 5). As in explained in point (f), the difference in fasting conditions do not invalidate this finding as it also predicts higher difference in serum levels.

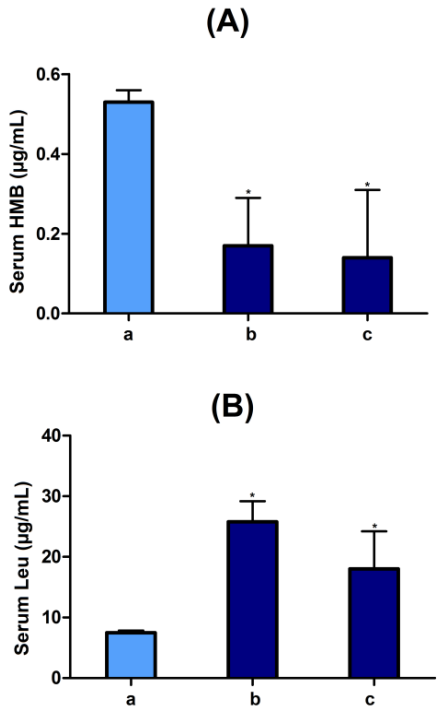

Fig. 5 Comparison of HMB (A) and Leu (B) in Serum from SD rats and ZLR (a). After one-way ANOVA test strong significant differences were found between male SD and male ZLR with similar ages in both cases, with an inverse behaviour between HMB and Leu.

Urine. Regarding urine the results are shown in Table 4. It was not possible to obtain all urine samples due to the difficulty of the sampling process itself. There were no samples for 13 and 70 weeks-old males, and only one time point in the mother during lactation (PND 19-20) could be sampled. For the rest of the groups, between 4 and 10 samples were analyzed. The following results were found:

Table 4. Basal levels of HMB and Leu in urine at different conditions and stages of life.

				Sample concentration (μg mL^{-1})	
Rat Strain	Gender /condition	Age (weeks)	Fasting	HMB	Leu
SD	Male/pup	1 (PND1-3)	3-4 h	7.86 ± 2.97	6.54 ± 3.27
		2 (PND12-14)		6.10 ± 2.84	6.43 ± 1.63
		3 (PND19-20)		16.8 ± 7.4	12.9 ± 4.3
SD	Female /pup	1 (PND1-3)	3-4 h	11.6 ± 6.8	7.48 ± 3.92
		2 (PND12-14)		6.46 ± 1.73	6.80 ± 2.07
		3 (PND19-20)		17.8 ± 11.4	13.1 ± 7.3
SD	Female	13	3-4 h	5.44 ± 2.58	5.70 ± 4.91
		13	Overnight	11.8 ± 4.7	5.84 ± 1.45
SD	Female/Gestating	13 (GD-0)	3-4 h	7.35 ± 2.48	2.06 ± 0.28
		14 (GD-5)		9.81 ± 1.73	2.28 ± 0.30
		15 (GD-12)		11.0 ± 3.6	1.79 ± 0.41
		16 (GD-18)		10.3 ± 0.9	2.14 ± 0.45
SD	Female	18 (PND19-20)	3-4 h	14.6 ± 6.4	6.72 ± 2.63
SD	Female/ OVX	24	Overnight	4.85 ± 2.94	0.49 ± 0.14
	Female/ SHAM	24		6.16 ± 3.29	0.48 ± 0.06
ZLR	Male	10	3-4 h	37.2 ± 11.0	10.4 ± 6.2
		15		34.4 ± 12.6	11.9 ± 1.9
		20		28.6 ± 19.5	10.9 ± 10.5

SD: Sprague-Dawle rats; PND: Postnatal day; GD: gestational day; OVX: Ovariectomized rats that were mothers at 13 weeks; SHAM: rats wich suffer the surgery but the ovaries are not removed and that were also mothers at 13 weeks; ZLR: Zucker Lean Rats

a) HMB and Leu concentrations were higher at PND 19-20, just the opposite to plasma in which they increased at the beginning of life. No differences were found in gestation and lactation and there were no difference with the level before gestation.

b) HMB excreted in ovariectomized animals was lower than in virgin animals with 13 weeks of life, being also coherent with the low HMB level found in serum.

c) The excretion of both compounds was higher in the ZLR than in SD rats.

Milk. Table 5 shows the HMB and Leu concentrations found in this biological fluid. No significant differences were found during the lactation period. It is interesting to remark that HMB levels in milk was in a concentration around 1.5 μg mL^{-1} milk and close to HMB concentration in serum from pups at PND1-3.

Table 5. Basal levels of HMB and Leu in milk from Sprague-Dawley Rats in different points of the lactation period.

			Sample concentration (μg mL^{-1})	
Rat strain	Age (weeks)	Feeding	HMB	Leu
SD	15 (PND1-3)	Unrestricted	1.35 ± 0.50	9.23 ± 2.05
SD	17 (PND12-14)	Unrestricted	1.37 ± 0.42	7.61 ± 4.39
SD	18 (PND19-20)	Unrestricted	1.84 ± 0.34	11.1 ± 5.7

PND: Postnatal day

Microdialysates from rat brain. Finally the analytical method was applied to microdialysates from rat brain in a study in vivo. The microdialysis technique was previously described in two recent publications by our group [29,34], and in the first of them HMB and Leu were also measured. The modifications of the method accomplished in this work, allowed us to detect basal levels of HMB and Leu in brain fluids, where we could not detect them before. The experiments were done in females SD rats with 18 weeks of life and under mild fasting, and the basal concentrations of HMB and Leu were 0.24 ± 0.09 μg mL^{-1} and 0.15 ± 0.19 μg mL^{-1}, respectively. Unlike other tissues, the concentrations of both compounds were of the same order of magnitude.

4. Conclusions

The proposed method is a powerful tool for the simultaneous determination of HMB and Leu in different biological fluids. The determination and quantification of these compounds using LC-MS/MS was successfully performed on an Acquity UPLC BEH Amide column, using pure water and 0.1% (v/v) formic acid in acetonitrile as mobile phases and triple quadrupole

detection in positive electrospray ionization mode. The sample treatment was adapted to each matrix, namely serum, urine, milk, and brain microdialysates, and was kept as simple as possible. The analytical performance of the method was validated providing a reliable tool for the simultaneous determination of these two compounds in several matrices, which in turn, would offer important information regarding the metabolic pathway of Leu and HMB at different stages of the life or in variety physiological situations.

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The authors are indebted to all the participants, without whom this work would not have been possible. We are grateful to the team of technicians of Abbott which helped in the tasks of care and maintenance of animals, and in the sampling protocol. The authors are grateful to Neile Edens, Ph.D. (Associate Research Fellow, Discovery Abbott Nutrition Columbus, Ohio) for his valuable suggestions in the manuscript. Finally, the authors also thank the anonymous reviewers, who provided useful comments on the manuscript.

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III.6 DETERMINACIÓN DE HMB Y LEU EN OTRA MATRIZ BIOLÓGICA MEDIANTE UHPLC-MS/MS

For reasons of confidentiality every detail can not be exposed in this section. Some parameters will be encrypted.

III.5.1 Animal Manipulation

A different biological matrix (BM) was measured in young virgin females SD rats of 13 weeks old. The samples were collected after sacrifice. They were stored at -80°C until analysis by UPLC®-MS/MS. Protocols for all experimental procedures were carried out according to ethical guidelines for animal experimentation at the Spanish National Research Council (RD 53/2013).

III.5.2 Analytical Method

The sample procedure for BM consisted on the following steps: 50 mg of BM samples were hydrolyzed with 0.4 mL of 20% FA in water, shaking in a vortex-mixer during 30 minutes at ambient temperature. Subsequent to the agitation 0.5 mL of MeOH LC-MS grade were added to continue in the vortex-mixer 20 minutes more. After that, the treated samples were centrifuged at 2400 x g (3000 r.p.m.) for 10 min at 5° C. The supernatant was collected into an eppendorf vial to dry in a rotary vacuum pump evaporator at 30°C. The dried samples were re–dissolved in a total volume of 250 µL of purified LC-MS water. To shake and sonicate was needed to total reconstitution. Finally, the liquid was filtered by 0.2 µm nylon filter. The filtrate was introduced in maximum recovery vials to inject into the UPLC®. A second dilution was done to measure Leu within LDR: 20 µL of the filtrate were taken in a new vial and was added 2 mL of purified LC-MS water, shaken and ready to inject also into the UPLC®.

The chromatographic and mass spectrometric detection conditions were the same as those mentioned above in published works.

III.5.3 Analytical Performance

For BM, different complex protocols were studied following strong hydrolysis with HCl at different concentrations (6N - 1N), with organic extraction additional using hexane, chloroform or methanol; another variant was weaker hydrolysis using formic acid (FA) at different concentrations (20-100% in water or in MeOH); and another variant was the evaluation of the combination of weak hydrolysis (20% FA in water) and organic solvent extraction additional (MeOH). The best results were obtained following the selected protocol described in the previous section.

III.5.4 Method Validation

As the previous methods the validation of linearity, accuracy (precision and trueness), sensitivity, and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for bioanalytical assay validation.

Linearity. Linearity of the calibration curves was evaluated using coefficients of determination (R^2 %) and P-values of the lack-of-fit test (%P_{lof}). The values obtained for R^2 were 99.0-99.6 % for Leu and 99.0-99.6 % for HMB, and P_{lof} values were higher than 5% in all the cases. This indicated a good linearity within the stated ranges (Table 1).

Table 1. *Analytical and statistical parameters of BM in water extract.*

	n	a	b (mL µg^{-1})	R^2 (%)	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	LDR (µg mL^{-1})	% P_{lof}
HMB	5	55	2091	99.6	0.007	0.022	0.030-4	23.1
Leu	5	1116	45939	99.0	0.0004	0.001	0.010-1	38.3

n, number of calibration levels; a, intercept; b, slope; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range; %P_{lof}, P-value for lack-of-fit test

Selectivity. The specificity of the method was determined by comparing the chromatograms of blank solvents with those corresponding to the samples. The two compounds of interest were found in all the cases to basal levels. Studied samples at basal levels named blank samples, showed these compounds in every experiment. No interferences from endogenous substances were observed at the retention times of each respective analyte (Figure 56), which eluted at 1.0 min and 1.6 min for HMB and Leu respectively. This finding suggested that the LC-MS/MS conditions provided sufficient selectivity.

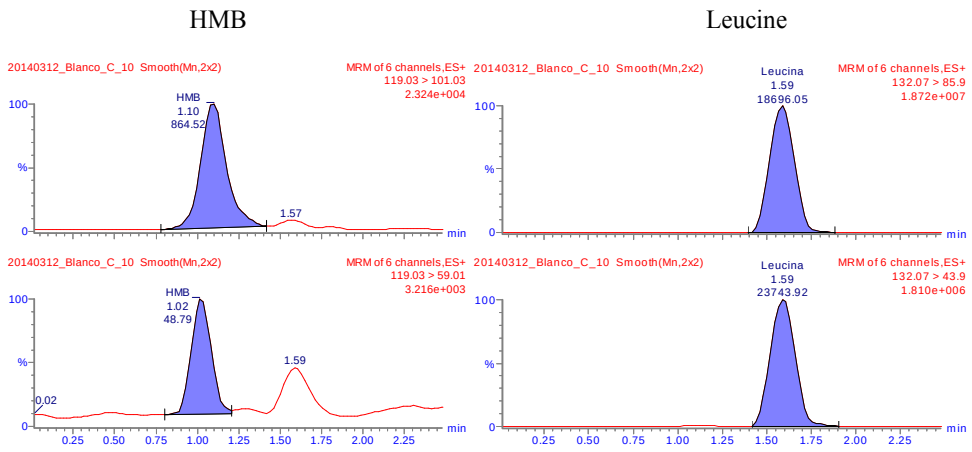

Figure 1. *BM extract in H_2O.*

Accuracy: precision and trueness. Due to the lack of certified reference materials, a spike/recovery assay was performed to validate the method in terms of trueness, which was evaluated by determining the recovery of known amounts of the compounds of interest spiked into blank samples. The samples were analyzed using the proposed method and the concentration of each compound was determined by interpolating from the standard calibration curve. Recoveries were calculated by comparing the interpolated amounts to the theoretical amounts, spiked amounts. As shown in Table 2, the recoveries were between 85% and 115% in all cases.

It must be mentioned that the use of the nylon filter was recommended to obtain a clean sample to avoid an obstruction into UPLC® Acquity system, but if the sample was centrifuged and supernatant collected unfiltered the results of recovery rates were similar. PTFE filters were also evaluated with similar results than nylon filters.

Table 2. *Recovery, precision and accuracy of target compounds in BM samples.*

		Intra-day				Intra-day			
	Spiked ($\mu g\ mL^{-1}$)	Found[a] ($\mu g\ mL^{-1}$)	R (%)	RSD (%)	n	Found[a] ($\mu g\ mL^{-1}$)	R (%)	RSD (%)	n
HMB	0.10	0.10	98.2	3.9	3	0.09	90.1	4.0	15
	0.81	0.70	85.9	6.5	3	0.70	86.4	5.7	15
	3.03	3.01	99.2	14.8	3	3.02	99.6	13.6	15
Leu	0.05	0.05	94.5	13.7	3	0.05	99.0	10.7	15
	0.50	0.50	100.2	13.2	3	0.52	104.9	13.1	15
	1.00	0.97	97.2	14.6	3	1.14	113.6	12.6	15

[a] *Mean value; R, % recovery; RSD, relative standard deviation; n, number of determinations.*

To evaluate the precision of the method, the intra- and inter-day precisions (as relative standard deviation, RSD) were assessed at three concentration levels for each compound. Three replicates at each level were analyzed on the same day in order to evaluate intra-day variability and were repeated for five days to determine inter-day variability. The results of repeatability and within-laboratory reproducibility are summarized in Table 8. RSD values were between 3.9% and 14.8%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered ≤ 15% of the actual value. Precision and trueness data indicated that the methodology to determine the target compounds in BM samples is highly accurate, precise, and robust, and that the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

Sensitivity. Limit of detection and LOQ are two fundamental aspects that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. The LOD is the minimum amount of analyte

detectable in the sample while the LOQ is the minimum amount that can be quantified. They are based on the theory of hypothesis testing and the probabilities of false positives and false negatives. In this work, these parameters were calculated from the signal-to-noise ratio. The calculated LODs (signal-to-noise ratio = 3) were 7 ng mL^{-1} for HMB and 0.4 ng mL^{-1} for Leu; and the corresponding LOQs (signal-to-noise ratio = 10) were 22 and 1 ng mL-1, respectively. The values obtained are shown in Table 7.

III.5.5 Method Application

Experiments focused on explore particular functions were performance with SD rats, specifically virgin female of 13 weeks of life. To evaluate basal levels of HMB and Leu in normal conditions, the animals were fasting during 12 h and previously they had been fed up with AIN-93-M normally. BM samples were analyzed according to protocol described above and the results obtained were referred in the units of µg of HMB or Leu per gram of BM: 5.66 ± 1.73 µg HMB/g and 90.87 ± 24.56 µg Leu/g.

III.7 PATENT APPLICATION: NUTRITIONAL COMPOSITIONS AND METHODS OF INCREASING ENDOGENOUS PRODUCTION OF β-HYDROXY-BETA-METHYLBUTYRATE (Publicación VII)

Additional experiments to evaluate basal levels of HMB and Leu in the different matrices exposed above when animals are fed with special diets rich in a specific ingredient were conducted and a patent application is subject to the patent office in the process of being published. Application No./Patent No.: 14382323.5 – 1357.

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Reference | Application No./Patent No.: 14382323.5 - 1357

Applicant/Proprietor: ABBOTT LABORATORIES

Designation as inventor - communication under Rule 19(3) EPC

You have been designated as inventor in the above-mentioned European patent application. Below you will find the data contained in the designation of inventor and further data mentioned in Rule 143(1) EPC:

DATE OF FILING : 22.08.14

PRIORITY : / /

TITLE : Nutritional compositions and methods of increasing endogenous production of beta-hydroxy-beta-methylbutyrate

DESIGNATED STATES : AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

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DECLARATION UNDER ARTICLE 81 EPC:
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Figura 32. *Carta de comunicación en la oficina de patentes*

Por motivos de confidencialidad, no es posible incluir más datos sobre la presente patente en esta Tesis Doctoral.

CAPÍTULO IV

Marcadores de oxidación

IV.1 OBJETIVOS

Los objetivos de los trabajos realizados y presentados en este capítulo son:

- ✓ Revisión del estado actual del conocimiento en relación al estudio de marcadores de degradación en productos nutricionales.

- ✓ Proponer metodología analítica para la determinación de marcadores de degradación en fórmulas infantiles mediante cromatografía de líquidos de ultra alto rendimiento acoplada a espectrometría de masas de alta resolución (UPLC®-TOF).

- ✓ Evaluación de una fórmula infantil mediante métodos clásicos.

- ✓ Relacionar los resultados obtenidos en UPLC®-TOF con los obtenidos clásicamente mediante estudios estadísticos.

- ✓ Proponer marcadores potencialmente útiles en la industria nutricional.

- ✓ Evaluar marcadores específicos de oxidación en un sistema biológico (bajo patente actualmente).

IV.2 ESTRATEGIA DE TRABAJO SEGUIDA

La estrategia seguida en último capítulo fue la siguiente:

A. Revisión de la evolución en la industria y el estado actual de conocimiento en el control alimentario mediante la determinación analítica de marcadores de oxidación y degradación en alimentos de diversa naturaleza. Estos compuestos son controlados para mantener los productos alimenticios con una calidad adecuada, y además evitar los efectos tóxicos que podrían afectar al ser humano.

B. Espectrometría de Masas de alta resolución con tiempo de vuelo (TOF). Se evaluaron productos nutricionales expuestos a ciertas condiciones de oxidación en una cámara de envejecimiento acelerado. Se realizó un barrido completo determinando todos los compuestos existentes en la matriz objeto de estudio, comparando así los distintos niveles de degradación del producto. Estadísticamente se puede identificar qué compuestos son críticos en cuanto a calidad y seguridad alimentaria.

C. Aplicación de metodologías clásicas consolidadas. Las técnicas clásicas ofrecen métodos validados y consolidados para controlar los procesos de oxidación de los productos nutricionales y evaluar su estabilidad. Se correlaciona estadísticamente las moléculas encontradas mediante TOF con los resultados obtenidos mediante las metodologías clásicas haciendo uso del análisis estadístico de componentes principales (PCA).

D. Análisis mediante cromatografía de líquidos-espectrometría de masas (TQD) en muestras biológicas. Se desarrolló específicamente un método analítico para determinar una serie de moléculas clásicamente conocidas como marcadores de estrés oxidativo. Malondialdehído, que es el biomarcador estudiado para cuantificar la peroxidación lipídica[190,191]; la

[190] *Esterbauer, H., Schaur, R. J., et al. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic. Biol. Med. 11 (1991) 81–128.*

orto-tirosina[192], que es el metabolito encontrado después de la oxidación de la fenilalanina; y la 8-oxo-deoxiguanosina, que es el compuesto de oxidación obtenido después del ataque por radicales libres a la base nitrogenada guanosina del ácido desoxirribonucleico[193].

E. Aplicación del método analítico. Para entender qué papel juega la nutrición en el deterioro o mejora de las funciones biológicas, incluida la función cognitiva, se llevó a cabo un experimento con ratas para estudiar la evolución de estos biomarcadores según la dieta utilizada.

Por motivos de confidencialidad, los puntos D y E no pueden ser expuestos en detalle, ya que en la actualidad son trabajos en fase de patente.

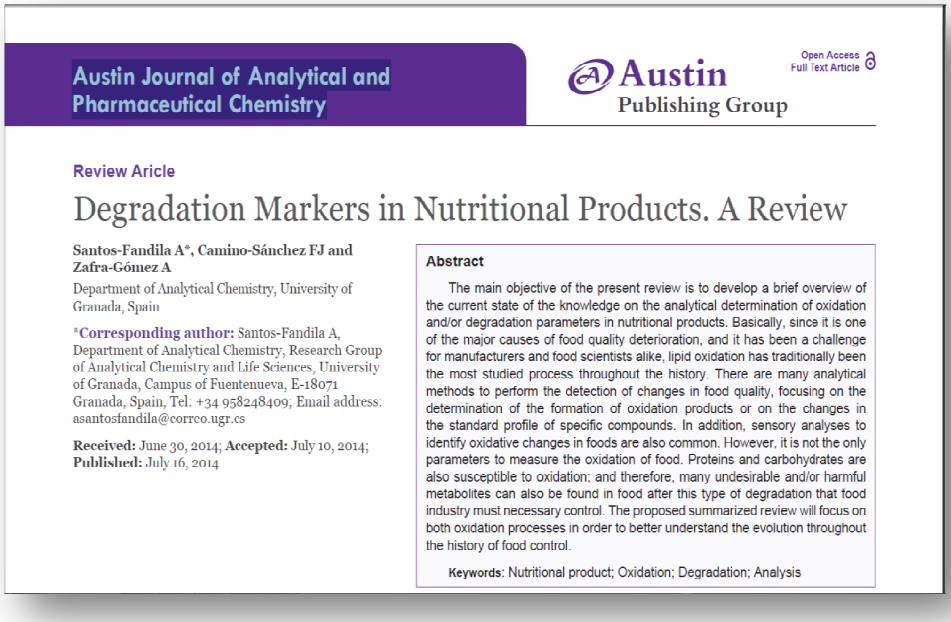
[191] *Martinovic, J., Dopsaj, V., et al. Oxidative stress biomarker monitoring in elite women volleyball athletes during a 6-week training period. J. Strength Cond. Res. 25 (2011) 1360-1367.*

[192] *Molnar, G. A., Nemes, V., et al. Accumulation of the hydroxyl free radical markers meta-, ortho-tyrosine and DOPA in cataractous lenses is accompanied by a lower protein and phenylalanine content of the water-soluble phase. Free Radic. Res. 39 (2005) 1359-1366.*

[193] *Puchades Montesa, M. J., Gonzalez Rico M.A., et al. Study of oxidative stress in advanced kidney disease. Nefrologia 29 (2009) 464-473.*

IV.3 INTRODUCCIÓN. A REVIEW. DEGRADATION MARKERS IN NUTRITIONAL PRODUCTS (PUBLICACIÓN VIII)

En este apartado se presenta un artículo de revisión sobre marcadores de degradación en productos nutricionales, publicado en la revista científica **Austin Journal of Analytical and Pharmaceutical Chemistry**, de la editorial Austing Publishing Group, donde la doctoranda participa en el panel de editores y fue invitada para publicar en el número inaugural. Ver Figura IV.1.



Austin Journal of Analytical and Pharmaceutical Chemistry

Austin Publishing Group

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Review Aricle

Degradation Markers in Nutritional Products. A Review

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Abstract

The main objective of the present review is to develop a brief overview of the current state of the knowledge on the analytical determination of oxidation and/or degradation parameters in nutritional products. Basically, since it is one of the major causes of food quality deterioration, and it has been a challenge for manufacturers and food scientists alike, lipid oxidation has traditionally been the most studied process throughout the history. There are many analytical methods to perform the detection of changes in food quality, focusing on the determination of the formation of oxidation products or on the changes in the standard profile of specific compounds. In addition, sensory analyses to identify oxidative changes in foods are also common. However, it is not the only parameters to measure the oxidation of food. Proteins and carbohydrates are also susceptible to oxidation; and therefore, many undesirable and/or harmful metabolites can also be found in food after this type of degradation that food industry must necessary control. The proposed summarized review will focus on both oxidation processes in order to better understand the evolution throughout the history of food control.

Keywords: Nutritional product; Oxidation; Degradation; Analysis

Figura IV.1. *Degradation Markers in Nutritional Products. A Review. Journal of Analytical and Pharmaceutical Chemistry de la editorial Austin Publishing Group, OPEN ACCESS, 1 (2014) 1-7 (no indexada)*

Degradation Markers in Nutritional Products. A Review

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Abstract

The main objective of the present review is to develop a brief overview of the current state of the knowledge on the analytical determination of oxidation and/or degradation parameters in nutritional products. Basically, since it is one of the major causes of food quality deterioration, and it has been a challenge for manufacturers and food scientists alike, lipid oxidation has traditionally been the most studied process throughout the history. There are many analytical methods to perform the detection of changes in food quality, focusing on the determination of the formation of oxidation products or on the changes in the standard profile of specific compounds. In addition, sensory analyses to identify oxidative changes in foods are also common. However, it is not the only parameters to measure the oxidation of food. Proteins and carbohydrates are also susceptible to oxidation; and therefore, many undesirable and/or harmful metabolites can also be found in food after this type of degradation that food industry must necessary control. The proposed summarized review will focus on both oxidation processes in order to better understand the evolution throughout the history of food control.

Keywords

Nutritional product; Oxidation; Degradation; Analysis

Introduction

In general, the oxidation of food stuffs is a process that must be avoided. However, the process occurs naturally when food is exposed to air and it is potentiated by heat, light, chemical catalysts or enzymatic processes. The

oxidation causes the loss of nutritional value of food and changes the chemical composition. For example, oxidation of fats and oils leads to rancidity and in fruits it can result in the formation of compounds which discolor damaging the product.

The food alterations could deteriorate the quality of the product and also generate undesirable compounds with direct consequences on human health. There are many reports published in the scientific literature about the possible toxicity of certain metabolites generated from lipid oxidation such as cholesterol oxidation products; COPs, commonly known as oxysterols; OS (1, 2); and phytosterol oxidation products; POPs; which have unhealthy effects at higher concentrations. Cytotoxicity, atherogenesis, mutagenesis, carcinogenesis, changes in cellular membrane and inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG-CoA reductase; activity have been widely described (3-6).

Therefore, it is mandatory to control the oxidation processes using the technology available. Throughout the history, several analytical methods have been proposed to keep under control the oxidation of nutritional products. There are very different techniques that can be applied depending on the final objective and the product under study. The oxidation parameters to measure can be classified in three important groups depending of the sample: lipids, proteins or carbohydrates. Another classification could be done taking into account the technique used to control the oxidation: chemical or sensory methods. Finally, a third classification could be showed comparing two modes: general oxidation or determination of target compounds.

This summary review consists in a globalized approach to know, from an analytical point of view, the evolution in food control in terms of oxidation parameters. In addition, the methods and techniques that are commonly employed today to determine the rate of oxidation of food are presented in more detail.

Lipid oxidation

Lipids are an important component of food being also used in a large amount of industrial applications. Lipids in food, either naturally occurring or added exogenously are used as nutrient and also provide a heat transfer medium for food processing and render desirable texture and flavor to the products. They are one of the major and essential macronutrients required for growth and maintenance of living organisms. However, overconsumption of lipids, especially certain saturated lipids and trans fats, has been associated with diseases such as obesity, hypertension, cardiovascular disease or cancer (3, 7-10), mainly when these lipids are oxidized generating other compounds. Although there are others hypothesis that differ regarding the issue of that fats are the unique responsible of cardiovascular disease (11, 12).

Lipid oxidation is an important cause of deterioration in quality of food both during manufacturing and product shelf life, and negatively affects the integrity of biological systems. The oxidative changes cause development of off-flavors, loss of nutrients and bioactives, and even the formation of potentially toxic compounds originating products unsuitable for the human consumption.

The overall mechanism of lipid oxidation consists of three phases: the initiation, with the formation of free radicals; the propagation, with the free-radical chain reactions; and the termination, with the formation of stable non-radical products.

The most important lipids involved in the oxidation process are the unsaturated fatty acid moieties, oleic, linoleic and linolenic. Lipid peroxidation generates a large number of by-products, including breakdown molecules resulting from cleavage of the oxidized fatty acyl chain as is shown in figure 1. The rate of oxidation of fatty acids increases with the degree of unsaturation. The free radicals reactions are thermodynamically difficult, for this reason the production of the first few radicals mandatory must occur by some catalytic

means such as hydroperoxide decomposition, light and heat exposure and metal catalysis. In addition, autoxidation is the most common process. It is defined as the spontaneous reaction of lipids with atmospheric oxygen through a chain reaction of free radicals.

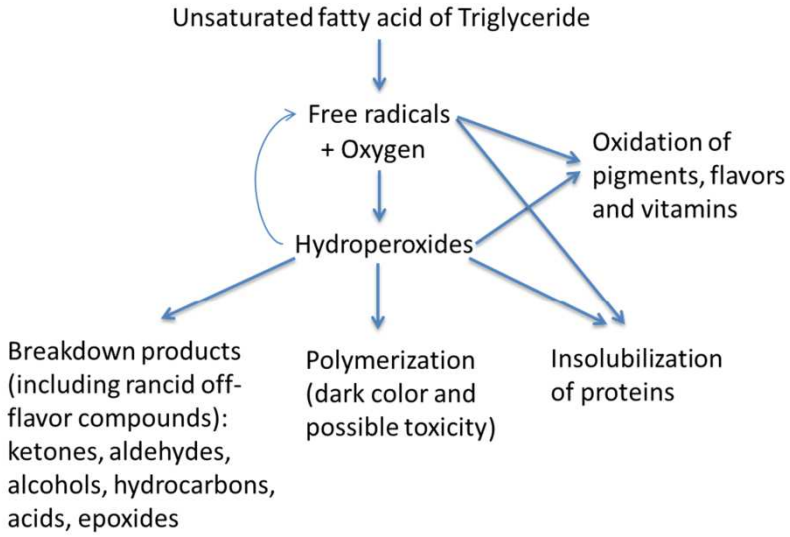

Fig. 1. *Lipid oxidation mechanism.*

Sensory evaluation

Sensory analysis is a scientific discipline that applies the principles of experimental design and statistical analysis to data obtained from human senses (sight, smell, taste, touch and hear) for the evaluation of consumer products. The discipline requires panels of human assessors, on whom the products are tested, and recording the responses made. By applying statistical techniques to the results it is possible to make inferences and insights about the products under test. Sensory analysis can mainly be classified into three sub-sections: effective testing (dealing with objective facts about products); affective testing (dealing with subjective facts such as preferences); and perception (the biochemical and psychological aspects of sensation). Sensory evaluation is used to study similarities or differences in a wide range of dishes/products; to analyze food samples for improvements; to gauge

responses for a dish/product, e.g. acceptable or unacceptable; to explore specific characteristics of an ingredient or dish/food product; to check whether a final dish/food product meets its original specification; and to provide objective and subjective feedback data to enable informed decisions to be made.

Depending on the product, different parameters are studied and specific preparation processes are applied. For example, to measure the quality of solid food, cold cuts parameters such as color intensity, juiciness, hardness, cohesiveness, rancid taste and general acceptability, are studied (13); in contrast, to evaluate meat it is required to prepare the food with a specific protocol where the temperature or the mode of cooking and serving to the panelists must be indicated. In that case, parameters such as aroma, appearance, color, flavor intensity, saltiness, moistness/juiciness, tenderness, and overall acceptability are evaluated (14, 15). In infant formulas are also evaluated similar parameters such as bitterness, saltiness, savoriness, sourness, sweetness, and pleasantness (16). In all cases an expert panel of at least 6-8 members is selected using a specific point number in the hedonic scale.

Oxygen absorption

In the initial stage of autoxidation a high consumption of oxygen occurs, the fat or oil increases the weight; therefore this event theoretically should reflect its oxidation level. The procedure based on weight gain is simple and cheap equipments are required. An oil sample is heated in a special oven with no air circulation, and periodically testing for weight gain. This method indicates oxygen absorption through mass change. It is one of the oldest methods for evaluating oxidative stability, but nowadays it is still used (17, 18). The method is only useful when highly unsaturated oils, such as marine or vegetable oils containing a high content of polyunsaturated fatty acids, are evaluated.

Oxygen consumption can be also measured directly by monitoring the drop of oxygen pressure. Using a headspace oxygen method, an oil sample is

placed in a closed vial that contains certain amount of oxygen at high temperature (100 °C). The pressure reduction in the vial, which is due to the oxygen consumption, is monitored and recorded automatically. Then, the oxygen uptake can be calculated (19). This method is simple and reproducible and could be the best method of analysis to assess the oxidative stability of fats and oils. However, it can be found interferences in products where proteins are present in a substantial ratio since proteins are also oxidized.

Measure compositional changes

Lipid oxidation may also be assessed by measuring the initial quantitative compositional change. In foods that contain fats or oils, unsaturated fatty acids are the main compounds that change during oxidation. Changes in fatty acid composition provide an indirect measure of the extent of lipid oxidation. In the majority of these methods the lipids are extracted, derivatized and measured by gas chromatography with different detectors; GC; (20-23) or GC coupled to mass spectrometry; GC-MS (24, 25). In recent years, ultrahigh performance liquid chromatography; UHPLC; has also widely been used. A recent published method determines the volatile carbonyls in oils by UHPLC coupled to diode array detector; DAD; and GC-MS (26) with dynamic headspace sampling and 2,4-dinitrophenylhydrazine; DNPH; as derivatisation agent. An alternative approach to the study of a complete profile is the use of these techniques to determine selective target compounds related with the oxidation of foods. An example is the determination of oxidative markers such as hexanal in vegetable oils by an automated dynamic headspace sampler coupled to GC-MS (27).

Measure of primary compounds of oxidation

The most traditional measurement of lipid oxidation is the Peroxide Value; PV. Peroxides are the main products that appear at the beginning of the autoxidation. They can be measured by techniques based on the generating iodine from potassium iodide, or the oxidation of ferrous ions to ferric ions (18, 28). The content is generally expressed in terms of milliequivalents of oxygen per kilogram of fat. Although PV is applicable at the early stages of oxidation, it

is highly empirical. The accuracy is questionable, the results vary depending on the procedure used, and the test is very sensitive to changes in the temperature. During the course of the oxidation, PV increases, reaching a maximum, and then decline.

A high number of methods have been developed for determination of PV, among which the iodometric titration, ferric ion complexes measurement spectrophotometry, and IR spectroscopy are most frequently used. Specifically, with Fourier Transform Infrared Spectroscopy; FTIR; the hydroperoxides can be quantitatively determined via measurement of their characteristic O-H stretching absorption band.

Initially, conjugated dienes and trienes were often used to control the level of oxidation. During the formation of hydroperoxides from unsaturated fatty acids conjugated dienes are typically produced, due to the rearrangement of the double bonds. The resulting conjugated dienes exhibit an intense absorption at 234 nm; similarly, trienes absorb at 268 nm. If UV absorption increases then it reflects the formation of primary oxidation products in fats and oils. In addition, there is a good correlation between conjugated dienes and PV.

Measurement of secondary compounds of oxidation

Measurement of malondialdehyde; MDA. The thiobarbituric acid; TBA; test was proposed over 40 years ago (29, 30) and it is one of the most widely used methods to measure the level of lipid peroxidation by determination of MDA in foods containing fats. It is simple and its results are highly correlated with scores obtained in the sensory evaluation (25, 31-35). The basic principle of the method is the reaction of one molecule of MDA and two molecules of TBA to form a red MDA-TBA complex (see figure 2), which can be quantified spectrophotometrically at 530 nm.

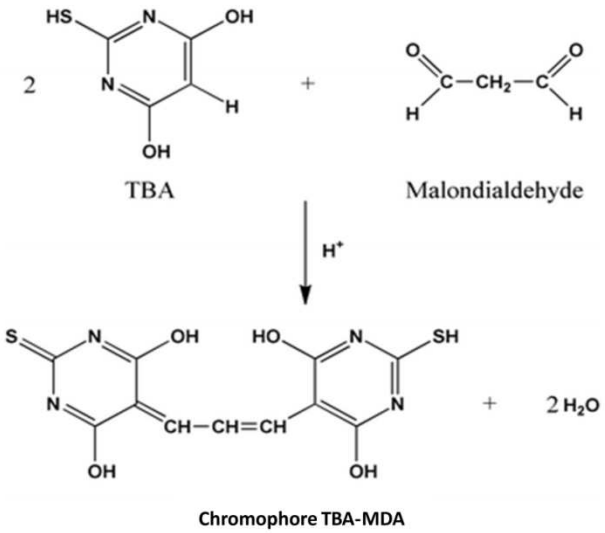

Fig. 2. *Reaction of 2-thiobarbituric acid; TBA; and malonaldehyde; MDA.*

It is noteworthy that this method has been criticized for several reasons. The method is not specific and selective; and the sensitivity could be further improved, since it has no acceptable limits of detection for measuring MDA (33, 36-38). Other TBA-reactive substances; TBARS, including sugars and other aldehydes, could interfere with the TBA reaction. Abnormally low values may result if MDA reacts with proteins in an oxidizing system (39). In many cases, the TBA test is recommended for comparing samples of a single material at different states of oxidation. Currently, it is known that TBARS assay is a general method for the detection of lipid peroxidation (40) and not only is measured MDA but also thiobarbituric acid reacts with other compounds to generate different colored species that interfere with the assay. Some studies have identified several compounds using mass spectrometry (41).

An interesting comparison between different methods to measure MDA by TBA derivatisation is presented in a work published in 2001 (42). The authors demonstrated that the quantification of MDA in milk powder samples using three variants of the TBA test, overestimated the MDA content because the high temperature used during the derivatisation step potentiated the oxidation, enhancing the MDA contents or promoting the formation of other by-products that interfere in the spectrophotometric assay. To solve this issue,

free MDA has also been determined using direct methods without derivatisation in biological systems (43, 44). There are others alternatives to measure MDA by derivatization with 2,4 dinitrophenylhydrazine; DNPH (42, 45-47).

p-Anisidine Value; p–AnV. It is a method to measure the content of aldehydes (mainly 2-alkenals and 2,4-alkadienals) generated during the decomposition of hydroperoxides. It is based on the color reaction of p-methoxyaniline and the aldehyde compounds (see figure 3 as example). The reaction of p-anisidine with aldehydes under acidic conditions provides yellowish products that absorb at 350 nm.

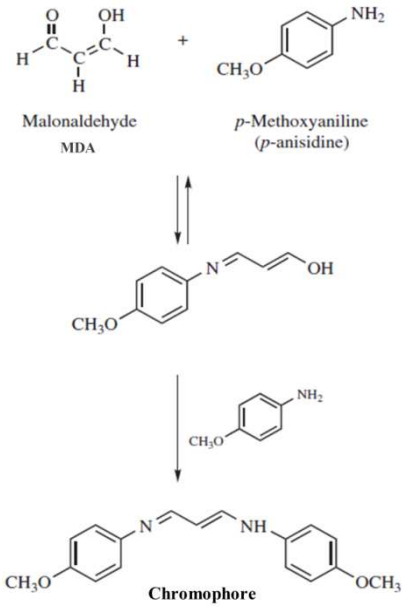

Fig. 3. *Reaction between p-anisidine and malonaldehyde; MDA.*

Measurement of 4-hydroxynonenal; 4-HNE; and 4-hydroxyhexanal; 4-HHE. These compounds are also secondary components of the lipid oxidation. They are both generated in tissue and food from polyunsaturated membrane lipids through a free radical-induced lipid peroxidation process. The biological properties of these aldehydes have been widely studied and some methods have been proposed with different techniques such as high performance liquid chromatography; HPLC; with ultraviolet detection; UV (48); TLC densitometry,

gas chromatography coupled to mass spectrometry; GC-MS (3, 49) or HPLC with mass spectrometry in tandem; MS/MS (10).

Measurement of Totox Value; TV. This parameter gives a measurement value of the total oxidation, including primary and secondary oxidation products (50-54). It is a combination of PV and p-AnV: TV = 2PV + p-AnV. This value reflects the oxidation level at early and later stages of oxidation reaction, respectively. With this equation, both hydroperoxides and their breakdown products are estimated and a more adequate result is offered to control the progressive oxidative deterioration of fats and oils. Since two very different values are combined, many authors disagree with this parameter.

Measurement of carbonyls content. The carbonyl compounds, including ketones and aldehydes, are the secondary oxidation products generated by the degradation of hydroperoxides, and are suggested to be the major responsible for off-flavors associated with the rancidity of food products. The total carbonyl content is measured by a colorimetric method. The carbonyl compounds formed during lipid oxidation are reacted with DNPH followed by the reaction of the resulting hydrazones with alkali (see figure 4).

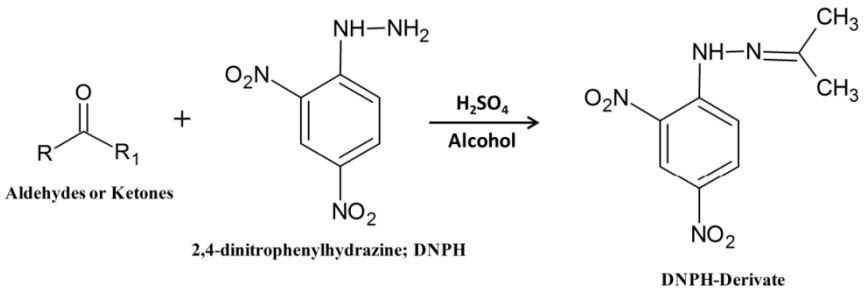

Fig. 4. *Reactions between carbonyls (aldehydes or ketones) and 2,4-dinitrophenylhydrazine; DNPH.*

The determination of total content of carbonyls has been frequently used in oxidative stability studies. Improved variants can be found recently (55). However, this procedure has been criticized because the determination

conditions cause degradation of hydroperoxides into carbonyl derivatives, obtaining overestimated results.

Oil stability Index; OSI. During lipid oxidation, volatile organic acids such as formic acid and acetic acid are produced at high temperatures together with hydroperoxides. In addition, other secondary products, including alcohols and carbonyl compounds, can be further oxidized to carboxylic acids. The OSI method measures the volatile acids by monitoring the change in electrical conductivity when effluent from oxidizing oils is passed through water. The OSI value is defined as the point of maximal change of the rate of oxidation, attributed to the increase of conductivity by the formation of volatile organic acids. This method shows the important disadvantage of requiring a high level of oxidation, i.e. the method has low sensitivity. There are commercially available equipments such as the Rancimat® (Metrohm Ltd., USA) and the Oxidative Stability Instrument® (Omnion Inc., USA), both are employed for determining the OSI value. Recently, a published article compares the oxidation in oils including OSI, and by several analytical methods above mentioned (56).

Hydrocarbons and fluorescence assay. Formation of saturated hydrocarbons such as ethane, propane and pentane, can be measured for monitoring lipid oxidation. Classically, these compounds have been determined by GC to assess rancidity of fats and oils as well as freeze-dried muscle foods. Usually, significant correlations exist between short-chain saturated hydrocarbons levels and rancid odor scores. Fluorescence assays are also helpful to assess lipid oxidation in muscle foods and biological tissues.

Others techniques. There are others techniques and different variants in the methodologies to measure the lipid oxidation such as Free Radicals, Differential Scanning Calorimetry; DSC, Nuclear Magnetic Resonance Spectroscopy; NMR, etc. In the fist case, the free radicals are short-lived intermediates which appear at the initial steps of the oxidation in fats and oils. Those compounds can be measured with different techniques such as Electron Spin Resonance; ESR; that is of great value for the study of the early stages of

lipid oxidation and prediction of oxidative stability of fats and oils. In addition, there is a strong linear correlation between ESR and Rancimat® or ESR and oxygen consumption analyses. The second technique, which is based on thermal release of oxidation reactions, has the potential of a nonchemical method for assessing oxidative stability of fats and oils, indicating the onset of advanced oxidation (termination). Finally, NMR is also a very useful technique for this purpose since the results obtained usually correlated adequately with the values obtained with Totox, conjugated diene and TBA assays.

Degradation of proteins and carbohydrates

During food processing, the molecular structure of proteins may suffer changes. In addition, chemical reactions such as lipid and protein oxidation, non-enzymatic browning and enzymatic activity are also responsible for color, flavor or odor alterations. As in the case of lipid oxidation, there are a large amount of analytical methods to detect these undesirable processes.

In addition to sensory tests, the protein oxidation is measured in terms of the appearance of carbonyl groups, normally using spectrophotometric techniques. Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDS-PAGE; and immunoblotting are also usually performed (57, 58) to study protein degradation (proteolysis) or protein oxidation.

Classically, the food safety procedures for determination of essential amino acids content of mechanically processed products from red meat animals and poultry is based on hydrolysis of a powder prepared by blending samples in acetone-chloroform. Samples are injected into a HPLC using gradient elution on an ion-exchange column for separation and with fluorescence detection. A variant of the elution program allows also the determination of tryptophan. In addition, β-alanine, 1-methyl-histidine, and 3-methyl-histidine from beef, pork and poultry products are determined to estimate muscle content of products. A colorimetric procedure for assay of hydroxyproline is also used as adjunct method for protein quality estimation (59). Cysteine as cysteic acid and methionine as methionine sulfone are also

determined with similar conditions (60). Currently, new methodologies provided by commercial companies allow determining a complete amino acids profile. The most used are named PicoTag (61) and more recent AcqTag (62) (Waters, Milford MA, USA).

On one hand, the classic methods for determination of protein by determination of nitrogen use Kjeldahl analysis. As example milk casein measurement is based on precipitation of casein at acid pH. Precipitated milk casein is removed by filtration and the nitrogen content of either the precipitate or filtrate is determined by Kjeldahl analysis (63). Furthermore, others methods have been devised to measure protein concentration based on UV-vis spectroscopy and HPLC (64). Few research groups have used HPLC coupled to mass spectrometer of high resolution such as time of flight; TOF (65).

In the case of carbohydrate, an important number of technologies and methodologies are also found in the literature to control its stability. The Maillard reaction, that generates various compounds with high toxicity, is well known. The study of those compounds is of a great interest. A typical compound to control is acrylamide. It has been demonstrated that acrylamide have neurotoxic and carcinogenic effects. It is widely recognized that acrylamide is mainly formed through the Maillard reaction from free asparagine and reducing sugars. The major sources of dietary acrylamide are potato products, processed cereals and coffee. This compound can be determined by LC-MS/MS after clean-up with solid phase extraction; SPE (66), by GC equipped with a nitrogen-phosphorus detector; NPD; with headspace solid-phase microextraction; SPME (67) and also by GC-MS/MS (68). Acrylamide is not the only compound generated during Maillard reaction. In the last years, others carboxylic acid and amides have been found and measured by HPLC-MS, GC-MS, GC coupled to flame ionization detector; FID; or enzymatically (69).

Additionally, furosine is a marker of the impairment of lysine residues in protein which is generated at the early stage of the Maillard reaction in thermally treated foods. This compound has been determined by HPLC (70).

Highlight gas chromatography-orthogonal acceleration time-of-flight mass spectrometry; GC-oaTOF; is an emerging technique used for quantifying furanones generated in model Maillard reactions (71). Finally, some studies based on the use of HPLC-TOF are recently appearing with remarkable results (72-74).

Conclusions

Food degradation may be controlled in many different ways, from controlling changes in the initial steps with the formation of primary oxidation products, to observe and study the last oxidation stages by measurement of secondary compounds. Sensory analysis, widely used, is able to evaluate the degradation mainly from a subjective point of view. To carry out quantitative evaluations and to control oxidative changes in foods, there are, in the scientific literature, a great amount of more objective methods. Each method shows both advantages and disadvantages, thus it is important to select the most adequate method, depending on the system under investigation and the state of oxidation itself. The use of two or more methods to determine different parameters is highly recommended. The information regarding to evaluate the food degradation in a global way with techniques of total screening using mass spectrometry of high resolution such as TOF is very limited. It is necessary to use these new techniques to expand the global knowledge about those degradation processes.

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IV.4 STUDY OF NOVEL AND CLASSICAL MARKERS IN NUTRITIONAL PRODUCT BY TIME-OF-FLIGHT MASS SPECTROMETRY (Publicación IX)

En este apartado se presenta el trabajo enviado para su publicación a la revista internacional **Food Analytical Methods** de la editorial Springer (Figura IV.2). Enviado 23/04/2015.

Food Analytical Methods

Study of novel and classical oxidation markers in nutritional products by time-of-flight mass spectrometry

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Figura IV.2. Study of novel and classical oxidation markers in nutritional products by time-of-flight mass spectrometry. *Revision Draft of Food Analytical Methods (Factor de Impacto: 1.802)*

Study of novel and classical oxidation markers in nutritional products by time-of-flight mass spectrometry

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ABSTRACT

The aims of this work were first to find new oxidation markers and relate them with the classical parameters used to control the oxidation; and second to find a new method of accelerated oxidation in a nutritional product for its quality control. In order to achieve these objectives, a study regarding the stability of the lipid and hydrosoluble fraction was developed in an infant formula being exposed to specific aging conditions. Ultra-high performance liquid chromatography coupled to electrospray ionization-time of flight mass spectrometry (UHPLC-ESI-TOF) was used for the elucidation of oxidation markers. Analytes were separated in an Acquity UPLC® BEH C18 column using water-methanol gradient in a 26 min run time. Classical methodologies for stability testing were also performed, such as peroxide value, liposoluble and hydrosoluble vitamins quantification and sensory analysis. Data were evaluated by Principal Component Analysis (PCA) to identify the most relevant compounds in our study which could be related to the aging process and could be used as aging markers.

Keywords:

Oxidation markers; Stability; Shelf life; Infant formula; accelerated aging process; UHPLC-MS-TOF; PCA

1. Introduction

Infant formulas (Ifs) are designed to fulfill the nutritional needs of infants and are considered the best alternative to breast milk during breast-feeding when it is impossible or insufficient. Feeding during the early stages of life is essential to ensure proper infant growth and prevent diseases. Optimal nutrition is vital due to the effects on the physical, mental, emotional and psychomotor development.

Conventional formulas are based on a mix of fatty acids of similar composition to those of human milk, proteins and specific carbohydrates that could be affected during the technologic process performed in the manufacture of them.

In fact, their composition can be modified during thermal processing treatments, as well as during storage, since the oxidizable fatty acids together with pro-oxidant minerals could increase the oxidative activity. Hence, it is essential to control lipid stability during storage to ensure their nutritional value and safety (Nasirpour, Scher, & Desobry, 2006). These changes depend on raw materials, formula composition, manufacturing process, and packaging and storage conditions. The food industry has made several attempts to improve the quality and the nutrient content of milk-base products, controlling the stability of oxidation by the addition of antioxidants. Reactions which can take place during storage include the denaturation of proteins, isomerization and crystallization of lactose, non-enzymatic browning reactions, lipid oxidation and interactions between micronutrients and other components. Those modifications may change the nutritional, physicochemical and sensory properties, and therefore may affect the shelf-life of the products (Pereyra Gonzáles, Naranjo, Malec, & Vigo, 2003).

Lipid oxidation is well recognized as a major cause of quality deterioration during processing or storage of lipid rich foods such as IFs (St. Angelo, Vercellotti, Jacks, & Legendre, 1996). Three different mechanisms of fatty acid

oxidation yielding different products have been described: autoxidation, photo-oxidation and lipooxygenase action. Lipid oxidation includes the oxidation of unsaturated fatty acids, particularly LCPUFAs, generating compounds which affect food quality due to changes in color, flavor, texture and even nutritional value and food safety. Molecular oxygen, UV light, transition metal ions and enzymes are commonly known as oxidation initiators. During the peroxidation of unsaturated fatty acids, hydroperoxides (ROOH) are formed; these primary products in turn rapidly are degraded, producing a complex mixture of secondary lipid oxidation products such as hexanal, 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) (Porter, Caldwell, & Mills, 1995). In addition, the rancidity is often a decisive factor for determining the shelf-life of food products through the development of off-flavours, which are caused by the formation of secondary reaction products.

Other compounds susceptible to oxidation are sterols, cholesterol oxidation products (COP) and the phytosterols (POP), which together are called sterols oxidation products (SOP) (Angulo, Romera, Ramirez, & Gil, 1997; Calvo, Ramos, & Fontecha, 2003). On the other hand, products of lipid oxidation react with non-lipid food components as proteins causing a loss of essential amino acids (François Fenaille, Parisod, Visani, Populaire, Tabet, & Guy, 2006).

The importance of measuring oxidation markers in IFs lies, not only on the impairment of nutritional properties, but also on the undesirable health effects produced by some degradation products during the aging process, which can generate health diseases. In recent years the relationship between intakes of oxidized fat with particular diseases has been studied in several publications and reviews (Esterbauer, 1993), finding variable results and some controversy depending on the experimental design. It is known that the generation of free radicals *in vivo* has an implication with certain pathologies such as atherosclerosis, rheumatoid arthritis, retinal degeneration, tumor promotion and aging (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003; Rice-Evans & Burdon, 1993). The direct relation between the intake of oxidized lipids and the mentioned diseases has been demonstrated in some recent works

(Grootveld, Silwood, Addis, Claxson, Serra & Viana, 2006; Jacobson, Price, Shamoo and Heald, 1985; Stringer, Gorog, Freeman and Kakkar, 1989).

Lipid oxidation is classically measured by monitoring secondary lipid oxidation products by different chromatographic methods such as MDA determination (Burg, Silberstein, Yardeni, Tavor, Blumenfeld, Zilbermann, et al., 2010; Cesa, 2004; François Fenaille, Mottier, Turesky, Ali, & Guy, 2001); saturated volatile aldehydes (Giammarioli, Bellomonte, Denaro, & Milana, 1995; Romeu-Nadal, Castellote, & López-Sabater, 2004); or thiobarbituric acid (TBA) assay (Turoli, Testolin, Zanini, & Bellu, 2004). Traditionally, peroxide value (POV) is used to determine primary lipid oxidation products (Velasco, Marmesat, Holgado, Márquez-Ruiz, & Dobarganes, 2008).

In a few studies based on the stability of the lipid fraction in milk-based Ifs, changes in lipid oxidation parameters during their storage have been reported, showing fluctuations or inconsistencies in oxidation measurements (Manglano, Lagarda, Silvestre, Vidal, Clemente, & Farré, 2005; Rodríguez-Alcalá, García-Martínez, Cachón, Marmesat, Alonso, Márquez-Ruiz, et al., 2007). Analysis of lipid oxidation products in foods is difficult due to several factors such as complex matrix, interfering substances in foods, complicated lipid extraction, the own complexity of the oxidation process and sometimes the lack of specificity of the analytical methods.

The Maillard reaction (MR) has been studied in great detail due to its role in the modification of food properties and stability during processing and storage. MR is also responsible for the decreasing protein digestibility (Van Boekel, 1998). The MR is a type of non-enzymatic browning reaction which comprises of a cascade of chemical reactions between a carbonyl compound and an amino compound. Several compounds are generated such as the ones called Amadori rearrangement products, the advanced glycation end products, furfurals, melanoidins, which are nitrogen-containing macromolecular materials, still little characterized (Martins, Jongen, & Van Boekel, 2000). Mutagenic and antimutagenic properties have been described for compounds formed during the MR (Oliver, Melton, & Stanley, 2006). Despite its complexity,

several methods have been described to determine the progression of MR. Some of these are related to the early stage by means of furosine determination using high performance liquid chromatography (HPLC) with different detection techniques, also measuring milk protein glycation by mass spectrometry (MS) techniques, or losses of essential amino acids by means of the identification of blocked lysine (Ferrer, Alegría, Farré, Abellán, & Romero, 2005). Other studies have been conducted on the effect of thermal treatment by determination of furfural derivates such as hydroxymethylfurfural (HMF) (Albalá-Hurtado, Veciana-Nogués, Izquierdo-Pulido, & Vidal-Carou, 1997). A classical procedure to determine furfural compounds is the spectrophotometric TBA method, although a lack of specificity was noted due to interferences with other furfurals and carbonyl compounds. Ne-(carboxymethyl)lysine (CML) is another parameter which has been related to advanced MR steps (Assar, Moloney, Lima, Magee, & Ames, 2009).

Furfural evolution during IFs storage gives a wide range of values ascribable to differences in the quality of the raw material, the heating treatments applied during processing, and the storage time and temperature. However, the reported results are only isolated measures which do not allow follow-up of the changes throughout product storage or shelf-life (Chávez-Servín, Castellote, & López-Sabater, 2006; Guerra-Hernández, León-Gomez, Garcáa-Villanova, Corzo-Sánchez, & Romera Gómez, 2002).

From a general point of view, thermal treatment improves food safety, sensory qualities and shelf-life of dairy products. However, heat treatment, light or oxygen exposure may adversely affect the quality of the product, safety and nutritional value by means of oxidative damage of lipids, carbohydrates and proteins. Several chemical markers are commonly used for assessing these modifications. Measurement of flavour by sensory methods, which determine the degree of consumer acceptability, together with chemical indices and separation techniques based on the analysis of specific compounds by liquid chromatography (LC) or gas chromatography (GC) are useful to define the degree of oxidation.

The aim of this study was to contribute to the knowledge about the degradation reaction by an accelerated aging process of a powdered IF through the study of oxidation markers by high resolution mass spectrometry in combination with conventional methods. This work aims to provide a preliminary vision to serve as a basis for the development of an analytical method of oxidation markers considered of interest to the food industry.

2. Experimental

2.1. *Chemicals and reagents*

All solvents and chemicals used were of LC–MS grade and obtained from Scharlab (Barcelona, Spain) unless otherwise stated. Water (18.2 MΩ cm) was purified and filtered by a specific LC–MS filter using a Milli-Q system from Millipore (Bedford, MA, USA). Certified Reference Materials (CRM) of vitamin A palmitate, α-tocopherol acetate, α-tocopherol, and L-(+)-ascorbic acid and USP Reference Standards of pyridoxine hydrochloride, riboflavin and thiamine hydrochloride were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions for different analysis were prepared according to internal Standard Operation Procedures of Abbott Laboratories S.A., (Chicago, IL, USA).

2.2. *Apparatus and software*

A chamber SOLARBOX 300e HR from Neurtek Instrument (Eibar, Spain) was used to accelerate the aging process. The HPLC system used for determination of fat-soluble vitamins consisted of a model 515 isocratic pump, a model 2707 injector, a 2489 UV/visible detector, an online degasser and Controller Pump Control Module II. The system used a cleaning column Chromegasphere Si60 3 cm × 3.0 mm and as analytical column was employed the Chromegasphere Si60 15 cm × 3.0 mm. HPLC equipment consisting of a 717 plus autosampler injector, a MS 600 pump, a second post-column Reagent Manager pump, and a 2475 Multi Fluorescence detector, was utilized for the determination of group-B hydrosoluble vitamins. All this equipment was from Waters (Manchester, UK). The equipment employed to determinate vitamin C

was an automatic Compact Titrator II from Crison Instrument (Barcelona, Spain) equipped with a platinum combination electrode and a magnetic stirrer. Analysis of unknown analytes was performed using a Waters UPLC® Acquity system equipped with a binary pump, vacuum membrane degasser, a thermostated column compartment and an automatic injector. The chromatograph was coupled to a time-of-flight mass spectrometric detector (TOF) model LCT Premier XE with an electrospray ionization (ESI) interface also from Waters. An Acquity UPLC BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm particle size) and Acquity UPLC BEH Amide 130 Å (1 mm × 100 mm i.d., 1.7 µm particle size) from Waters were tested as chromatographic columns. Empower 2.0 software was used for data acquisition for the determination of vitamins and MassLynx version 4.1 together with UNIFI version 1.7 from Waters were used for LC-TOF analyses. Minitab statistical software 17 (Pennsylvania, USA) was used for statistical analysis.

2.3. *Accelerated aging process*

Two batches of infant milk were homogenized and equally distributed into 16 sterile 50 ml bottles. One bottle was stored as control (free of aging process) and the rest were subjected to an accelerated aging process (Sample 1 to 14) using a chamber SOLARBOX 300e HR. The aging chamber was programmed to 55 °C, 60% humidity and UV light, based on the parameters established by Labuza (Labuza, 1971). Samples remained there for two weeks taking out one every 24 hours, with the exception of the first sample which was collected at 12 hours (0, 12, 36, 60, 84, 108, 132, 156, 180, 204, 228, 252, 276, 300, and 324 hours). Subsequently, samples were stored at -32 °C in inert atmosphere together with the control until analysis. The same aging process was applied to two samples of an oil mixture used as ingredient of infant formula (Oil 1 and 2) with their respective control for complementing the experiments. Oil samples were taken from the aging chamber at 7 and 14 days. The sample names were: Control, Sample 1-14, Oil Control, Oil Sample 7 and Oil Sample 14. Two determinations (n=2) for each sample were carried out.

2.4. *Peroxide value*

A method based on IDF Standard 74A (International Dairy Federation IDF, 1991) was applied to determine the degree of fat oxidation, consisting on a liquid-liquid extraction (LLE) of 1 g of product reconstituted in 100 mL of water with a 2:1 (v/v) mixture of isooctane:methanol. The peroxide value was obtained by measuring the oxidation of ferrous iron (Fe^{2+}) to ferric (Fe^{3+}) spectrophotometrically.

2.5. *Liposoluble vitamins*

The determination of vitamins A and E was performed by HPLC based on the method from the AOAC 2001.13 (Association of Analytical Communities, 2001) where all-trans and 13-cis vitamin A was determined as retinol palmitate and vitamin E as α-tocopherol acetate and α-tocopherol. Calibration standards were freshly prepared from the original stock solution in each experiment. The lipid extraction was carried out per POV determination.

2.6. *Hydrosoluble vitamins*

The measurement of thiamine (vitamin B1), riboflavin (vitamin B2) and pyridoxine (vitamin B6) was carried out by an isocratic HPLC method in reverse mode chromatography and using the fluorescence detection system for quantitation. The HPLC method for the determination of the three vitamins was based on the European Norms: EN 14122, EN 14152 and EN 14164, respectively (UNE-EN 14152:2003, 2003; UNE-EN 14122:2003, 2003; UNE-EN 14164:2008, 2008). Work standard solutions for calibration purposes were daily prepared to obtain the appropiate calibration curves.

Vitamin C determination as ascorbic acid was performed by potentiometric titration with indophenol (2,6 dichloroindophenol) , from an acidic solution of the reconstituyed powder sample or diluted oil sample. Ascorbic acid is oxidized to dehydroascorbic acid using indophenol solution after

deproteinization of the sample. Method is based on the Official Method AOAC, 985.33 (Association of Analytical Communities, 1990).

2.7. *Sensory analysis*

A sensory analysis was conducted for the stability study by an expert panel. This test can mainly be classified into three categories: objective testing, based on real facts about products; subjective testing, about observations or appreciations like preferences of the analyst; and perception, encompassing biochemical and psychological aspects of sensation. Statistical techniques are recommended for tasting results obtained by correlating the different possible viewpoints on the products subject to experiment (Santos-Fandila, Camino-Sánchez, & Zafra-Gómez, 2014). Different parameters are studied depending on the product. In our case, parameters such as the intensity of the aroma, appearance, color, taste, texture and acceptability were evaluated. The expert panel was composed of six people who were trained to recognize these parameters in both control and oxidized samples. Powdered milk samples were reconstituted with water as recommended by the manufacturer in each product. In the studied product: 15 g of product were reconstituted in 100 mL of natural water at 40°C in opaque plastic cups. After 30 min to get a tempered product, parameters such as optimum state, moderate oxidation or paint flavor among others (described in Annex I from supplementary material) were essentially evaluated. Each assessed parameter is encoded with "Yes" or "No", to be introduced in the statistical study of PCA as a score of 1 or 2.

2.8. *UHPLC-TOF analysis*

2.8.1. Sample treatment

Water-soluble fraction: 5.0 g of powder were reconstituted into 50 mL of deionized water. Then, an aliquot (500 µL) was taken into a 50 mL flask and water was added up to the final volume. 1 mL of this solution was prior filtered with a 0.2 µm nylon filter and was added to a maximum recovery LC topaz vials for subsequent analysis in the UHPLC-TOF system.

Lipid soluble fraction: was obtained from POV determination, prepared by LLE with isooctane:methanol (2:1, v/v) using the IDF Standard 74A:1991 (International Dairy Federation, 1991). An aliquot of 1 mL was evaporated in a nitrogen stream and reconstituted with 1 mL of methanol to inject into UHPLC-TOF system. For oil, 0.2 g of the lipid mixture of the IF were weighed and 10 mL ethanol were added. After homogenizing, an aliquot (1 mL) was placed into a chromatographic vial to be injected into the UHPLC-TOF system.

2.8.2. Liquid chromatographic and mass spectrometric conditions

Chromatographic analysis was performed using a UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size) from Waters. Components of the sample were separated using a gradient consisting of water as solvent A and methanol as solvent B. Gradient conditions, at a 0.4 mL min^{-1} flow rate, were: 0.0-2.5 min, 95% to 5% B; 2.5-16 min, 95% to 5% B; 16-19 min, 65% to 35% B; 19-22 min, 5% to 95% B; 22-22.5 min, 95% to 5% B; and maintaining 5% B until 26 min. Total run time was 26 min. An injection volume of 5 µL (in partial loop mode) was used. The column temperature was maintained at 25°C, and the sample temperature at 8°C. Weak solvent was a mixture of water: methanol (95:5, v/v) and strong solvent was water: methanol (1:1, v/v). These solutions were stable for at least a week at room temperature.

Regarding MS conditions, ESI was performed in positive ion mode. TOF detector covered a scan range of 100–1000 Da. The equipment was previously calibrated for this mass range. The measurement outside this range would involve loss of sensitivity and lack of accuracy. Electrospray ionization spray voltage was 3 kV and the cone voltage was 50 V. Nitrogen was used as a desolvation gas at 800 L h^{-1} and as auxiliary gas in the cone at 40 L h^{-1}. Nebulizer pressure was set at 2 bars. The temperature of the source was 120 °C and the desolvation temperature was 200 °C. Other instrumental parameters were: skimmer 1 and 2 at 50 and 30 V, respectively; hexapole RF at 30 V; and transfer time of 0.1 s. Compounds were identified using their exact mass with a mass

accuracy of ±0.001 Da, and by comparing data with those published in scientific literature.

MassLynx and UNIFI software from Waters were used to locate and identify the compounds.

2.9. *Statistical analysis*

In order to reduce the number of response variables, the Principal Component Analysis (PCA) was performed. PCA is used as exploratory data analysis and involves a mathematical procedure that transforms a number of (potentially) correlated variables into a (smaller) number of uncorrelated variables called principal components (Shrestha and Kazama, 2007). The main objective of PCA is to reduce the dimensionality of the data set and to identify new meaningful underlying variables. The purpose of PCA is to recognize simple patterns of correlation between variables. In particular, to recognize if the observed variables can be explained largely or entirely in terms of a much smaller number of variables called Principal Components (PC) or factors.

To carry out the statistical analysis, several variables were taken into account. Content of water-soluble and fat-soluble vitamins, sensory analysis results and ions extracted with UNIFI and Masslynx software from TOF screening data were selected as experimental variables.

Data obtained by means of LC-TOF was evaluated with the UNIFI software, extracting the ions which showed differences among samples and may be correlated with the aging process. B-group vitamins were excluded from PCA, they did not show a clear variation along the aging process; moreover, in the preliminary PCA analysis they did not had significant contribution to any PC. PCA compute a large number of PC, and not all of them are significant. The criteria for retaining the PC were the eigenvalue and the variability that each PC explained. Only those PCs with and eigenvalue higher than 1 and an explained variability higher than 5% were retained as significant.

3. Results and discussion

Results of the vitamins at different oxidation states are detailed in table 1 and POV value in Figure 1.

Table 1
Concentration of fat-soluble and water-soluble vitamins and Peroxide value in the aged samples and controls

Sample	Vitamin A (IU 100 g^{-1})	Vitamin E acetate (IU 100 g^{-1})	Vitamin E total (IU 100 g^{-1})	Vitamin C (mg 100 g^{-1})
Control	3264.5	17.60	38.55	130.55
Sample 1	2195.0	15.55	32.80	122.25
Sample 2	2123.5	16.00	32.25	134.50
Sample 3	1990.0	16.35	31.15	129.10
Sample 4	1700.5	16.00	28.40	117.50
Sample 5	1526.0	14.85	25.30	123.35
Sample 6	1538.0	15.00	25.55	113.70
Sample 7	1487.0	14.65	24.90	117.25
Sample 8	1391.5	11.50	22.90	119.70
Sample 9	1286.0	12.10	22.75	111.35
Sample 10	1452.0	12.05	24.25	107.20
Sample 11	1408.5	12.10	23.90	104.40
Sample 12	1333.0	11.35	22.20	105.75
Sample 13	1448.5	11.55	22.75	120.75
Sample 14	1133.0	10.90	18.95	116.05
Oil control	6266.0	31.35	65.60	ND
Oil Sample 1	0.00	37.80	37.80	ND
Oil Sample 2	0.00	13.40	13.40	ND

ND: Not detected

Table 1 continuation
Concentration of fat-soluble and water-soluble vitamins and Peroxide value in the aged samples and controls

Sample	Vitamin B6 (µg 100 g^{-1})	Vitamin B2 (µg 100 g^{-1})	Vitamin B1 (µg 100 g^{-1})
Control	339.5	917.5	655.5
Sample 1	458.5	995.5	665.0
Sample 2	509.0	928.0	644.0
Sample 3	573.5	894.0	637.0
Sample 4	431.5	896.5	642.0
Sample 5	533.0	854.5	625.0
Sample 6	573.0	870.5	611.5
Sample 7	459.0	1004.5	622.5
Sample 8	303.0	914.5	638.0
Sample 9	436.0	912.0	653.5
Sample 10	361.0	891.5	620.5
Sample 11	318.5	925.5	651.0
Sample 12	423.5	849.5	592.0
Sample 13	789.5	978.5	686.0
Sample 14	502.5	864.0	601.0
Oil control	ND	ND	ND
Oil Sample 1	ND	ND	ND
Oil Sample 2	ND	ND	ND

ND: Not detected

3.1. *Peroxide value*

In view of the results presented in the Figure 1, samples experienced a significant increase in the content of peroxides in the lipid fraction with respect to control, as well as in the oil mixture. The peroxides formed are mainly hydroperoxides which are stable for weeks, or even months, at room temperature; a period in which there are slight variations in the content of peroxides. After overcoming this stage, POV increases rapidly until there comes a point at which the rate of peroxide decomposition relative to its formation increases considerably.

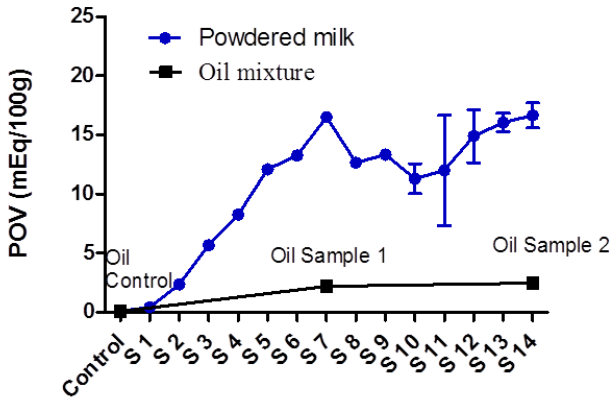

Fig. 1. *Peroxide value in the samples during the aging process*

3.2. *Fat-soluble vitamins*

Fat-soluble vitamins were gradually degraded as the oxidation process continued. Vitamin A (Table 1) in the product was reduced to almost 50% of its initial content at the end of the oxidation study. In the oil mixture, vitamin A was not detected in any of the oxidized samples, being its degradation 100% in the sample aged during 7 and 14 days. In the case of vitamin E, a greater degradation of α-tocopherol (62%) was observed with respect to α-tocopherol acetate (41%), as it is also shown in Table 1. Despite of that, vitamin content after 14 days of oxidation was still within the required specifications for an IF. A similar behavior was observed in the oil samples for Vitamin E. On day 7 of the study, all the content of α-tocopherol disappeared while α-tocopherol acetate remained constant. At the end of the study, α-tocopherol was reduced to 40% of its initial content. These results are also summarized in Table 1.

3.3. *Water-soluble vitamins*

Hydrosoluble vitamins of the oxidized samples fluctuated significantly in comparison to control. As it is shown in Table 1, slightly higher levels of vitamins were obtained at some points during the experiment. These differences could be attributed to product heterogeneity and method variability, which was acceptable since the difference in the peak response was less than 4% between

replicates. Fat-soluble vitamins are initially added into the solution during the manufacturing of the IF, then the IF is dried and subsequently B-vitamins are added in a dry-blending process. Therefore, concentration of B-vitamins may show a higher variability within the samples than the rest of vitamins.

3.4. *Sensory analysis*

According to the results of the tasting analysis, the product would be unsuitable for consumption past 48 hours, since from the second sample the product was rejected as non-compliant tasting. Sensory analysis results are included in supplementary material.

3.5. *Liquid chromatographic and mass spectrometry analysis*

Two liquid chromatography columns were tested in order to achieve a good separation of peaks: an Acquity UPLC BEH C18 and an Acquity UPLC BEH Amide. The BEH C18 column provided the best resolution of the peaks. Consequently, this column was selected for further experiments. Mobile phases composed of 100 % water as solvent A and 100% (v/v) as solvent B were applied. Two gradients were studied, one shorter against other longer (15 minutes and 26 minutes). The long gradient described previously was used for obtaining better results in terms of quality and amount of information.

The detection method by TOF mass spectrometry in full scan mode was also optimized based on the methodology for the identification of unknown compounds in foodstuffs operating in ESI positive mode. After acquiring the data, the chromatograms were studied to determine differences and trends by comparing samples and controls. The identification of compounds from TOF mass spectrometer was performed by the information available in scientific literature and the database SciFinder® (Washington, DC, EE.UU.). In some cases, it was not possible to assign the exact identity of a measured ion; therefore molecular weight was used as unknown compound for analysis in PCA. Variations in area of each compound in different samples show the behavior of the substance in the developed experiments. One limitation for this

procedure is that TOF mass analyzer can be used only for the calibrated mass range which was calibrated (100 – 1000 m/z). In addition, it is advisable to prepare work solutions, standards and processed samples freshly for each experiment in order to prevent other oxidation process.

3.6. *PCA Analysis*

The statistical model obtained by PCA with 3 principal components explained 79.3% of the total variability. The eigenvalue of the principal components PC1, PC2 and PC3 were 33.5, 10.9 and 4.8 respectively. The variability explained for each PC was 54.0%, 17.6% and 7.7%, respectively. The rest of the calculated PCs were not retained.

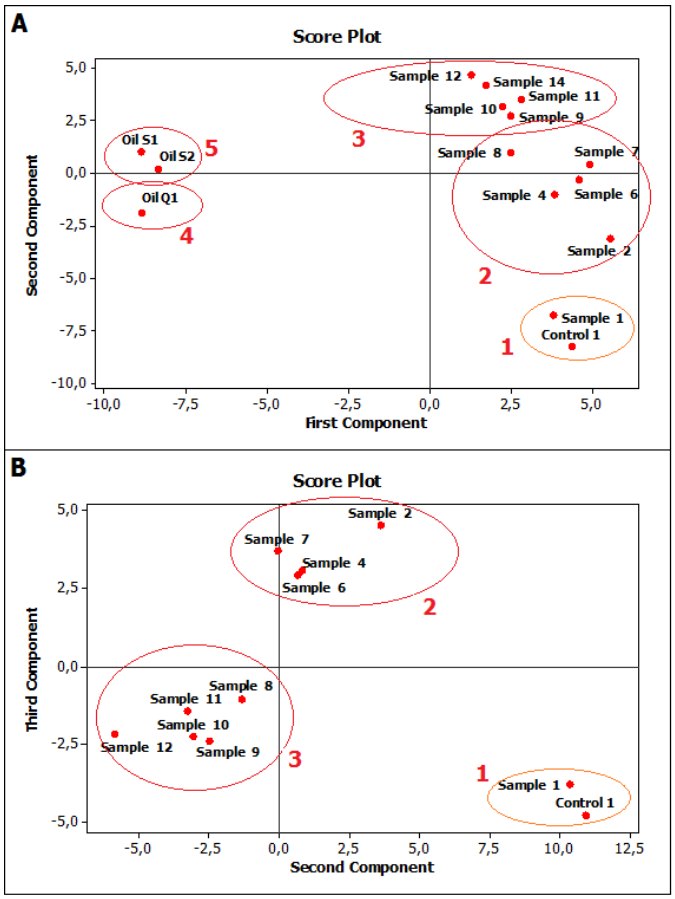

Fig. 2 *Score plot of Principal Component 1 and 2 (A). Score plot of Principal Component 2 and 3 (B)*

Figure 2A shows the score plots of each sample in the model. Several groups of samples can be clearly identified in the graph. Oil samples are located far on the left part of the graph, over the axis correspondent to PC-1; on the other hand, all product samples were located on the right of the graph. Only a slightly variation over the horizontal axis is observed in the position of the samples according to their aging. This indicates that PC-1 is related to the differences between the oil samples and product samples; therefore, PC-1 did not explain aging processes. PC-2 is projected over vertical axis. For this Component, a clear differentiation among samples is observed in the graph. Samples were located upward along the vertical axis the longer they were aged.

Table 2
Correlation matrix of the Principal Components Analysis

Component 1		Component 2		Component 3	
Eigenvalue	33.5	Eigenvalue	10.9	Eigenvalue	4.8
Proportion	54.00%	Proportion	17.60%	Proportion	7.70%
Cumulative	54.00%	Cumulative	71.60%	Cumulative	79.30%
Variable	Loading values	Variable	Loading values	Variable	Loading values
338.339 o.p	0.168	Refused tasting	0.257	411.351 o.p	-0.247
279.160 o.p	0.167	Refused flavor	0.257	157.222 o.p	-0.188
355.287 o.p	0.166	383.308 o.p	0.215	282.275 o.p	-0.185
177.124 a.p	0.164	Peroxide value	0.210	401.637 o.p	-0.169
282.275 o.p	0.164	282.299 a.p	0.151	350.335 o.p	-0.162
288.276 o.p	0.162	340.276 a.p	0.141	453.345 a.p	0.184
205.223 a.p	0.160	360.319 o.p	-0.132	475.325 a.p	0.230
Vitamin C	0.156	Vitamin A	-0.143	387.246 a.p	0.243
387.246 a.p	0.152	379.426 o.p	-0.150	227.232 a.p	0.257
299.225 o.p	0.151	257.207 o.p	-0.179	546.358 a.p	0.264
360.319 o.p	0.149	Vitamin E. total	-0.209		
340.276 a.p	0.142	350.335 o.p	-0.218		
379.426 a.p	0.132	284.240 o.p	-0.235		
309.328 a.p	0.128	309.328 o.p	-0.238		
257.207 o.p	0.119				
475.325 a.p	0.116				
Peroxide value	0.106				
227.232 a.p	0.105				
Vitamin E. acetate	-0.127				
244.254 o.p	-0.142				
288.296 o.p	-0.166				
316.318 o.p	-0.169				

a.p: aqueous phase; o.p: organic phase

Table 2 summarizes the loading values of the variables with a significant contribution to each of the three retained Components. Only these variables with a loading value higher than 50% of the absolute value of the highest loading value were considered as significant within the component. Each loading value expresses the weight of the variable in the correspondent PC.

Variables with a different sign within the same PC indicate that they have an opposite effect over this variable. Masses in the table correspond to the positive adduct of the molecule as were obtained in the TOF mass spectrometer.

Regarding PC-1, those variables with negative weight are projected over the left side of the x-axis; therefore they are characteristic of oils: high content of Vitamin E and ions 244.254, 288.296 and 316.318. The rest of variables (ions) described the powder products. PC-2 shows a strong correlation among negative attributes of tasting, peroxide value and ion 383.308; being these two variables mainly responsible of bad organoleptic properties. Both variables are also positively correlated with other two ions whose amount increased in the aged samples (282.299 and 340.276). These variables are oriented in the same direction than the aged samples, thus indicating that aged samples have higher content of them. On the other hand, the content of the vitamins (variables) with negative loading values within PC-2 was decreasing in the aged samples. This is the case of Vitamin A and E, which have a negative loading value and therefore they were degraded during the aging process. Apart from these two vitamins, whose oxidation behavior is well established, concentration of other six compounds significantly decreased during the aging process. PC-2 was present in both the finish product and oil samples, being related with fat oxidation, since, in both, the variable peroxide values were increased along experiments. Fat-soluble vitamins have a negative contribution in the Component, therefore, their concentrations decrease during the aging process while peroxides and undesirable tasting appears. Variable Vitamin E acetate was not present in this component because it was not degraded significantly. This fact is due to the degradation of α-Tocopherol which contributes to the variable Vitamin E (total). In addition to fat-soluble vitamins, other six ions with negative contribution were recognized, thus having a similar behavior of these vitamins.

PC-3 is also related with the chemical and physical differences in the product due to the aging process.

Figure 2B shows the Score Plot for PC-2 and PC-3. Samples are now divided into three groups: 1) Control sample and Sample 1; 2) Samples 2 to 7; and 3) Samples 8 to 14.

Ions 411.351, 282.275 and 350.335 are the main characteristic of group 1. Information regarding the identity of these ions for any of the possible adducts (H^+, Na^+, NH_4^+, Li^+) could not be found. Group 2 is described due to an increase in the intensity of the following ions: on one hand 157.222 identified as 4-Hydroxynonenal and 401.637 as 7-Ketocholesterol from organic phase; and on the other hand, the ions 387.246, 453.345, 475.325 and 546.358, and the identified ions 227.232 as histidinalanine and 379.426 as pentosidine, all those last in the aqueous phase. Then, the intensity of these ions decrease after sample 7 as the aging process continues. It should be highlighted that these ions were detected mainly in the aqueous extract of the samples; therefore they have a polar nature unlike those ions in group 1 which were present in the organic extract. In addition, in Group 1 as organic extract the ion 379.426 or Pentosidine and 284.240 identified as 8-hidroxydeoxyguanosine (8-OH-dG) had no influence on the oxidation process along the time. Group 3 contained the most oxidized samples, being this group also described by several ions in the aqueous phase such as 282.299, 340.276 and 309.328 which was identified as Fructosyllysine, and furthermore 383.308 in the organic phase. It is remarkable that this last group was not identified by differences in the content of the vitamins in respect to the others.

According to the PCA evaluation, aged samples can be grouped in three main groups. Significant differences after 24 hours at high temperature and exposure to UV light were evidenced. Ions which describe each of the three groups, for both PC-2 and PC-3, are summarized in table 3.

Table 3
Main ions for the description of the aging model. Ions in the columns are characteristic of each group of samples.

Not aged (m/z)	**Middle aged (m/z)**	**Highly aged (m/z)**
360.319 o.p	157.222 o.p	282.299 a.p
379.426 o.p	227.232 a.p	309.328 a.p
257.207 o.p	379.426 a.p	340.276 a.p
350.335 o.p	387.246 a.p	383.308 o.p
284.240 o.p	401.637 o.p	
309.328 o.p	453.345 a.p	
411.351 o.p	475.325 a.p	
282.275 o.p	546.358 a.p	
350.335 o.p		

a.p: aqueous phase; o.p: organic phase

4. Conclusions

Some reactions are produced during the storage of foodstuffs and nutritional products, modifying their physicochemical and nutritional properties and affecting their quality.

With this multidisciplinary study we achieved to describe the oxidation process and separating different samples and degrees of oxidation. The process of accelerated oxidation could be useful as a predictive model to control the expiry date of the nutritional product. The time saving in comparison with standard methods which require months to oxidize different products is remarkable. Future guidelines may be the following: using an instrument of mass spectrometry with higher accuracy as LC-Q-TOF; compare different types of products to improve and have more control on the expiry date; the implementation of a deconvolution software and libraries would be recommended to allow the identification of the compounds through their exact mass and optionally isotope profile assess; and validate the selected markers of oxidation for each nutritional product to study.

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Compliance with Ethics Requirements

Authors state that they have written an entirely original work, and if the authors have used the work and/or words of others, they have been appropriately cited or quoted**.** Authorship has been limited to those who have made a significant contribution to the conception, design, execution, or interpretation of the reported study. There is not any conflict of interest that might be construed to influence the results or interpretation of their manuscript. This article does not contain any studies with human or animal subjects.

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IV.5 DETERMINATION OF BIOMARKERS RELATED TO AGING IN BIOLOGICAL TISSUES FROM RAT BY UHPLC – PDA – MS/MS (Publicación/Patente X)

The main objective of this work was to develop and validate a multicompound method to measure biomarkers related to aging named reactive oxidative species (ROS) in biological tissues and fluids such as liver, brain and serum.

An ultra high performance liquid chromatography–tandem with photodiode array and mass spectrometry (UHPLC–PDA–MS/MS) method for the identification and quantification of the following ROS malondialdehyde (MDA), orto-Tyrosine (o-Tyr) and 8-oxo-deoxiguanosine (8-OHdG) in liver, brain and serum samples from rats was set up. These analytes were separated in an Acquity UPLC BEH C18 column using methanol–water gradient with formic acid as additive, in a 3 min run, and were detected and quantified using PDA and a triple quadrupole (QqQ) mass spectrometer. The mass spectrometric conditions were individually optimized for each analyte to obtain maximum sensitivity in the Multiple Reaction Monitoring (MRM) mode. Selection of two specific fragmentation reactions for each compound allowed simultaneous quantification and identification in one run, ensuring a high specificity of the method. The limits of detection (LODs) ranged from 5 to 70 ng mL^{-1} and the limits of quantification (LOQs) from 20 to 200 ng mL^{-1}. The inter– and intra–day variability was lower than 15% and the recoveries ranged from 85–115%. The biological application of the method was also developed, specifically to evaluate the role of the nutrition in the profile of these ROS after an oral administration of different diets with ingredients potentially importants.

MDA, o-Tyr and 8-OHdG were selected due to they are related to different oxidation vias such as lipid oxidation, protein oxidation and DNA oxidation, respectively. These three compounds were selected to measure the detriment or damage in the organism in the fluid or target tissues in the study of the natural aging. In addition, the effects of the different diets with specific

ingredients were evaluated in the same fluids and tissue targets. Brain was chosen to be related with natural degeneration or Alzheimer's disease, for example. Liver is chosen as accumulative organ to see easier these compounds. And serum was also interesting to evaluate basal levels of the selected ROS in different groups de animals feeding with different diets.

The results showed that some diets affected positively to the biological degradation process. That is, the oxidation parameters were influenced by some ingredients, decreasing the rate of oxidation or aging in the biological systems studied, in this case the rodent.

As result of these experiments a patent application has been submitted to the patent office. For confidential reasons, more details can not be exposed.

Por motivos de confidencialidad, no es posible incluir más datos sobre la presente patente en esta Tesis Doctoral.

CONCLUSIONES DE LA TESIS

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

I. Se han desarrollado y validado varios métodos analíticos para la determinación de diferentes biomoléculas en diversas matrices, relacionadas con diferentes funciones en un sistema vivo, fundamentalmente la cognitiva, sistema músculo-esquelético e inmune.

II. Los métodos propuestos ofrecen análisis multicomponentes con moléculas pertenecientes a distintas familias, aportando mejoras importantes en relación a métodos ya existentes, e incluso herramientas novedosas para afrontar nuevas necesidades bioanalíticas.

III. La metodología desarrollada ha sido aplicada, en cada caso, en muestras reales de suero, orina, leche materna, microdializados de cerebro de rata y otras matrices biológicas. De esta manera, los métodos aportan información bioquímica sobre los estados naturales del animal y después de una intervención nutricional, encontrando importantes hallazgos potencialmente útiles en la industria alimentaria. Además se evaluó una fórmula infantil desde el punto de vista de estabilidad.

IV. Se han explicado especialmente los procedimientos de recogida y tratamiento de muestra. Las matrices estudiadas son diversas y en algunos casos complejas, de modo que fue necesario optimizar cada caso individualmente para evitar ciertos inconvenientes como bajas recuperaciones de los analitos durante la extracción o posibles efectos de supresión de la señal en el espectrómetro de masas, logrando así obtener tratamientos de muestra efectivos y extracciones cuantitativas.

V. El empleo de la espectrometría de masas-masas con dos transiciones MRM por cada analito, ha permitido la cuantificación y confirmación de los resultados en un mismo análisis, mejorando y optimizando aún más la productividad del método.

VI. La resolución de los problemas analíticos presentados en esta Tesis han permitido obtener métodos con elevada exactitud, sensibilidad, selectividad, amplios rangos dinámicos lineales, y tiempos de análisis más cortos con la consiguiente reducción de costes y de generación de residuos.

VII. Los hallazgos bioquímicos obtenidos han aportado información muy valiosa en la industria nutricional tanto en líneas de pediatría como en adultos, para la futura generación de nuevas fórmulas infantiles y productos nutricionales o mejorar los ya existentes en el mercado.

VIII. Ha sido posible llevar a cabo un estudio *in vivo* en ratas para demostrar la absorción de los HMOs desde el intestino a la circulación sistémica, lo que constituye uno de los primeros estudios que muestran este aspecto *in vivo.* El tiempo de absorción era dosis-dependiente y generalmente los niveles de HMOs permanecieron elevados respecto al basal después de 300 minutos de su administración.

IX. La administración oral de 2'-FL afectó a las concentraciones de otras moléculas como la Fuc, en suero y especialmente en orina.

X. Las crías de rata presentaron mayores niveles circulantes de SA, 6'-SL, 2'-FL y especialmente 3'-SL, así como Fuc y Lact que en los animales adultos.

XI. Con el método propuesto en el capítulo 3 hemos sido los primeros en demostrar que el HMB traspasa la barrera hematoencefálica cuando se administra oralmente.

XII. Se ha observado que los niveles de HMB disminuyen con la edad, sin embargo los de Leu aumentan. Esto estaba alineado con el hallazgo encontrado en el experimento con ratas ovarectomizadas, donde el HMB era menor en las ratas donde se había combinado ovarectomización y edad. Una explicación razonable sería que el catabolismo del HMB podría reducirse con la edad y los procesos asociados a la misma, como puede ser

la menopausia en una mujer. Así seríamos los primeros en relacionar el vínculo de HMB – pérdida de hueso/músculo en mujeres menopáusicas.

XIII. Por último, además de todos los hallazgos expuestos anteriormente, se propone un método de oxidación acelerada para establecer modelos de predicción de estabilidad en productos nutricionales con un estudio preliminar identificando marcadores potencialmente interesantes en la industria alimentaria.

ANEXO