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TESIS DOCTORAL

ANALYTICAL METHODOLOGY FOR THE DETERMINATION OF POTENTIALLY EFFECTIVE COMPOUNDS FROM NATURAL PRODUCTS TO CONTROL *NOSEMA CERANAE* INFECTION IN HONEY BEE (*APIS MELLIFERA*)

Presentada por Dª Ana María Ares Sacristán para optar al grado de doctora por la Universidad de Valladolid

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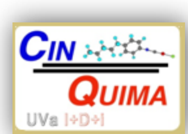

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Analytical methodology for the determination of potentially effective compounds from natural products to control *Nosema ceranae* infection in honey bee (*Apis mellifera*)

Presentada por Dª Ana María Ares Sacristán alumna del programa de doctorado en Química Analítica, Contaminación y Medio Ambiente impartido por el Departamento de Química Analítica, autoriza la presentación de la misma, considerando que cumple todos los requisitos establecidos en la legislación vigente.

Valladolid, a 15 de Mayo de 2015

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La autora de la presente memoria ha disfrutado de una ayuda para becas y contratos de Formación de Profesorado Universitario del Programa Nacional de Formación de Recursos Humanos de Investigación, en el marco del Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008-2011, así como una ayuda para una estancia predoctoral breve por la misma entidad, a quién expreso mi agradecimiento.

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“Soy de las que piensan que la ciencia tiene una gran belleza. Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas”

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A mi familia

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FOREWORD

This PhD Thesis consists of a series of original research papers, according to the Spanish national (R.D. 99/2011, BOE 35/2011) and regional (BOCYL 243/2012) regulations.

An extended introduction is included in this PhD Thesis for contextualization and linkage of the original research publications, and to help understanding the relevance of the findings. A brief description of the main methods provides an overview of the range of techniques applied, and it is not intended to give exhaustive details of the work done, which is properly related in specific papers (publications). Likewise, the compilation of the introduction, specific objectives and methodology, provide just a synthesized version of the entire work related in the publications.

Prior to his defense, this PhD Thesis has been evaluated by two experts from International research centers directly related to its subject:

- **Dr. Susanne K. Wiedmer**, Department of Chemistry, University of Helsinki (Finland).
- **Dr. Almudena García Ruíz**, Departamento de Ciencias de Alimentos y Biotecnología, Escuela Politécnica Nacional de Quito (Ecuador).

ABBREVIATION INDEX

CCD	collony colapse disorder
DAD	diode array detection
DCM	dichloromethane
ESI	electrospray ionization
ESI-MS	electrospray-mass spectrometry
FLD	fluorescence detection
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
GLS(s)	glucosinolate(s)
GRA	glucoraphanin
HPLC	high-resolution liquid chromatography
ITC(s)	isothiocyanate(s)
LC	liquid chromatography
LC-DAD-MS	liquid chromatography coupled to diode array detection and mass spectrometry
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
MRL	maximun residue limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl *t*-butyl ether
PLE	pressurized liquid extraction
RPLC	reversed-phase liquid chromatography
SFE	supercritical fluid extraction
SFN	sulforaphane
SLE	solid-liquid extraction
SPE	solid phase extraction
UHPLC	ultra high performance liquid chromatography
UHPLC-MS/MS	ultra high performance liquid chromatography coupled to tandem mass spectrometry
UV	ultraviolet

RESUMEN

En los últimos años se ha venido observando una extraña desaparición de las abejas melíferas (*Apis mellifera*) que está afectando drásticamente a un elevado número de colmenas por todo el mundo, causando serios problemas no sólo a la apicultura sino a todas las actividades agrícolas relacionadas con la polinización. Una de las hipótesis que puede explicar estos problemas es la propagación de la nosemosis, que es una enfermedad digestiva de las abejas causadas por los microsporidios *Nosema apis* y *Nosema ceranae*. Estas pérdidas importantes de abejas se solucionaron inicialmente con la autorización provisional de la fumagilina. Hoy en día, el uso de este compuesto no está autorizado, y por consiguiente es necesario encontrar compuestos alternativos que puedan ser efectivos contra la infección por *Nosema*. El resveratrol y sus compuestos derivados, que han sido positivamente relacionados con beneficios para la salud de humanos y también de animales, han demostrado su potencial para controlar la infección por *Nosema* en abejas melíferas. Por otro lado, continúa la búsqueda de nuevos compuestos obtenidos de productos naturales que puedan ser utilizados para controlar la infección por Nosema. En la actualidad, una gran parte de la investigación relacionada con los productos naturales se ha centrado en el broccoli, y en concreto, los glucosinolatos (GLSs) y sus productos relacionados, que parecen tener una cierta actividad anticancerígena. En particular, la mayoría del estudio ha estado focalizado en un único compuesto bioactivo del broccoli, llamado sulforafano (SFN), que posee un gran potencial frente a numerosas enfermedades. Por estos motivos, puede ser interesante el investigar su potencial para controlar la infección por *Nosema*, y además, será necesario el extraer estos compuestos de la manera más compatible posible con el medio ambiente, si se pretende administrarlo a las abejas melíferas. En resumen, si estos compuestos (isómeros del resveratrol, GLSs y SFN) van a ser administrados a abejas melíferas, es de suponer que residuos de estos compuestos se puedan encontrar en productos apícolas, tales como la miel, la cera o el polen de abeja. Además, el análisis de los compuestos anteriormente citados en productos apícolas puede ser también de interés para verificar si han sido adecuadamente administrados, y para saber si estos compuestos pueden estar presentes en productos que serán consumidos/usados por personas y abejas.

Por todo ello, el principal objetivo de esta tesis doctoral ha sido el desarrollo de metodologías analíticas para determinar resveratrol, GLSs y SFN en productos apícolas (miel, cera y polen). Para poder alcanzar esta meta, se han propuesto una serie de objetivos secundarios: **i**) el desarrollo, optimización y validación de métodos analíticos específicos para determinar isómeros de resveratrol y piceidos en cera y polen: **ii**) el desarrollo, optimización y validación de métodos de extracción eficaces, selectivos y compatibles con el medio ambiente para obtener GLSs del brócoli; **iii**) el desarrollo de, optimización y validación de métodos analíticos específicos para determinar GLSs y SFN en productos apícolas.

Ha de comentarse que debido a las características físico-químicas de los compuestos recogidos en este estudio, la técnica analítica más adecuada paras llevar a cabo su separación y análisis es la cromatografía de líquidos (LC). Además, es posible utilizar varios detectores acoplados al LC dependiendo de cada objetivo. Por ejemplo, los GLSs y SFN son encontrados normalmente en altas concentraciones en brócoli, por lo que un detector de absorción molecular con diodos alineados (DAD) sería suficiente para detectar y cuantificar a la mayoría de los compuestos. De cualquier forma, detectores de espectrometría de masas (MS) pueden ser también utilizados para confirmar la presencia de los analitos en las muestras, y para cuantificar aquellos compuestos encontrados a concentraciones bajas en alguna de las muestras. Por otro lado, cabe esperar cantidades bajas de isómeros de resveratrol, GLSs y SFN en productos apícolas, y consecuentemente, son requeridos detectores sensibles como la MS ó MS en tándem (MS/MS) para realizar una correcta cuantificación y confirmación de los compuestos estudiados. En todos los casos, la metodología seguida para alcanzar las metas propuestas se ha desarrollado de manera análoga y con etapas similares: **i**) se ha llevado a cabo una exhaustiva búsqueda bibliográfica con el fin de saber el estado actual de cada tema; **ii**) se han seleccionado de manera preliminar las condiciones cromatográficas y de detección; **iii**) se han propuesto y optimizado un tratamiento de muestra eficiente; **iv**) las condiciones cromatográficas y de detección se han seleccionado y optimizado; **v**) el método propuesto ha sido adecuadamente validado y aplicado al análisis de muestras.

Como resultado del trabajo realizado en esta tesis, dos métodos analíticos nuevos (cromatografía de líquidos acoplado a la espectrometría de masas, LC-MS) han sido desarrollados y validados para determinar isómeros de resveratrol y piceido (*cis* y *trans*) en cera y polen, respectivamente. En ambos casos, se han propuesto procesos de extracción eficaces, que consistían en una extracción sólido-líquido (SLE) y una posterior etapa de concentración. Se han analizado muestras de diferentes orígenes, y han sido detectados residuos de algunos de estos compuestos, lo que es un descubrimiento interesante ya que no había sido publicado con anterioridad. Ha de puntualizarse que no ha sido necesario investigar la presencia de estos compuestos en miel, debido que este estudio ya había sido realizado con anterioridad.

Por otro lado, la determinación de GLSs intactos y SFN en brócoli ha sido llevada a cabo en otra parte de esta tesis. En primer lugar, se ha realizado una extensa búsqueda bibliográfica con el fin de conocer las tendencias actuales para la extracción y caracterización de compuestos potencialmente beneficiosos para la salud del brócoli, prestando especial atención a los GLSs y SFN. A continuación, se han desarrollado y optimizado, por medio del diseño de experimentos, dos métodos de extracción distintos, rápidos, eficientes y compatibles con el medio ambiente (agua caliente y extracción con líquidos presurizados) para obtener GLSs intactos de hojas de brócoli. Además, también han sido propuestas nuevas condiciones de separación (cromatografía de líquidos acoplado a detectores de diodos alineados y a la espectrometría de masas, LC-DAD-MS; y cromatografía de líquidos de ultra alta presión acoplada a la espectrometría de masas en tandem, UHPLC-MS/MS). Se ha de especificar que los experimentos con UHPLC-MS/MS han sido realizados en el *Green Technology Group* de la Universidad de Lund (Suecia) bajo la supervisión de la Prof. Charlotta Turner. Además, los resultados de los estudios de estabilidad han demostrado que los extractos de hojas de brócoli deben ser almacenados a bajas temperaturas (4ºC) y protegidos de la luz con el fin del mantener el mayor contenido total de GLSs intactos en estos extractos. Finalmente, se ha desarrollado y validado un método LC-DAD para analizar SFN en brócoli. El procedimiento de extracción propuesto, basado en la extracción con disolvente utilizando metil *t*-butil eter (MTBE) seguido de una extracción en fase sólida

(SPE) con cartuchos de sílice, ha demostrado ser eficaz y más compatible con el medio ambiente que las propuestas previas.

La última sección de esta tesis doctoral se ha centrado en el desarrollo de nuevas estrategias analíticas para determinar GLSs intactos y SFN en productos apícolas como la miel, cera y polen. En todos los casos, se han desarrollado y optimizado métodos de cromatografía de líquidos acoplado a la espectrometría de masas en tándem (LC-MS/MS) para separar, identificar y cuantificar los compuestos estudiados. Los GLSs intactos han sido eficazmente extraídos de polen de abeja por medio de la SLE con agua caliente, seguida de SPE con cartuchos rellenos de amino. Estos cartuchos también han sido usados para la miel, pero en este caso, la miel fue previamente disuelta en agua caliente. Un método de extracción diferente basado en tres etapas (disolución, extracción y concentración) ha demostrado ser eficaz, simple y relativamente barato (bajo consumo de disolventes) para obtener GLSs intactos en cera. Ha de señalarse que residuos de GLSs intactos han sido detectados en algunas de las muestras de polen y miel analizadas. Este es un hecho especialmente relevante en el caso de las muestras de polen, ya que no ha sido publicado con anterioridad. Finalmente, se ha desarrollado y optimizado un método de extracción eficaz de SFN en la miel, basado en una SPE con cartuchos poliméricos. Además, se ha detectado por primera vez SFN en dicha matriz de colmenas no tratadas.

Como resumen, se puede decir que esta tesis doctoral ha estado dedicada a desarrollar metodologías analíticas basadas en LC acoplada a diferentes detectores (DAD; MS; MS/MS), tan rápidas, simples, compatibles con el medio ambiente y sensibles como ha sido posible, para determinar isómeros de resveratrol, GLSs y SFN en productos apícolas (miel, cera y polen), debido a que está siendo investigado el potencial de estos compuestos para controlar la infección por *Nosema ceranae* en abejas melíferas (*Apis mellifera*). Además, se han propuesto nuevos métodos de las mismas características que las anteriormente expuestas para extraer y determinar GLSs y SFN del brócoli. Finalmente, se ha demostrado que es necesario el desarrollo de métodos analíticos específicos para determinar isómeros de resveratrol, GLSs intactos y SFN en productos apícolas; dichos métodos deben ser, tal y como hemos propuesto, muy sensibles, ya que

el contenido encontrado en la mayoría de los casos fue cercano al µg/kg. Además, la determinación de alguno de estos compuestos en productos que pueden ser consumidos por humanos, como la miel y el polen, les proporcionan un valor añadido, ya que se ven afectados positivamente por las propiedades beneficiosas de estos compuestos, y al mismo tiempo incrementan el valor nutricional y bioactivo de estos productos naturales.

SUMMARY

In the last few years the strange disappearance of honey bees (*Apis mellifera*) has been devastating a great number of beehives worldwide, causing serious problems not only for apiculture but also for all agricultural activities related to the action of these pollinators. One of the hypotheses that could explain such problems is the spread of nosemosis (*Nosema* infection), which is a digestive disease in honey bees caused by the microsporidia *Nosema apis* and *Nosema ceranae*. This large-scale bee loss was initially solved by the provisional authorization of fumagillin. Nowadays, the use of this compound is not authorized, and consequently it is necessary to search for alternative compounds which could be effective against this *Nosema* infection. It has been demonstrated that resveratrol and its related compounds, which have all been positively linked to health benefits for humans and also animals, have the potential to control *Nosema* infection in honey bees. Moreover, the search for new compounds from natural products that could be used to control *Nosema* infection continues. Recently, a large amount of research into natural products has been related to broccoli, and particularly glucosinolates (GLSs) and their related compounds, which appear to be protective against chemical carcinogens. Most of the study has been focused on a single bioactive component within broccoli, namely, sulforaphane (SFN), which potentially is very effective against several diseases. Consequently, it could also be of interest to investigate the potential of GLSs and SFN to control the *Nosema* infection; for this reason, it is necessary to extract those compounds in the most environmentally friendly way if they are going to be administered to honey bees. In conclusion, if such compounds (resveratrol isomers, GLSs and SFN) are administered, it may be surmised that residues will be found in bee products, such as honey, beeswax, or bee pollen. Furthermore, an analysis of resveratrol isomers, GLSs and SFN in bee products could be of significant interest to verify that these compounds have been properly administered, and whether they might be present in products to be consumed/used by bees and humans.

The main goal, therefore, of this PhD Thesis is to create analytical methodologies to determine resveratrol, GLSs and SFN in bee products (honey, beeswax and bee pollen).

For this purpose several secondary goals are proposed: **i**) developing, optimizing and validating specific analytical methods to determine resveratrol and piceid isomers in beeswax and bee pollen; **ii**) developing, optimizing and validating efficient, selective and environmentally friendly extraction methods to obtain GLSs and SFN from broccoli; **iii**) developing, optimizing and validating specific analytical methods to determine GLSs and SFN in bee products.

Due to the physico-chemical properties of the compounds involved in this research, the most suitable analysis technique to perform separation and analysis is liquid chromatography (LC). In addition, it is possible to use several detectors coupled to LC depending on each goal. For example, GLSs and SFN are usually found in large concentrations in broccoli, so a diode array detector (DAD) would be sufficient to detect and quantify most of those compounds. However, mass spectrometry (MS) detectors could also be used to confirm their presence in the samples and to quantify compounds detected at lower concentrations in some samples. Smaller amounts of resveratrol isomers, GLSs and SFN were expected in bee products, and as a result sensitive detectors such as MS or tandem mass spectrometry (MS/MS) were required for correct quantification and to confirm the presence of the compounds being studied. In all cases, the methodology followed to achieve the goals was applied in the same way and in similar stages: **i**) an exhaustive search of the bibliography was conducted for state-of-the art knowledge of each subject; **ii**) a preliminary selection was made of the chromatographic and detection conditions; **iii**) an efficient sample treatment was proposed and optimized; **iv**) the chromatographic and detection conditions were selected and optimized; **v**) the proposed method was properly validated and applied to sample analysis.

As a result of the work carried out on this PhD Thesis, two new analytical methods (LC-MS) were developed and validated to determine resveratrol and piceid isomers (*cis* and *trans*) in beeswax and bee pollen, respectively. In both cases, efficient extraction procedures were proposed, which involved a solid–liquid extraction (SLE) and a concentration step. Samples from different origins were analyzed, and residues from several of these compounds were detected, which is an interesting finding as this has

hitherto never been reported. It should also be mentioned that it was not necessary to investigate these compounds in honey, as this research had previously been carried out.

In addition, in another part of this PhD Thesis the determining of intact-GLSs and SFN in broccoli has been undertaken. Firstly, an exhaustive bibliographical search has been conducted in order to know the current trends in extracting and characterizing health promoting compounds in broccoli, with special attention being paid to GLSs and SFN. Also, two different rapid, efficient and environmentally friendly extraction procedures (heated water and pressurized liquid extraction) to obtain intact-GLS from broccoli leaves have been developed, optimized by means of experimental designs, and validated. Moreover, new separation conditions (liquid chromatography coupled to diode array detection and mass spectrometry, LC-DAD-MS; and ultra high performance liquid chromatography coupled to tandem mass spectrometry, UHPLC-MS/MS) have been proposed. The UHPLC-MS/MS experiments were performed in the Green Technology Group at the University of Lund (Sweden) under the supervision of Prof. Charlotta Turner. The results of stability studies have shown that broccoli leaf extracts should be stored at lower temperatures (4ºC) and protected from light exposure in order to maintain the highest total intact GLS content in the extracts. Finally, an LC-DAD method to analyze SFN in broccoli has been developed and validated. The proposed extraction procedure, based on a solvent extraction with methyl *t*-butyl ether (MTBE) followed by a solid phase extraction (SPE) with silica cartridges, has proven to be efficient and more environmentally friendly than previous proposals.

The final section of this PhD Thesis focuses on the development of new analytical strategies to determine intact-GLSs and SFN in bee products, such as honey, beeswax and bee pollen. In all cases, LC-MS/MS methods were developed and optimized to separate, identify and quantify the compounds being studied. Intact-GLSs were efficiently extracted from bee pollen by SLE with heated water, followed by SPE with an amino sorbent; meanwhile, the same sorbents were used with honey, but in this case the latter was previously diluted in heated water. A different extraction method based on three steps (dissolution, liquid-liquid extraction, and concentration) has proven to be efficient, simple and relatively cheap (a low consumption of solvents) to obtain intact-

GLSs from beeswax. It must be said that residues of intact-GLSs were detected in some of the honey and bee pollen samples analyzed. This finding is especially relevant in the case of the bee pollen samples, as prior to this study it had never been reported. Finally, an efficient procedure for extracting SFN from honey has been developed and optimized, based on an SPE with polymeric sorbents. SFN was detected by LC-MS/MS in non-treated beehives for the first time.

To sum up, this PhD Thesis has aimed at creating rapid, simple and environmentally-friendly analytical methodologies, with maximum possible efficiency and sensitivity, which are based on LC coupled to several detectors (DAD; MS; MS/MS) to determine resveratrol isomers, GLSs and SFN in bee products (honey, beeswax and bee pollen); this is in line with its investigation of the potential of these compounds to control *Nosema* infection in honey bees (*Apis mellifera*). In addition, new methods with the same characteristics as those described above have been proposed to extract and determine GLSs and SFN from broccoli. Finally, it has been demonstrated that there is a need to develop specific analytical methods to determine resveratrol isomers, intact-GLSs and SFN in bee products; such methods should, as this PhD Thesis has proposed, be very sensitive, as the content observed in most cases was at the μg/kg level. Moreover, the detection of some of these compounds in bee products that would be consumed by humans, for instance, honey and bee pollen, implies that they could be positively affected by the beneficial health properties of such compounds, with an increase in the nutritional and bioactive values of these natural products.

LIST OF THE ORIGINAL PAPERS AND AUTHOR´S CONTRIBUTION

This PhD Thesis is based on the following papers, which are referred to in the text of the manuscript by their Roman numerals as indicated below:

Paper I: Ares, **A. M.**, González, Y., Nozal, M. J., Bernal, J. L., Higes, M., Bernal. J. Development and validation of a liquid chromatography with mass spectrometry method to determine resveratrol and piceid isomers in beeswax. *Journal of Separation Science* 32 (2015) 197. Impact factor (2013): 2.594. The final publication is available at Wiley via http://dx.doi.org/10.1002/jssc.201400955.

Paper II: Ares, **A. M.**, Soto, M. E., Nozal, M. J., Bernal, J. L., Higes, M., Bernal. J. Determination of resveratrol and piceid isomers in bee pollen by liquid chromatography coupled to electrospray ionization-mass spectrometry. *Food Analytical Methods*. Impact factor (2013): 1.802. The final publication is available at Springer via http://dx.doi.org/10.1007/s12161-011-9231-3

Paper III: Ares, **A. M.**, Nozal, M. J., Bernal. J. Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds. *Journal of Chromatography A* 1313 (2013) 78. Impact factor (2013): 4.258. The final publication is available at Elsevier via http://dx.doi.org/10.1016/j.chroma.2013.07.051.

Paper IV: Ares, **A. M.**, Bernal, J. L., Nozal, M. J., Bernal. J. Optimized extraction, separation and quantification of twelve intact glucosinolates in broccoli leaves. *Food Chemistry* 152 (2014) 66. Impact factor (2013): 3.259. The final publication is available at Elsevier via http://dx.doi.org/10.1016/j.foodchem.2013.11.125.

Paper V: Ares, **A. M.**, Bernal, J., Martín, M. T., Bernal. J. L., Nozal, M. J. Optimized formation, extraction, and determination of sulforaphane in broccoli by liquid chromatography with diode array detection. *Food Analytical Methods* 7 (2014) 730. Impact factor (2013): 1.802. The final publication is available at Springer via http://dx.doi.org/10.1007/s12161-013-9766-6.

Paper VI: Ares, **A. M.**, Bernal, J. L., Nozal, M. J., Bernal. J. Effect of temperature and light exposure on the detection of total intact glucosinolate content by LC-ESI-MS in

Broccoli Leaves. *Food Analytical Methods* 7 (2014) 1687. Impact factor (2013): 1.802. The final publication is available at Springer via http://dx.doi.org/10.1007/s12161-014-9806-x.

Paper VII: Ares, **A. M.**, Bernal, J., Nozal, M. J., Turner, C., Plaza, M. Fast determination of intact glucosinolates in broccoli leaf by pressurized liquid extraction and ultra high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. (Under Review).

Paper VIII: Ares, **A. M.**, Nozal, M. J., Bernal. J. Development and validation of a liquid chromatography-tandem mass spectrometry method to determine intact glucosinolates in bee pollen. (Under Review).

Paper IX: Ares, **A. M.**, Bernal, J. L., Nozal, M. J., Bernal. J. A specific and validated method to quantify intact glucosinolates in honey by liquid chromatography coupled to tandem mass spectrometry. (Under Review).

Paper X: Ares, **A. M.**, Bernal, J. L., Nozal, M. J., Bernal. J. Analysis of intact glucosinolates in beeswax by liquid chromatography-tandem mass spectrometry. (Under Review).

Paper XI: Ares, **A. M.**, Valverde, S., Bernal, J. L., Nozal, M. J., Bernal. J. Development and validation of a LC–MS/MS method to determine sulforaphane in honey. *Food Chemistry* 181 (2015) 263. Impact factor (2013): 3.259. The final publication is available at Elsevier via http://dx.doi.org/10.1016/j.foodchem.2015.02.085.

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Papers not included in this PhD Thesis:

Paper: Bernal, J., **Ares**, **A. M.**, J. Pol, J., Wiedmer, S. K. Hydrophilic interaction liquid chromatography in food analysis. *Journal of Chromatography A* 1218 (2011) 7438.

Paper: Soto, M. E., **Ares**, **A. M.**, Bernal. J., Nozal, M. J., Bernal, J. L. Simultaneous determination of tryptophan, kynurenine, kynurenic and xanthurenic acids in honey by liquid chromatography with diode array, fluorescence and tandem mass spectrometry detection. *Journal of Chromatography A* 1218 (2011) 7592.

Paper: Ares, **A. M.**, Nozal, M. J., Bernal, J. L., Martín-Hernández, R., Higes, M., Bernal, J. Liquid chromatography coupled to ion trap-tandem mass spectrometry to evaluate juvenile hormone III levels in bee hemolymph from Nosema spp. infected colonies. *Journal of Chromatography B* 899 (2012) 146.

Paper: Ares, **A. M.**, Bernal, J. Hydrophilic interaction chromatography in drug analysis *Central European Journal of Chemistry* 10 (2012) 534.

Author´s contribution

Paper I: The author performed part of the experimental work and wrote major parts of the paper.

Paper II: The author performed part of the experimental work and wrote major parts of the paper.

Paper III: The author performed the bibliographic revision and wrote major parts of the paper.

Paper IV: The author performed the bibliographic revision, planned and performed the experimental work and wrote major parts of the paper.

Paper V: The author performed the bibliographic revision, planned the experimental work, performed most of experiments, and wrote major parts of the paper.

Paper VI: The author performed the bibliographic revision, planned and performed the experimental work, and wrote major parts of the paper.

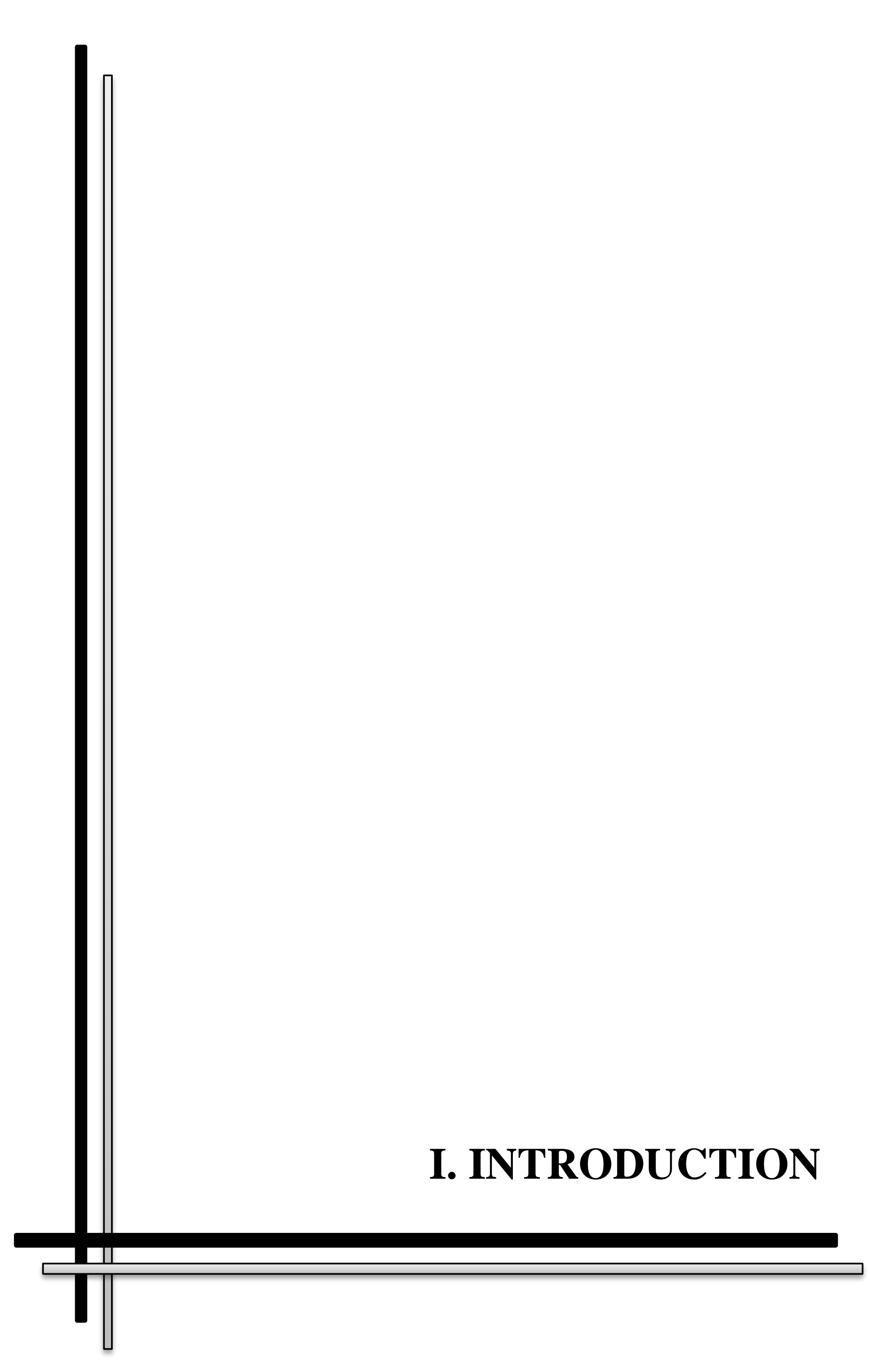
Paper VII: The author performed the bibliographic revision, participated in the planning of the experiments, performed all the experimental work, and assisted in writing of the paper.

Paper VIII: The author performed the bibliographic revision, planned and performed the experimental work, and wrote major parts of the paper.

Paper IX: The author performed the bibliographic revision, planned and performed the experimental work, and wrote major parts of the paper.

Paper X: The author performed the bibliographic revision, planned and performed the experimental work, and wrote major parts of the paper.

Paper XI: The author performed the bibliographic revision, planned the experimental work, performed some of experiments, and wrote major parts of the paper.



I. INTRODUCTION

I. INTRODUCTION

Beekeeping is an important activity within the agrarian structure of our country, with the number of Spanish productive colonies (more than 2.5 million) being approximately 21% of total registered hives in the European Union. Besides the value of their products (honey, bee pollen, royal jelly, beeswax, etc.), bee colonies have an important role as pollinators, and both cultivated and wild plant species have a huge influence on the conservation and stability of ecosystems, genetic variation of plants and developing wildlife diversity. It is important to emphasize that beekeeping also allows the use of marginal agricultural areas, which are not amenable to other uses, and contributes to bees settling in rural areas. Therefore, anything that affects them negatively has a significant impact on agriculture and the environment. It is significant, therefore, that in recent years there has been a substantial weakening of bee colonies and mass death in Europe as well as in other countries[1-10]. Regarding Spain, the most serious cases are located in regions like Castilla-León, Extremadura, Andalucía, Castilla-La Mancha and Galicia, where more than 75% of national hives are situated, and this represents a serious problem of both severe economic and ecological dimensions. This phenomenon has become known as Colony Collapse Disorder (CCD)[11-14] and has caused continuing global concern.

In order to develop their productive potential, it is necessary for honey bee (*Apis mellifera*) colonies to have a suitable health status, one which also ensures its viability as a producing species. Among the diseases affecting honey bees, varroosis and nosemosis (*Nosema* infection) are significant. A rough estimate of the prevalence of *Nosema*, conducted by the Regional Centre for Research into Beekeeping and Honey Production, at Marchamalo (Guadalajara, Spain), states that this disease has worsened since 2000 to the extent that currently there is considered to have been a drastic reduction in the number of individual specimens and the permanent loss of many hives. This situation has led to a decrease of over 50% in Spain.

Moreover, the possibility of synergistic interaction such as certain systemic toxic pesticides for agricultural use and which are toxic for bees[15-19], or acaricide substances

used in varroosis control[20], which can affect the immune status of bees whilst favoring the pathogenic action of parasites and infectious agents, are one of the factors believed to influence mortality, along with certain practices of beekeeping overexploitation. Other possible causes of bee depopulation can be attributed to the presence of *Nosema ceranae*, which is a microsporidian parasite frequently detected in affected apiaries[2,12,13].

These situations of depopulation, whose common denominator was the presence of *Nosema ceranae*, have been resolved with similar treatments against *Nosema apis* by means of antibiotics, amongst which fumagillin is the best known[21], and this has been successfully used in the past years under veterinary prescription. However, there is no maximum residue limit (MRL)[22], and this compound possesses genotoxic effects[23]. Consequently, the European authorities have decided to revoke this temporary authorization, and its use is currently forbidden.

The trend, therefore, of recent research is aimed at finding an alternative, in the form of either synthetic or natural compound that could be effective to control the *Nosema ceranae* infection. Because of the excellent antifungal properties that certain natural compounds provide, some studies have been conducted to investigate their potential effectiveness in combating nosemosis and ones which do not display incompatibilities with the possible presence of their residues in bee products or with the honey bees. In this regard, various extracts from plants[24,25] or components from natural products[26,27] have been evaluated by *in vitro* studies against *Nosema ceranae*, proving their usefulness.

Recent experience with phytoalexins, used for centuries in traditional medicine, and specifically with resveratrol, suggests that their antifungal activity could be useful in the treatment of *Nosema ceranae* infection[26,27]. In fact, it was previously observed that resveratrol has had a similar effect against *Sacharomices cerevisae* as amphotericin B[28], and that resveratrol had displayed the potential to control *Nosema ceranae* infection in honey bees in previous research conducted in collaboration with the Regional Centre for Research into Beekeeping and Honey Production at Marchamalo[28]. Recently, a large amount of research into natural products has focused on broccoli and GLSs, which

appear to be protective against chemical carcinogens. These compounds, which are produced by plants as a defense mechanism against pathogens and plagues[30], are considered natural pesticide metabolites. GLSs undergo enzymatic hydrolysis by myrosinase to glucose and a variety of degradation products (isothiocyanates, ITCs; nitriles, thiocyanates; epithiocyanates; epithionitriles; and oxazolidines), which are responsible for flavour and most of the biological activities of GLSs, and they have shown significant antifungal and antibacterial properties, inhibiting the growth and development of many pathogens including fungi, bacteria, insects and parasites[31,32]. In particular, most of the research has been focused on a single bioactive component within broccoli, SFN, formed by the hydrolysis of a GLS (glucoraphanin, GRA). Thus, it could also be of interest to investigate the potential of GLSs and SFN to control *Nosema ceranae* infection.

Finally, it must be mentioned that a multidisciplinary team is required in order to determine the effectiveness of such compounds against *Nosema ceranae* infection. The TESEA research group is developing a suitable analytical methodologies, while the Regional Centre for Research into Beekeeping and Honey Production at Marchamalo is responsible for performing the field assays and microbiological analyses.

This PhD Thesis has focused on creating analytical methodologies which are fast, simple and environmentally friendly, and ones which display maximum possible efficiency and sensitivity, to determine resveratrol isomers, GLSs and SFN in bee products (honey, beeswax and bee pollen), whilst at the same time investigating the potential of these compounds to control *Nosema ceranae* infection in honey bees (*Apis mellifera*). In addition, new methods with the same characteristics as those described above have been proposed to extract and determine GLSs and SFN from broccoli in order to obtain the compounds in a way which is the most compatible with their potential administration to honey bees. Due to the physico-chemical properties of the compounds involved in this research, the most suitable analysis technique to perform separation and analysis is LC coupled to several detectors (DAD; MS; MS/MS), in accordance with the corresponding goals.

I.1. HISTORICAL BACKGROUND AND STATE OF THE ART

Honey bees are easily amongst the most important insects for humans on Earth. The usefulness of honey bees is due to their great number, their social life and their ability to pollinate a broad variety of different flowers. It is also well known the bee is very important for agriculture, because roughly one third of all plants or plant products eaten by humans are directly or indirectly dependent on bee pollination. The great value of bees as pollinators for crops and wild plants has been known for many years[32], but unfortunately this knowledge is not widely appreciated and understood. The lack of bees for pollination can mean a loss for farmers of maybe 75% of their crops. Bee pollination not only results in increased seed or food production, but it may also produce better crop quality, as well as sustainability, because an efficient pollination of flowers may also serve to protect the crops against pests[34-37]. No less important are the economic activities, as the global economic cost of bee decline causes lower crop yields and increases production costs. It should also be mentioned that the value of pollinated crops is greater than that of those crops which are not directly dependent on pollination[38]. The consumption of natural products, which is currently increasing due to acceptance by the consumers, is also related with the productive activities of bees. Sustainable and environmentally friendly production of honey, bee pollen, beeswax, propolis and royal jelly contributes to economic development in rural areas where other activities are difficult to plan[36,39,40].

Unfortunately, the health status of beehives has worsened in recent decades, producing a high loss and mortality all over the world[41,42]. This fact was reflected for the first time when the United States lost more than 70% of its colonies in 2006[8,9,43]; this loss was paralleled in Europe and even exceeded in certain countries[1-7,10,44]. In most cases, the high level of death occurs in the autumn-winter period, but over time deaths have been increasingly detected over the whole year. This devastating phenomenon (CCD)[11-14], is characterized by the acute weakening of adult bees, whilst the brood and queen are apparently healthy and all of them remain in the hive. On the other hand, worker bees abandon it, disappearing and dying in the vicinity of the place without returning there[45]. Usually this global weakening of the hive often brings about increased opportunities for

parasitic invasions, like the wax moth (*Galleria mellonella*), the small hive beetle (*Aethina tumida*) or a parasitic theft from neighboring hives[46].

There are so many factors that affect bee health and which may be related to CCD. More than 60 influential variables have been described and considered even regarding bee loss[46]. As they constitute such a large number, and as a result of possible interactions between them, control, prevention and identification are greatly hindered[47,48].

One of the factors studied is the lack of control and presence of mites, such as *Varroa destructor*[49], sometimes associated with other diseases[50,51], in relation to the incorrect application of the treatments or to the development of drug-resistant populations[52-54]. Its presence is also associated with unregistered acaricide substances (fluvinate, amitraz, coumaphos, chlorpyrifos, etc.) whose use has been ineffective or improperly administered[20], or whose toxicity has not been tested in bees, affecting the immune status and favoring parasitism[55]. Furthermore, susceptibility to different infectious agents, parasites and toxic substances, increases when the *Varroa* parasitism exceeds a certain critical epidemic threshold[56]. Poor nutrition in the beehive due to low quality pollen especially in monocultures, nutritional stress[57,58], and even the climate change[37] with either extreme drought or rainfall[59], are other possible factors associated with the successive loss of colonies. In addition, bad beekeeping practices resulting from improper handling of the colonies and those relating to zoo-technical problems are the causes of some deaths[60,61]. The development of a disease is favored by shortages in food reserves during winter or the increase in forced demand productivity (transhumance colonies producing a high yield all year round) [1].

The use of agricultural pesticides is one of the most important hypotheses related to CCD. Environmental pollution by pesticides continues to be an increasing problem, and their distribution throughout the plant, including pollen, nectar and fluids, raises particular concern about exposure to pollinators. Bees are living hazardous lives, as farmers all over the world use more synthetic pesticides and many bees are killed by the careless use of pesticides and herbicides. Most of these chemicals are poisonous for bees and some of them are extremely dangerous both for bees and for humans.

Numerous toxic compounds to which bees are exposed (neonicotinoids, fipronil, chlorvenvinphos, etc.) and which even spread in very small amounts, can result in serious destruction of many bee colonies. Although they do not directly kill bees, they cause significant effects on the normal function of the colony, such as their ability to orientate correctly, memory, feeding behavior or the ability to communicate, both in adults and the developing brood[15-19]. Furthermore, some recent research presents evidence that bees are more attracted to and prefer nectar with neonicotinoids[62].

However, a further major variable in the massive loss of bees concerns the presence of different species of microsporidia[3,12,13,63]. It is considered that about half the known genera of microsporidia prefer insects as a host to invade[64], and their infection leads to reduced fertility, a decline in longevity and a loss of strength[65]. As for this group of parasites affecting honey bees (*Apis mellifera*), a clearly recognized pathology called either nosemosis, "*Nosema* disease" or "*Nosema* infection" [66], which is caused by *Nosema* apis, has often affected hives in Spain[2] and around the world[67]. The symptoms of swelling belly and diarrhea by gastrointestinal impairment (see **Figure 1**) is characteristic of *Nosema* infection caused by *Nosema apis*, which is not found in the new clinical manifestations of beehives affected by *Nosema spp.*[68].

Figure 1. *Nosema apis* infected bee (left); uninfected bee (right). Image source: LPO, Diario el Mundo, http://www.elmundo.es/suplementos/magazine/2007/396/1177697408.html

The new symptomatology is distinguished by a progressive weakening of the hives, depopulation or even death inside the hive, the latter being the most severe case[2,11]. These new cases of "dry" *Nosema* infection have increased in recent years, to the extent that the analyses of Spanish apiaries conducted by the Regional Centre for Research into Beekeeping and Honey Production at Marchamalo[2,3,12,13] have resulted in the detection of *Nosema ceranae*[2] for the first time in Europe and continuing at world level[69,70,71]; this makes this microsporidium one of the greatest current pathogens for bees. This type of *Nosema* infection caused by *Nosema ceranae* is termed nosemosis

type C or *Nosema ceranae* infection. It has also been proposed that this weakening of hives may increase by the synergistic effect caused by the existence of this microsporidium with pesticides[72].

There has been an attempt to resolve these depopulation situations, in which the presence of *Nosema ceranae* is evident (see **Figure 2**), with treatments similar to those used for *Nosema* infection caused by *Nosema apis*; these are based on the use of antibiotics, among which fumagillin utilization is preponderant[21,73-75]. Fumagillin is the only antibiotic approved for dealing with *Nosema* infection in North America, and it has been extensively used in apiculture for more than 50 years in the control of *Nosema apis.* However, it must be applied seasonally and with caution in order to avoid residues in honey, due to its toxicity in mammals[76]. Nowadays, the European authorities have suspended its use within its territory, since it also lacks of a MRL[22], and has genotoxic effects[23].

Figure 2. Healthy honeycomb (left); honeycomb infected with *Nosema ceranae* (right).
Image source: http://esa.ipb.pt/cia2014/images/apr/Nanettietal.pdf

In addition, in research carried out mention is made of the current application protocol for fumagillin possibly exacerbating *Nosema ceranae,* because this microsporidium is apparently released from the suppressive effects of fumagillin at concentrations that continue to have an impact on honey bee physiology[76].

In view of the current situation and the need to mitigate the effects caused by *Nosema ceranae*, priority should be given to searching for new compounds to control this microsporidium. In this regard, antifungals are not the best option as very often certain toxicities appear in patients due to their clinical use. Consequently, an alternative and

potentially effective option is that of certain compounds from natural products. Here, various plant extracts rich in polyphenols, such as laurel[24], formulas formed with vitamins and dry plant essences (Api-Herb)[25] and isolated natural compounds[26], have been evaluated by *in vitro* or *in vivo* studies, in order to check their potential utility in controlling nosemosis type C.

One of the natural compounds currently being considered is resveratrol, which has shown antifungal potential in humans, among many other properties[77]. Recent tests with this compound suggest that it could be successfully used to control *Nosema ceranae* infection[26]. Furthermore, GLSs and their hydrolysis products, particularly SFN, which have been previously considered natural pesticides[30], have also shown significant antifungal and antibacterial activities[31,32], but have never been tested in the control of *Nosema ceranae* infection.

I.1.1. RESVERATROL AND PICEID

I.1.1.1. Features and biological activity

Phenolic compounds, which are ubiquitous in plants, are one of the most widely occurring groups of phytochemicals. These compounds play an important role in plant growth and reproduction, providing protection against pathogens and pests, and contributing to the color and sensory characteristics of fruits and vegetables[78]. In addition, phenolic compounds exhibit a wide range of physiological properties, such as antioxidant or cardioprotective attributes, and they have been usually associated with the health benefits derived from consuming high levels of fruits and vegetables. Phenolic compounds include a large group of several hundred chemical compounds, for example, phenolic acids, stilbenes, flavonols, anthocyanins, flavanols, among others. Stilbenes, which are not as abundant in foods as other phenolic compounds, are currently gaining interest due to one specific compound, namely, resveratrol. Resveratrol (3,4',5-trihydroxy-stilbene) is the most representative stilbene compound and is produced by plants in response to fungal infection and a variety of stress (biotic and abiotic) conditions[79]. In nature, it can be found in two isomeric forms (*cis* and

trans; **Figure 3**), which may be transformed into the corresponding *cis* or *trans* piceids (resveratrol-3-O-β-D-glucopyranoside, **Figure 3**), respectively[80].

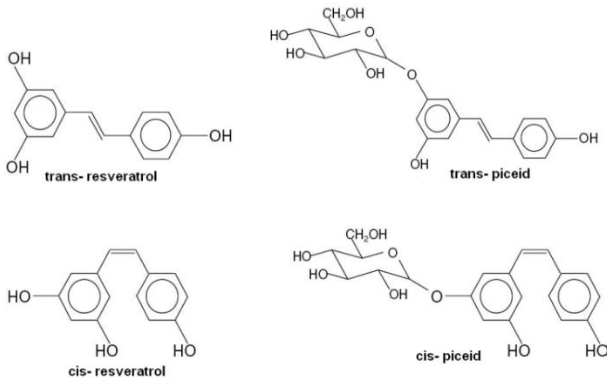


Figure 3. Chemical structures of resveratrol and piceid isomers.

In the recent decades, there has been an increasing interest from health professionals and scientists in bioactive compounds obtained from natural products such as resveratrol. This compound is present in a wide variety of plants, including grapes and fruits[81-84], nuts[85-87] or chocolate[88]. Grapes are probably the most important source of resveratrol for humans, as it is also found in one of the final products of grapes, wine[89]. Indeed, resveratrol has been considered as the source of many of the health benefits associated with moderate consumption of red wine, in particular, it has been mentioned as a possible contributor to cardiovascular protection conferred by red wine consumption, the so-called French paradox, and it is one of the most extensively studied natural compound[82,90].

Resveratrol has shown excellent health promoting properties such as anti-inflammatory, antioxidant, antimicrobial and anti-aging attributes[78,80,90-94]. Moreover, one of the most promising health benefits is its anticancer activity, in which many of the assays have demonstrated the efficacy inhibiting the initiation and development of different types of tumors[95-97], fibroblasts[98] or colon cancer[99]. Likewise, it has also shown positive effects against diabetes or obesity[100]. It is also important to mention that no proven toxic or negative side effects have been reported regarding its use/consumption. Finally, it must be specified that the biological activity of *trans*-resveratrol has been more widely

researched than the other isomers (*cis*-resveratrol, *trans*-piceid, and *cis*-piceid), but it appears that all of them may also have health benefits[80]. It is clear that the inclusion of resveratrol and related compounds into the daily diet seems really promising and worthwhile.

The supposed benefits of resveratrol have been investigated not only in humans, but also in mammals, due to the fact that it is a precursor of viniferins, which are potent antifungal agents[101,102]. For example, resveratrol has been shown to be as powerful as amphotericin B against *Sacharomices cerevisiae*[28], and has also been successfully used to inhibit the growth of other fungi such as *Candida albicans* or *Encephalitazoon cunicoli*, among others[77,103]. Furthermore, the potential benefits of the use of resveratrol have been observed not only in mammals, but also in other animals, such as bees. Some studies, for example, have focused their attention on observing in laboratory conditions the potential of resveratrol for controlling *Nosema ceranae* infection in honey bees (*Apis mellifera*)[24,26,27], while two further studies have examined whether treatment with resveratrol affected the longevity of honey bees[26] or lengthened the average lifespan in wild-type honey bees[104]. In addition, laboratory and field studies conducted in collaboration with the Regional Centre for Research into Beekeeping and Honey Production at Marchamalo have verified the usefulness of resveratrol to control the infection caused by *Nosema ceranae*. On the basis of this collaboration, a PhD Thesis was written[28] and a study published relating to the determination of resveratrol and piceid isomers in honey[105]. Therefore, an analysis of resveratrol and its related compounds in bee products could be of significant interest to verify that these compounds have been properly administered, and whether they might be present in products that would be consumed/used by bees and humans. In the present PhD Thesis, it has been decided to investigate the potential presence of these compounds in beeswax and bee pollen for several reasons; **i**) to complete the work initiated with the analysis of honey; **ii**) beeswax could be considered a hive reservoir, and the compounds present in beeswax could directly affect the bee colony or be transmitted to other bee products, with, in the case of the stilbenes under study, positive health effects; **iii**) the nutritional value of bee pollen, which is a widely consumed natural food supplement, would be

enhanced by the potential presence of resveratrol and related compounds. For all these reasons, the need to develop new methods to analyze resveratrol and piceid isomers in beeswax and bee pollen is justifiable.

I.1.1.2. Analytical background

Resveratrol and related compounds have never been investigated in beeswax. There was a preliminary investigation in bee pollen as a part of a previous PhD Thesis from the TESEA group, but the development, validation and application of method was not completed. In fact, these compounds have been scarcely investigated in other bee products, as they have been only reported two research studies in propolis[106,107], and one in honey[28,105,]. In addition, resveratrol and piceid isomers were only determined in one of these works by using LC coupled to several detectors (DAD; fluorescence, FLD; MS) after performing a previous SPE with polymeric cartridges.

Stilbenes have normally been determined in grapes[108-112] and wine[113-117], although further investigations have been conducted in plants[118], pistachios[85], strawberries[82], tomatoes[119], beer[120], fruit juices[83,84], peanuts[86,87] or chocolate and cocoa[88]. SPE, which is often chosen as a cleaning and/or sample concentration step, has also successfully been used in some of these studies, especially in wine and grapes[84,111,113,115]. However, several other sample treatments have been employed to analyze stilbenes, such as solvent extraction[88] or even microwave assisted extraction or ultrasound-assisted extraction[112,117]. The current trend in sample preparation and enhanced environmental safety, for instance, supercritical fluid extraction (SFE)[121] and pressurized liquid extraction (PLE)[110] have also been employed. While in the first case SFE has the limitation of using large amounts of organic solvent, which means subcritical conditions, PLE has proven effective in terms of time extraction time.

The determination of resveratrol and its related compounds has usually been achieved by LC in reverse phase mode (typically silica C_{18} columns) with different detectors like ultraviolet (UV) or DAD[85,105,108,109,110,118], FLD[85,105,111,113,114] or MS[105,118,109,112,115,116,]. As an alternative technique to LC, GC[107,117] and capillary electrophoresis[106,122] have likewise been employed. However, because of the physico-chemical properties of the

analytes, LC is the technique of choice. In relation to the detector, it must be said that MS is currently preferred choice due to the high sensitivity and identification and confirmation capabilities in relation to FLD and DAD.

I.1.2. GLUCOSINOLATES AND SULFORAPHANE

I.1.2.1. Features and biological activity

Plant-based foods, in particular vegetables from the cruciferous family, are prominent and are known to have healthy beneficial properties, such as protection against different types of cancer, cardiovascular and neurodegenerative diseases[123]. These positive effects are associated with the phytochemical content and the bioactive secondary metabolites that cruciferous plants possess, among which are included the phenolic compounds, vitamin C and GLSs[125-127]. Within cruciferous vegetable family, one of the most widely produced and consumed in the world is the *Brassica oleracea* L. var *italica*, commonly known as broccoli (see **Figure 4**)[123,128]. Numerous varieties of broccoli are identified, such as the first cultivated (var. *Calabrese*), the most widely consumed (var. *Marathon* and *Parthenon*), or the more recent hybrids (*Peto# 7*). The high concentration of its bioactive compounds in seeds during cultivation facilitates distribution by all the plant parts (inflorescences, sprouts, stems and leaves)[129,130], albeit in different amounts, which also depends on their variety[125,131].

Figure 4. Broccoli plant (left); broccoli cultivars (right).
Image source: http://www.laopiniondemurcia.es/comunidad/2013/03/24/superficie-cultivo-brocoli-multiplico-12-1980/459977.html; http://www.regmurcia.com/servlet/s.Sl?sit=c,543,m,2714&r=ReP-20161-DETALLE_REPORTAJESPADRE

One of the most important physico-chemical groups of compounds in broccoli are, due to their excellent properties, GLSs[128]. At least 120 different GLSs have been identified in plants to date[127], although closely related taxonomic groups typically contain only a

small number of such compounds, and their presence is mainly limited to plants of the Brassicaceae family[125]. These secondary plant metabolites are β-thioglucoside N-hydroxysulfates with a side chain (R) which determines the GLS class: aliphatic (derived from methionine) and the most abundant, indole (derived from tryptophan) and aromatic (derived from phenylalanine and tyrosine) (see **Figure 5**)[131,132]. Their biosynthesis occurs mainly in three stages: elongation of the side chain from the precursor amino acid, followed by the synthesis of the basic structure of GLS (aglycone), and ending with the modification of the side chain[131].

These compounds are found in plant cells with an enzyme called myrosinase (thioglucoside glucohydrolase EC 3:2:3:1), responsible for catalyzing their hydrolysis. When plant tissue are damaged (by insect attack, chewing, cutting...etc.), and in the presence of water, GLSs and myrosinase contact is stablished in the cell, which breaks the β-thioglucoside bond, producing glucose, sulfate and a diverse group of products derived from aglycone. This aglycone is an unstable intermediate, which undergoes nonenzymatic structural reorganization to give thiocyanates, ITCs, nitriles or oxazolidines, among others[132].

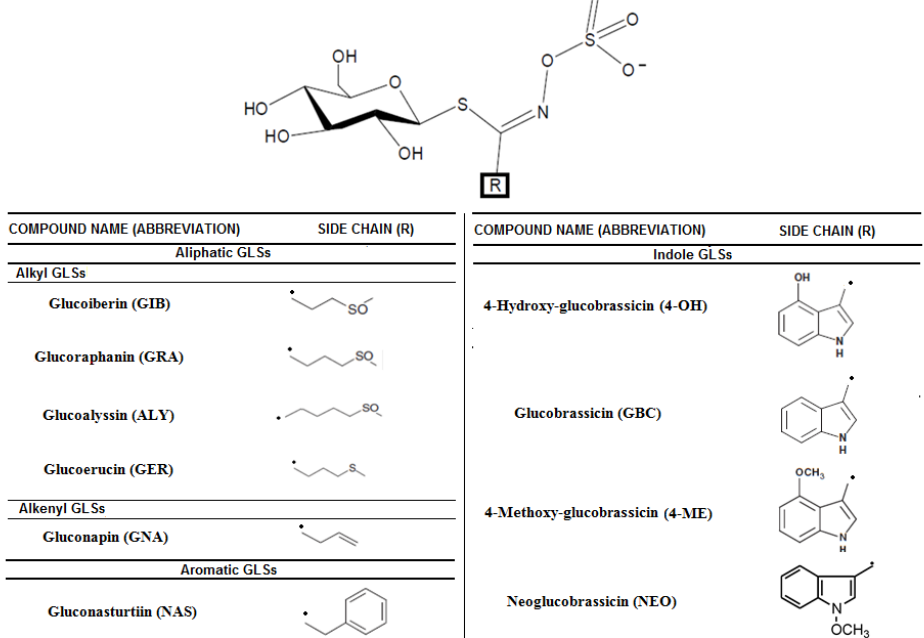


Figure 5. Classification and structure of the most common side chain of GLSs in broccoli.

The ITCs and nitrites, which are usually the majority after hydrolysis, contribute to the characteristic broccoli odor and flavor[125]. The formation of the GLS breakdown compounds will depend on a variety of factors such as the presence of water, the structure of the side chain, the reaction conditions (temperature and hydrolysis time), pH, the presence of ascorbic acid, the availability of metal ions (especially Fe (II)) or the presence of specific proteins[130,133]. It has been also reported in the related literature that the generation of nitriles is specially favored in the presence of the specific proteins and an acidic pH (3-4), meanwhile ITCs are easily created at higher pH values (4-7) (see **Figure 6**)[134].

Figure 6. GLSs hydrolysis by myrosinase[134].

It should be added that industrial or domestic processing may inactivate myrosinase, normally due to the thermal process, although after human consumption hydrolysis may be carried out with the myrosinase that is present at low levels in the intestinal flora[125]. Some studies have shown that the enzyme activity in broccoli increases with the temperature up to 30°C, subsequently decreasing from this temperature up to 50ºC, at which the enzyme is practically inactivated[135,136]. However, myrosinase activity and stability depend on certain other factors, such as the water content of the sample[136] and pressure, the effect of which combine with temperature decreases myrosinase activity[138].

GLSs have limited biological activity. Some of them appear to be protective against chemical carcinogens, while others could be considered natural pesticides because they are produced by plants against pathogens and pests, altering their growth[30]. In addition, certain anti-nutritional effects have been also described in animals, for instance, those of a goitrogenic nature in mammals[131,140], although no negative effects have been reported in humans.

As it previously mentioned, most of the health promoting effects attributed to GLSs are related to one of their hydrolysis products, ITCs. These compounds are known for their numerous properties as fungicides, bactericides, nematicidal or allelopathic and recently gaining interest because of their activity against different cancers[124]. Indeed, nowadays a large body of research on functional foods, especially regarding anticarcinogens, has focused on a single bioactive ITC, sulforaphane (1-isothiocyanato-4-methylsulfinylbutane; SFN)[129], which is formed by hydrolysis of GRA, a GLS that is usually found at high concentrations in broccoli[139] (see **Figure 7**).

Figure 7. GRA hydrolysis by myrosinase.

In relation to the biological activities of SFN, it must be pointed out that this compound has shown itself to be useful as a chemopreventive agent in colon cancer with

inactivated or lost p53[86], as an inhibitor of pancreatic cancer cell growth in vitro and in vivo tumour suppressor in mouse models[141], as well as also showing beneficial effects in relation to prostate[142] and breast cancer[143]. In addition, SFN has demonstrated its potential to reduce the risk of diabetes, atherosclerosis, respiratory diseases, ocular neurodegenerative disorders and cardiovascular diseases[144]. Numerous studies have been conducted with the aim of examining the antimicrobial activity of the ITCs, including SFN, and it is well known that these compounds display activity against microorganisms such as fungi and bacteria. In particular, some researches have been carried out into SFN susceptibility patterns to different types of bacterial and fungal pathogens. The results concluded that SFN inhibits the development and growth of bacteria as *Helicobacter pylori, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella, Escherichia coli* and the fungus *Candida albicans*[31,32,146-151].

Finally, as mentioned in the previous paragraphs, GLSs and SFN have demonstrated their potential against fungi, bacteria, and pests. Therefore, it could be of interest to research their potential to control the *Nosem*a *ceranae* infection. For this reason, if they are going to be administered to honey bees, it is necessary to develop the most environmental friendly extraction methods, and to propose analytical methods for determining residues of these compounds in bee products.

I.1.2.2. Analytical background

I.1.2.2.1. Glucosinolates in broccoli

Although most of the investigation has focused on broccoli edible parts (sprouts, florets, heads, stems, roots), some research has been conducted with the aim of determining GLSs from broccoli leaves in order to give them a certain economic value, such as nutraceutical reservoirs, whilst at the same time reducing the enviironmental impact[152-156]. Different methods for analysing of GLSs in broccoli leaves in accordance with the presence (intact or non-intact desulfo-derivatives) of a sulfate group have been reported in literature. Although the desulfation step reduces the polarity of GLSs and improves their chromatographic separation in reversed-phase liquid chromatography (RPLC), it is a time-consuming procedure, which involves a greater number of

steps[152,153], and faster methods have been proposed for the direct analysis of intact-GLSs[154-156]. Typically, extraction of intact-GLSs is carried out in heated methanol and water mixtures, usually (70:30, v/v)[154,155], or 0.1% formic acid in water and methanol (20:80 v/v), subsequent to autoclaving with boiling water[156]. As can be seen, all these treatments include heating, which is necessary to inactivate myrosinase and avoid degradation of the intact-GLSs. Similar sample treatments (SLE protocols) were used in most cases to extract the compounds from other broccoli parts. Nowadays, traditional extraction techniques, which usually require long extraction times, large amounts of samples, sorbents and organic solvents, are being replaced by novel extraction techniques that are known to be more environmentally friendly[157]. PLE, especially using water as a solvent, is an emerging greener technology compared to conventional extraction methods, and it has been already successfully used to obtain bioactive compounds in foods and plants. PLE has been never applied to extract intact-GLSs from broccoli leaves, but has been previously used to extract intact-GLSs in *Isatis tinctoria* leaves[158] and has also been tested to extract phytochemicals, including GLSs in vegetables (broccoli heads) and fruits[158,159]. Analysis of intact-GLSs in broccoli leaves have been usually performed by LC with UV or DAD[155] and MS[154-156] detectors; meanwhile silica C_{18}[154,155] and C_{30}[156] based analytical columns were used in all cases. Among these, MS and in particular MS/MS has emerged as one of the preferred detection techniques, offering sufficient sensitivity together with an unambiguous identification and quantification of GLSs[159]. In addition, conventional RPLC analysis of GLSs requires 20 min or longer. To reduce this time, ultra high performance liquid chromatography (UHPLC) could be used instead, as it might be possible to obtain better resolutions and sensitivities as well as shorter running times[159]. The coupling of UPLC with different detectors (DAD, MS) has barely been employed to determine GLSs[159-161], and broccoli (accessions and heads) has been analyzed in few of these studies[159,162], but GLSs content in broccoli leaves has never been investigated with UPLC.

I.1.2.2.2. Glucosinolates in bee products

As previously stated, most studies have focused their attention on determining GLSs in Brassica vegetables, due to the fact that these plants are rich in these bioactive compounds. However, it must be said that GLSs have been investigated and detected in honey as a result of their potential use as botanical biomarkers[163] or to evaluate their degradation products[164]. In the first of these studies[163], the methodology applied involved a dilution of the sample with acidified water and a further SPE, with a silica C_{18} sorbent of the analytes prior to their analysis by LC, coupled to several detectors (DAD; MS/MS); however, the method proposed was not specifically developed for GLS analysis and was employed only for identification purposes. Meanwhile, in the other analysis[164], GLS and its degradation product were extracted with a mixture of organic solvents; quantitative examination was by the palladium chloride method. In this case, GLSs absorbance was measured after incubation with the palladium chloride reagent; then, the analytes were isolated and identified by gas chromatography coupled to mass spectrometry (GC-MS). GLSs have never been investigated in bee pollen or beeswax, although they have only been studied, albeit once, in plant pollen from rapeseed and Indian mustard[165]. In this study, GLSs were determined by LC and UV detection following by overnight SLE, partition of the resulting extracts, precipitation of the protein material and an enzymatic desulfation; meanwhile, GC-MS detection was only used to identify and confirm the presence of certain GLSs.

I.1.2.2.3. Sulforaphane in broccoli

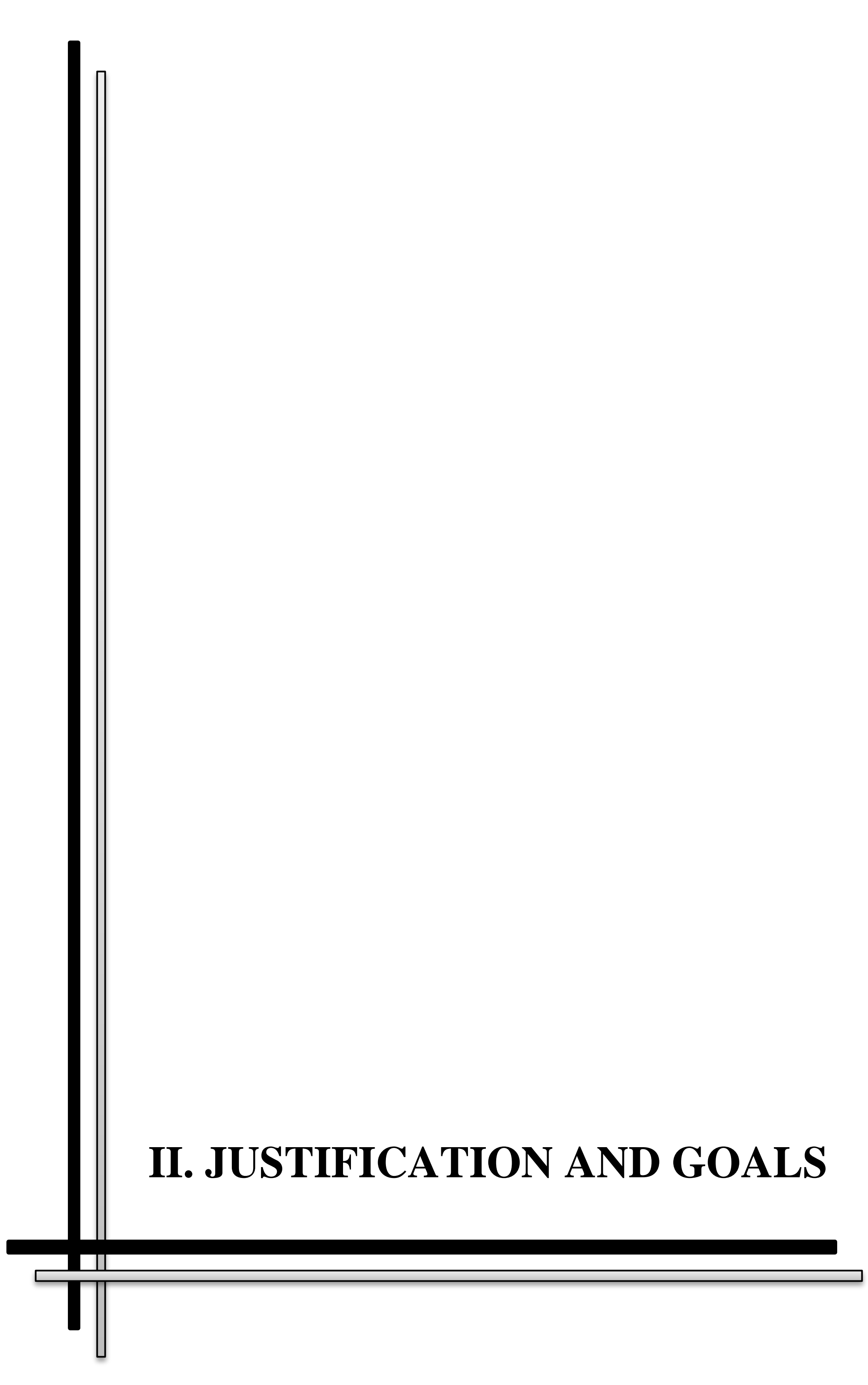
As may be expected, many methodologies have been reported concerning the extraction and determination of SFN in broccoli, but by the time this PhD Thesis was began no specific and exclusive study had been published in which SFN was determined in broccoli leaves. Most of the studies in which this compound was analyzed were based on the hydrolysis of its precursor (GRA) by using acidic water at different pHs[159,166-174]. A further extraction step with an organic solvent such as dichloromethane (DCM)[159,166-174], ethyl acetate[175,176], or chloroform[177], was performed in all cases. In some cases, SFN was incubated with 2-(N-morpholino) ethanesulfonic acid[178], or it was conjugated with

2-mercaptoethanol[179]. In addition, it should be said that in many of the above-mentioned studies SPE with silica based cartridges was performed to purify the broccoli extracts[167,169,170,175,180], while high-speed countercurrent chromatography[177], preparative LC[133], and low pressure column chromatography[181] have been also used to purify the broccoli extracts. In all these cases above mentioned endogenous SFN is determined, using the myrosinase present in the plant, while in others cases, the exogenous SFN determination is carried out adding the enzyme externally[182].

Several analytical methods such as LC[168-171,179,182] and gas chromatography (GC)[175,176,180,183] have been used to determine SFN in broccoli. However, it has been postulated that in some cases SFN was thermally degraded in the injection ports of the GC equipment[169], and usually the GC analysis times were longer than those of LC. For these reasons, it was decided that LC should be employed in this study. Furthermore, almost all these LC studies employed silica C_{18} based analytical columns and UV or DAD as detectors. The scan use of MS detectors could be due to the large amount of SFN detected in broccoli, which makes it unnecessary to develop extremely sensitive methods.

I.1.2.2.4. Sulforaphane in bee products

As with GLSs analysis in bee products, the presence of SFN in those products has received very little attention. For instance, only one publication can be found dealing with an analysis of SFN in honey[184]; here the authors used an SFN sugar extract to feed the bees. SFN was determined in herbhoney, which was previously diluted in a mixture of ethanol and water, and then extracted with chloroform by LC and UV detection. Moreover, no research has been conducted relating to the analysis of SFN in a bee product different than honey.



II. JUSTIFICATION AND GOALS

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II.1. JUSTIFICATION

Honey bees (*Apis mellifera*) play an important role in food production (honey, pollen etc.), and their pollinating activity is not only essential to maintain world agricultural production but also to ensure biodiversity in different ecosystems. In the last few years a strange disappearance of bees has been devastating a great number of beehives worldwide, causing serious problems not only for apiculture but also for all agricultural activities related to the action of pollinators. One of the hypotheses that could explain these problems is the dissemination of nosemosis, which is a digestive disease of honey bees caused by the microsporidia *Nosema apis* and *Nosema ceranae*. In particular, *Nosema ceranae* is a highly prevalent worldwide pathogen for honey bees that has been related to colony losses. Such severe bee losses were resolved by the provisional authorization of fumagillin, thanks to exceptional temporary authorization given for using this compound under veterinary supervision to treat *Nosema* infection in positively diagnosed apiaries. Nowadays, this authorization has expired, and therefore it is necessary to seek alternative compounds which could be effective against *Nosema* infection. This is the reason why the potential of natural compounds for controlling *Nosema* infection in honey bees is being investigated. Resveratrol and its related compounds, which have all been positively linked to health benefits in both humans and, have demonstrated their potential for controlling *Nosema ceranae* infection in honey bees. Moreover, the search continues for new compounds from natural products that could be used to control *Nosema ceranae* infection. Recently, a large amount of research into natural products has focused on broccoli and GLSs, which appear to be protective against chemical carcinogens. These compounds undergo enzymatic hydrolysis by myrosinase to glucose and a variety of degradation products (ITCs, nitriles, thiocyanates, epithiocyanates, epithionitriles and oxazolidines), which are responsible for flavour and most of the biological activities of GLSs. A large part of the research has centered on a single bioactive component within broccoli, SFN, formed by the hydrolysis of a GLS (GRA), which has the potential to reduce the risk of various types of diseases (cancers, diabetes, atherosclerosis, etc.). Consequently, it could be

also of interest to investigate the potential of GLSs and SFN to control *Nosema* infection.

In conclusion, if the compounds in question (resveratrol, GLSs and related compounds) are administered to honey bees, it may be surmised that residues of these compounds can be found in bee products, such as honey, beeswax, or bee pollen. Thus, an analysis of resveratrol, GLSs and related compounds in bee products could be of significant interest to verify that such compounds have been properly administered and whether they could be present in products that would be consumed/used by bees and humans.

II.2. GOALS

The main goal of this PhD Thesis is to create analytical methodologies to determine resveratrol, GLSs and related compounds in bee products (honey, beeswax and bee pollen). Certain secondary goals are proposed:

- Developing, optimizing and validating specific analytical methods to determine resveratrol and piceid isomers in beeswax and bee pollen. In this case, it was not necessary to investigate these compounds in honey, as this research had previously been conducted.
- Developing, optimizing and validating efficient, selective and environmental friendly extraction methods to obtain GLSs and SFN from broccoli. As these can be administered to honey bees, they should be obtained in the most environmental friendly way, in order to avoid subsequent treatment of the extracts to make them compatible with the honey bees, thus increasing the cost and complexity of the methods.
- Developing, optimizing and validating specific analytical methods to determine GLSs and SFN in bee products (honey, beeswax and bee pollen).
-

II.3. THESIS STRUCTURE, SPECIFIC GOALS AND OBJECTIVES

The main methods applied in the PhD Thesis, results, some discussion and conclusions were described and synthesized in the summary, as well as the methodologies employed are summarized in section III. This PhD Thesis is based on 11 papers, each one holding a self-contained investigation published or under review in peer reviewed international journals. Each paper focuses on different aspects of analytical methodologies developed and dedicated to determine resveratrol, GLSs and related compounds in different matrices. **Figure 8** shows a schematic overview of the main topics covered by each paper. Moreover, **Table 1** synthesizes the goals and objectives of each paper.

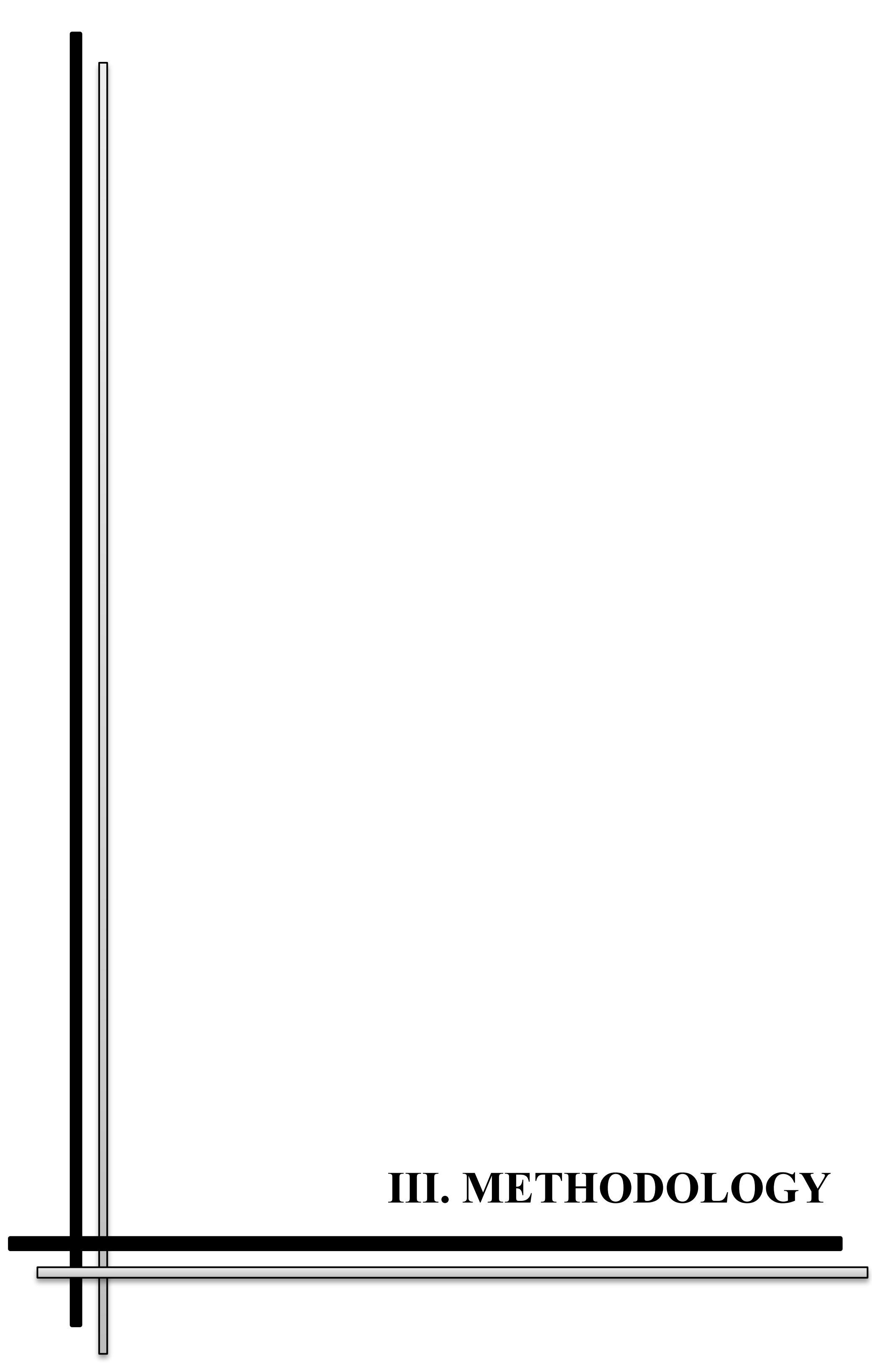
Figure 8. Schematic overview of topics covered in the thesis.

Table 1. Summary of the goals and specific objectives of each paper.

	GOAL	SPECIFIC OBJECTIVES
PAPER I	Analysis of resveratrol and piceid isomers in beeswax	➢ Development and optimization of an efficient and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS for their determination. ➢ Validation and application of the proposed method.
PAPER II	Analysis of resveratrol and piceid isomers in bee pollen	➢ Development and optimization of an efficient and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS for their determination. ➢ Validation and application of the proposed method.
PAPER III	Overview of broccoli health promoting compounds	➢ Presenting an overview on the extraction, separation techniques and biological activity of the main broccoli health-promoting compounds.
PAPER IV	Extraction and determination of 12 intact-GLSs from broccoli leaves	➢ Development and optimization of an efficient and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-DAD-ESI-MS for their characterization. ➢ Validation and application of the proposed method
PAPER V	Extraction and determination of SFN from broccoli florets, stems and leaves	➢ Optimization of SFN endogenous formation by GRA hydrolysis. ➢ Development and optimization of efficient, selective and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-DAD-ESI-MS for its determination. ➢ Validation and application of the proposed method.
PAPER VI	Determination of intact-GLSs stability in broccoli leaf extracts	➢ Detection potential differences in GLS content due to the effect of the: Drying temperature and working and storage conditions (temperature and light exposure).

Table 1. (continued).

	GOAL	SPECIFIC OBJETIVES
PAPER VII	Extraction and determination of 15 intact-GLSs from broccoli leaves	➢ Explore the potential of PLE as an alternative and environmental friendly extraction technique. ➢ Development and optimization of a specific PLE extraction procedure ➢ Development and optimization of an analytical method using UHPLC-ESI-MS/MS for the 15 intact-GLSs. ➢ Validation and application of the proposed method.
PAPER VIII	Analysis of 12 intact-GLSs in bee pollen	➢ Development and optimization of an efficient, specific and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS/MS for their determination. ➢ Validation and application of the proposed method.
PAPER IX	Analysis of 12 intact-GLSs in honey	➢ Development and optimization of an efficient, specific and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS/MS for their determination. ➢ Validation and application of the proposed method.
PAPER X	Analysis of 12 intact-GLSs in beeswax	➢ Development and optimization of an efficient, specific and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS/MS for their determination. ➢ Validation and application of the proposed method.
PAPER XI	Analysis of SFN in honey	➢ Development and optimization of an efficient, specific and environmental friendly extraction procedure. ➢ Development and optimization of an analytical method using LC-ESI-MS/MS for SFN determination. ➢ Validation and application of the proposed method.



III. METHODOLOGY

III. METHODOLOGY

As might be expected different experiments were performed in accordance with the matrix and the analytes, but the same methodology was employed in all the studies, with the exceptions of **Paper III** and **Paper VI**. These involved the following stages: **i**) an extended and exhaustive review of the bibliography; **ii**) preliminary selection of the chromatographic and detection conditions, taking into account the previous publication devoted to the analysis of resveratrol and piceid isomers in honey[105]; **iii**) proposing and optimizing an efficient sample treatment; **iv**) optimizing the chromatographic and detection conditions; **v**) appropriate validation of the proposed method and its application to the analysis of the samples.
Once the general methodology that was followed in most of the studies had been outline, the next paragraphs would be dedicate to briefly summarizing the different specific stages of the method development, as a detailed explanation could be found in the corresponding publications.

III.1. RESVERATROL AND PICEID

As stated above, it is necessary to develop new analytical methods to determine resveratrol and piceid isomers in bee products, as so far no method has been published to perform this task. After an examination of the published literature relating to resveratrol and piceid analysis, it was decided that LC-MS should be used to perform all the experiments which were summarized in **Papers I** (beeswax) and **II** (bee pollen). Method optimization began with the chromatographic and detection conditions that were proposed in a previous study[105], where stilbenes were analyzed in honey. In view of the absence of specific procedures to determine resveratrol and piceid isomers in beeswax and bee pollen, it was decided that, as a result of its simplicity, a solvent extraction should be developed and optimized as the sample treatment. Following optimization, the final chromatographic and detection conditions were selected. Separation of the analytes was achieved in gradient (**Paper I**) or isocratic (**Paper II**) elution modes; regarding MS, electrospray ionization (ESI) in negative mode was selected. Next, validation of the methods were carried out in line with various

international guidelines determining selectivity, limits of detection and quantification, as well as linearity, precision, and accuracy. Finally, the utility of the methods was verified by an analysis of several beeswax and bee pollen samples of different origins.

III.2. GLUCOSINOLATES AND SULFORAPHANE

III.2.1. BROCCOLI

As previously stated, GLSs and SFN have been widely studied in broccoli: this, however, does not mean that they are the only bioactive compounds of this vegetable. Broccoli contains substantial amount of health-promoting compounds such as vitamins, GLSs, phenolic compounds, and dietary essential minerals; thus, it benefits health beyond providing simply basic nutrition, and consumption of broccoli has been increasing over the years. For this reason, a review was written (**Paper III**) giving an overview of the extraction and separation techniques, as well as the biological activity of some of the compounds given above. As has already been mentioned, a part of this PhD Thesis is focuses on the study of GLSs and SFN in broccoli leaves to investigate these by-products as a source of the compounds, with the aim of reducing environmental impact whilst at the same time, increasing their economic value. In addition, as the compounds would be administered to honey bees to check their potential to control *Nosema ceranae* infection, they should be obtained in the most environmental friendly way. Thanks to the bibliographic review undertaken in **Paper III**, it was decided that LC-DAD should be as the analysis technique, due to the nature of the analytes and the large amounts expected in this matrix. However, it should be added that MS detectors were used to confirm and identify the presence of the compounds under study (**Papers IV-VII**), to quantify the less abundant GLSs (**Papers IV** and **VI**), and, in one case (**Paper VII**) as it was a good choice when using UHPLC.

The chromatographic conditions selected in **Paper IV** were also employed in **Paper VI,** and the mobile phase components were selected to perform the experiments in **Paper VII**. Separation of intact-GLSs was conducted in all cases in gradient elution mode (**Papers IV**, **VI**, **VII**); SFN, meanwhile, was determined in isocratic elution mode (**Paper V**). In all cases, optimizing the extraction conditions aimed at proposing rapid,

efficient and environmental friendly extraction procedures, and in two cases (**Papers IV** and **VII**) the extraction procedures were optimized by means of experimental designs. Solvent extraction followed by a SPE procedure was employed to extract SFN from broccoli (**Paper V**), while a solvent extraction (**Paper IV**) and PLE (**Paper VII**) were used to extract intact-GLSs from broccoli leaves. In the case of MS, ESI in negative mode was used to detect intact-GLSs, and in positive mode for SFN analysis. Finally, the methods were validated as described in the previous section, and applied to the analysis of broccoli leaves from different cultivars. On the other hand, the methodology employed in **Paper VI** was quite different from that described above, since here the objective was not related to method development but to the study of the effects of temperature and light exposure on total intact-GLSs content. As mentioned before, the chromatographic and detection conditions were the same as those for **Paper IV**, and these were employed to analyze broccoli leaf extracts stored at different conditions, or oned which were obtained by different drying conditions.

III.2.1. BEE PRODUCTS

Firstly, it should be said that LC was employed as the analysis technique for the above mentioned reasons. Moreover, due to the low concentrations of GLSs and SFN expected in bee products, MS detectors were employed in all cases. GLSs have been the object of scant research in bee products, and therefore the decision was taken to use the chromatographic conditions developed and optimized in **Paper IV** to begin examining bee pollen (**Paper VIII**), honey (**Paper IX**) and beeswax (**Paper X**). As the proposed sample treatments for honey analyses were tedious and not specific for determining intact-GLSs, new procedures were required. Intact-GLSs were efficiently extracted from bee pollen (**Paper VIII**) by performing solvent extraction followed by SPE. A similar extraction procedure was proposed for the analysis of intact-GLSs in honey (**Paper IX**). In this case honey was firstly dissolved, and afterwards SPE was carried out. Meanwhile, intact-GLSs were extracted from beeswax (**Paper X**) with a sample treatment based on three steps (dissolution, liquid-liquid extraction, and concentration). Subsequently, the LC-MS/MS conditions were optimized, and the same LC conditions and slightly different MS/MS parameters were selected, with ESI in negative mode in

all cases. The proposed methods were validated, as described above, and several samples from different origins were analyzed. Finally, an LC-MS/MS method was developed and optimized to analyze SFN in honey (**Paper XI**). As occurred with GLSs in bee products, the chromatographic and detection conditions selected in **Paper V** were used to begin with the experiments. Once again, as in the case of GLSs, the best results in terms of extraction efficiency, solvent consumption and clean chromatograms were obtained with SPE. Next, the chromatographic and detection conditions were optimized, and the proposed method was validated and applied to the analysis of SFN in honey from different botanical origins.

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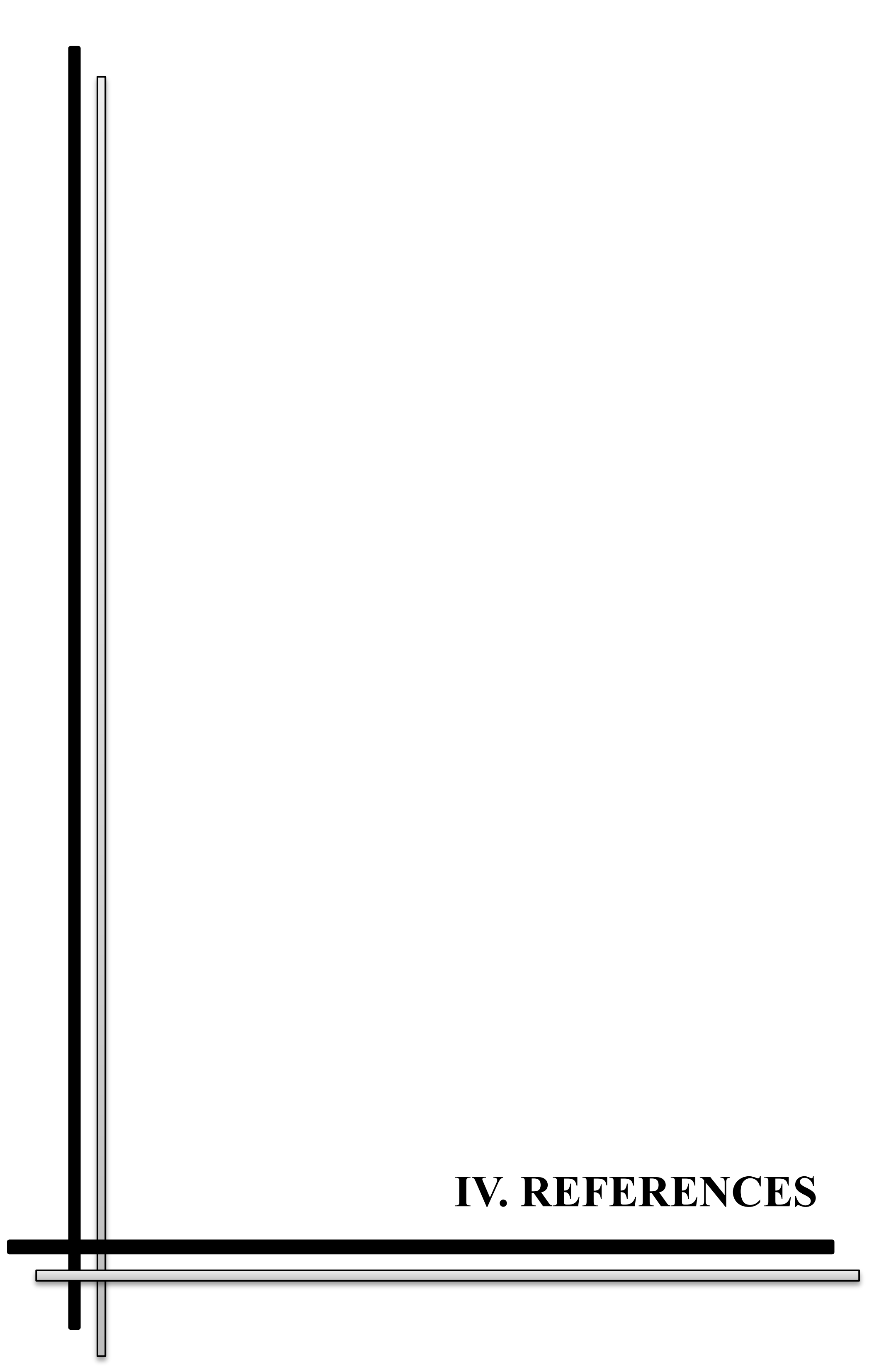

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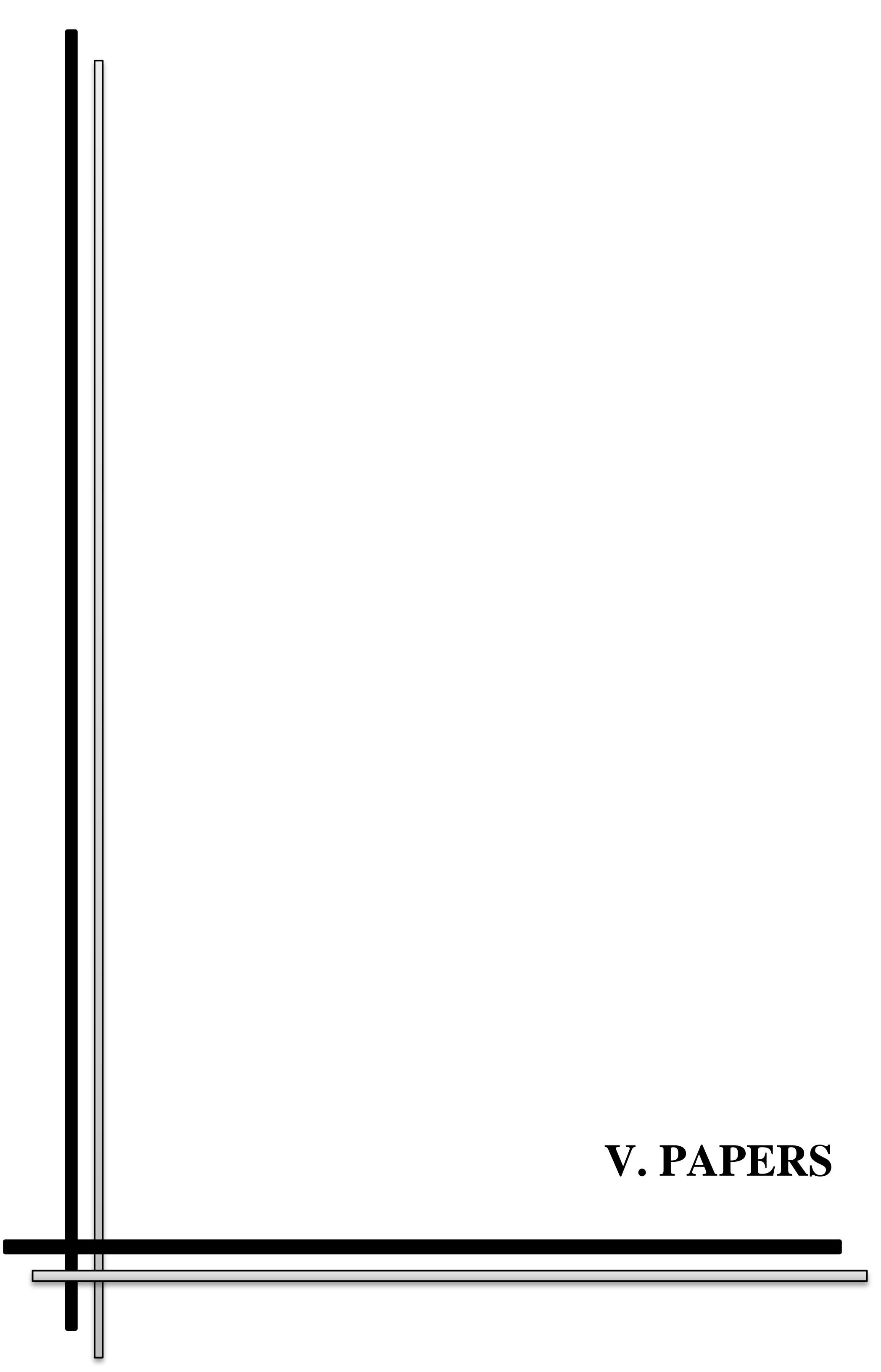

V. PAPERS

V.1. PAPER I. Development and validation of a liquid chromatography with mass spectrometry method to determine resveratrol and piceid isomers in beeswax

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Research Article

Development and validation of a liquid chromatography with mass spectrometry method to determine resveratrol and piceid isomers in beeswax

This paper represents the first report of a liquid chromatography coupled to electrospray ionization mass spectrometry method for simultaneously analyzing resveratrol and piceid isomers (*cis* and *trans*) in beeswax. An efficient extraction procedure has been proposed (average analyte recoveries were between 89 and 95%); this involved a solid–liquid extraction using a mixture of ethanol and water (80:20, v/v) and a concentration step in a rotary evaporator. The separation of all the compounds was achieved using a C_{18} column and a mobile phase composed of ammonium formate 0.03 M in water and acetonitrile in gradient elution mode at a flow rate of 1 mL/min. The method was fully validated in terms of selectivity, limits of detection and quantification, linearity, precision, and accuracy. The limits of detection and quantification ranged from 1.0 to 1.7 and 3.5 to 5.5 μg/kg, respectively. Finally, the proposed method was applied to analyze beeswax samples collected from experimental and organic apiaries.

Keywords: Beeswax / Liquid chromatography / Mass spectrometry / Piceid isomers / Resveratrol isomers
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1 Introduction

Resveratrol (3,4′,5-trihydroxystilbene), which is the most representative member of the stilbenes, can be found in two isomeric forms (*cis* and *trans*; see Supporting Information Fig. S1) that may be transformed into the corresponding *cis* or *trans* piceids (resveratrol-3-*O*-β-D-glucopyranoside), respectively [1]. These compounds have been widely studied, especially *trans*-resveratrol, because their potential health benefits, including anticancer, anti-inflammatory, antioxidant, anti-microbial, and anti-aging properties [2–4]. It must be commented that the potential of resveratrol for controlling *Nosema* infection in honeybees have been studied in laboratory conditions (*Apis mellifera*) [5–7], while other studies have investigated whether treatment with resveratrol affected the longevity of honeybees [5, 8]. Consequently, it may be supposed that residues of these compounds can be found in bee products, after the stilbenes are supplied to the beehive. Moreover, an analysis of resveratrol and its related compounds could be of significant interest to verify that those compounds have been adequately administered, and whether these compounds could be present in products that would be consumed/used by bees and humans. We have decided to investigate the potential presence of these compounds in beeswax, as it could be considered a beehive reservoir, and the compounds present in beeswax could directly affect the bee colony or be transmitted to other bee products, and in the case of the stilbenes, with positive health effects. Resveratrol and its related compounds have been studied in only one bee matrix, namely, honey [9]. The methodology involved SPE of the analytes before analysis by LC. SPE has been also successfully used with other matrices, especially wine and grapes [10–12]. However, several other sample treatments have been employed to analyze stilbenes, such as solvent extraction [1, 13–15], MAE or ultrasound-assisted extraction [16], SFE [17], or PLE [18]. Furthermore, the current trend in sample preparation techniques is focused on the simplification of those procedures to reduce the number or reagents and time spent on this step [19]. Thus, the use of microextraction [20] or stir bar sorptive extraction [2] has become popular in recent years to determine stilbenes. Some of those sample treatments could be used in beeswax, but taking into account our experience and the good results obtained in recent research [19], we decided to use solvent extraction as the sample treatment.

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The determination of resveratrol and its related compounds has usually been achieved by means of LC in RP mode (C_{18} columns) with UV or diode array [9,13,15,17,20–22], fluorescence [9,10,18,21,23], or MS detectors [9,14,15,22,24–26]. It should be also mentioned that the hyphenation of RP-LC and zwitterionic hydrophilic interaction LC with MS has been successfully employed in the last years for the analysis of phenols, including *cis*- and *trans*-resveratrol [25, 26]. As an alternative to LC, GC [27] and CE [28] have likewise been employed.

The main goal of this paper is to develop and propose an analytical method based on LC–ESI-MS to determine resveratrol and piceid isomers (*cis* and *trans*) in beeswax. We have also developed a simple and efficient sample treatment to analyze these compounds in beeswax. The proposed method was validated and applied to an examination of resveratrol and piceid isomers in beeswax from experimental and organic apiaries.

2 Materials and methods

2.1 Reagents and materials

trans-Resveratrol and *trans*-piceid standards, as well as ammonium formate, ammonium hydroxide, and formic acid, were supplied by Sigma–Aldrich Chemie (Steinheim, Germany). Ethyl acetate, acetonitrile, and methanol (LC grade) were obtained from Lab Scan (Dublin, Ireland), while ethanol (LC grade) was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained in a Milli-RO plus system together with a Milli-Q system from Millipore (Bedford, MA, USA). A Vibromatic mechanical shaker, a drying oven, and an ultrasonic bath (Ultrasons), all supplied by J.P. Selecta (Barcelona, Spain), a 5810 R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany), a Moulinette chopper device from Moulinex (Paris, France), and a R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were also employed for the extractions. A B100AP UV lamp from Cole-Parmer (Vernon Hills, IL, USA) and nylon syringe filters (0.45 μm) from Nalgene (Rochester, NY, USA) were also used.

2.2 Standard solutions

Stock standard solutions of each *trans* compound, at a concentration of 1000 mg/L, were prepared in a mixture of water and methanol (50:50, v/v). The *cis* isomers were obtained from *trans* isomers with the same procedure developed in a previous publication [9]. Briefly, mixtures of *trans* compounds were irradiated for 24 h with a Cole-Palmer B100APUV lamp at 254 nm. The *cis* isomer ratios were higher than 90% in both cases (93% for *cis*-resveratrol and 94% *cis*-piceid), and subsequently the concentration values for the *cis* isomer standards were assigned on the basis of the disappearance of *trans* isomers following irradiation. Once conversion had been carried out, a mixture of the four analytes was prepared by diluting with methanol, at a concentration of 50 mg/L for each compound. This was tested on a daily basis and diluted with methanol to produce a set of working standards.

Beeswax samples (1.0 g) were spiked before (A samples) or after (B samples) sample treatment, with different amounts of the standards to prepare the matrix-matched standards for validation (QC samples and calibration curves), matrix effect, and sample treatment studies. To obtain appropriate spiking of the A samples, beeswax was melted at 60°C before the addition of the corresponding concentration of stilbenes. Next, the beeswax was cooled at room temperature and stored in the dark at −20°C until analysis. Meanwhile, the stilbene standards were included in the reconstitution solution when preparing the B samples. Each QC sample was prepared with 1.0 g of beeswax samples spiked with three different concentrations of the compounds within the linear range: low QC level, 10 μg/kg; medium QC level, 300 μg/kg; and high QC level, 2500 μg/kg. All standard solutions were stored in amber glass containers and kept in the dark at −20°C; they were determined stable for more than three weeks.

2.3 Sample procurement and treatment

2.3.1 Samples

Beeswax samples ($n = 10$, E1–E10; where E means beeswax samples from experimental apiaries) were taken from experimental apiaries in which resveratrol was used to feed beehives under controlled conditions (Centro Apícola Regional of Marchamalo, Castilla la Mancha, Spain). Beeswax samples ($n = 6$, O1–O6; where O means, beeswax samples from organic apiaries) collected from organic apiaries where no resveratrol treatment had ever been applied, and also supplied by the Centro Apícola Regional of Marchamalo, were the object of a preliminary analysis by LC–ESI-MS to verify the presence of resveratrol and piceid isomers. Once the absence of residues from these compounds was confirmed, subsamples of the corresponding beeswax pools were used as blank samples to prepare matrix-matched samples for validation, matrix effect, and sample treatment studies. Finally, the beeswax samples collected were mixed and frozen at −20°C. Following this, these frozen samples (spiked and nonspiked) were cut into thin layers, ground and pooled for optimum sample homogeneity; subsequently, they were stored in the dark at −20°C until analysis.

2.3.2 Sample preparation

Briefly, 1 g of ground and homogenized beeswax together with 30 mL of an ethanol and water (80:20, v/v) mixture were transferred to a centrifuge tube. The resulting mixture was shaken mechanically for 20 min at 960 oscillations per minute in a Vibromatic shaker and then centrifuged for 10 min at 25°C and 10 414 × *g*. Following this, the supernatant was collected and transferred to a 50 mL conical flask and evaporated

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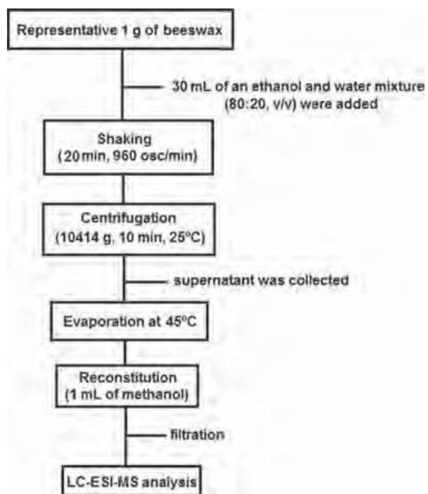

Figure 1. Analytical procedure work-up flow chart.

to dryness in a rotary evaporator at 45°C; the dry residue was reconstituted with 1 mL of methanol, filtered through a nylon filter, and injected into the LC–ESI-MS system. Figure 1 illustrates the scheme of the entire analytical method used during the present study.

2.4 Chromatographic system

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC–MS system was used, consisting of a vacuum degasser, a quaternary solvent pump, a programmed autosampler, a column oven, and a single quadrupole MS analyzer with an ESI interface, all of which were controlled by Agilent Chemstation software (Rev.A.10.02 (1757)). The analytical column was a Gemini 5 μm C_{18} 110 Å (150 × 4.6 mm id) protected by a Gemini C_{18} guard cartridge (4 × 3.0 mm), both from Phenomenex (Torrance, CA, USA). Subsequent to the optimization study, the mobile phase selected was a mixture of (A) ammonium formate 0.03M in water (pH 8.0) and (B) acetonitrile applied at a flow rate of 1 mL/min in the following gradient mode: (i) 0 min (A–B, 75:25, v/v); (ii) 12 min (A–B, 75:25, v/v); (iii) 20 min (A–B, 40:60, v/v); (iv) 23 min (A–B, 0:100, v/v); (v) 26 min (A–B, 0:100, v/v); (vi) 29 min (A–B, 75:25, v/v); (vii) 32 min (A–B, 75:25, v/v). In addition, a post-time of 3 min was included between injections. The injection volume and column temperature were set at 20 μL and 35°C, respectively. Operating conditions of the ESI interface in positive mode that provided the greatest sensitivity for the stilbenes, which were obtained after conducting FIA tests of the MS parameters, were as follows: fragmentor voltage, 140 V (resveratrol isomers) and 180 V (piceid isomers); drying gas (N_2) temperature, 325°C; drying gas flow (N_2), 12 L/min; nebulizer gas (N_2) pressure, 50 psi; capillary voltage, 3500 V; gain, 20. Full-scan spectra were obtained by scanning from m/z 100 to 600. The selected-ion monitoring mode for the most abundant ion of the compounds was used for quantifying resveratrol and piceid isomers, 227 and 389, respectively; two other ions (resveratrol isomers, 161 and 212; piceid isomers, 227 and 435) were employed to confirm the presence of those compounds (see Supporting Information Fig. S2). Chromatograms were treated with the Data Analysis program included in the ChemStation (Rev.A.10.02 (1757)) software from Agilent Technologies.

3 Results and discussion

3.1 Optimization of the extraction from beeswax

3.1.1 Selection of the extraction procedure

In view of the absence of specific procedures in which resveratrol and piceid isomers were determined in beeswax, it was decided that a solvent extraction should be developed and optimized as the sample treatment as a result of its simplicity, and because it has provided good results in previous research [19]. First, a study was made of the amount of beeswax that would be used in the experiments (0.5–2.0 g). It was found that 1.0 g of sample was the maximum weight that could be used, as recoveries were adequate and the S/N ratios showed improvement in relation to lower weights, despite being quite similar to those obtained for greater amounts of sample. Different solvents, some of which had been previously used to extract resveratrol and related compounds in other matrices [1, 13–15], were tested, namely methanol, ethanol, ethyl acetate. After several preliminary experiments had been performed, it was observed that the best recoveries (>60%) were obtained with ethanol or methanol. Different agitation sources were also checked (Vibromatic, magnetic agitation, or sonication), and the best results in terms of recoveries were obtained with the Vibromatic shaker at maximum speed (960 oscillations per minute). To increase extraction efficiency, and consideration being given to the existing scientific literature [1, 13, 14], it was decided that different mixtures of methanol and ethanol with water should be tested (100:0, 90:10, 80:20, 70:30, 50:50, v/v). The best results (higher recovery percentages) were achieved with ethanol and water mixtures, and more concretely for the 80:20, v/v solution, while the recoveries were lower for all the methanol and water solutions assayed. Thus, the ethanol and water mixture (80:20, v/v) was selected to continue the experiments. In addition, the volume of extractant solution (10–40 mL), and the extraction time with the Vibromatic (10–30 min) were also studied to check their influence on extraction recoveries (Supporting Information Tables S1 and S2). The results showed that 30 mL of the ethanol and water mixture chosen (80:20, v/v), and 20 min of extraction time provided adequate recoveries percentages (≥ 90%); conversely, larger volumes or longer extraction did not significantly affect those values. In addition, a centrifugation step (10 min at 25°C and 10 414 × g)

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Table 1. Evaluation of the efficiency of the sample treatment and the matrix effect

QC sample	Evaluation of the sample treatment Mean (%) ± RSD (%)			Evaluation of the matrix effect Mean (%) ± RSD (%)		
	Low	Medium	High	Low	Medium	High
trans-Piceid	95 ± 5	92 ± 3	94 ± 5	60 ± 4	63 ± 5	65 ± 5
cis-Piceid	92 ± 3	91 ± 2	89 ± 2	70 ± 5	68 ± 4	72 ± 5
trans-Resveratrol	92 ± 3	90 ± 4	91 ± 4	52 ± 5	55 ± 5	51 ± 6
cis-Resveratrol	91 ± 4	93 ± 3	90 ± 5	60 ± 6	62 ± 5	58 ± 5

Data obtained as described in Subsections 3.1.2 and 3.3.2 ($n = 6$).
Low QC, 10 μg/kg; medium QC, 300 μg/kg; high QC, 2500 μg/kg.

was included to facilitate separation between the solid and liquid phases. Afterwards, the supernatant was collected, transferred to a conical flask, and gently evaporated to dryness in a rotary evaporator at 45°C. Next, reconstitution was deemed appropriate so as to improve extraction efficacy. Different solvents (methanol, ethanol, and an 80:20, v/v ethanol and water mixture) and volumes (0.5–2.0 mL) were assayed to obtain the best results. It was observed that the recoveries were similar in all cases, and that solvent volumes higher than 1 mL did not improve the recovery percentages. Moreover, the peak shapes (width and height) were enhanced when methanol was used. Thus, it was decided that 1 mL of methanol should be employed to reconstitute the dry residue.

3.1.2 Extraction efficiency

To assess the efficiency of the proposed sample, a comparison was made of the results obtained for blank beeswax samples spiked at three different stilbene concentrations (low, medium, and high QC levels), either before (A samples) or following (B samples) sample treatment. The resulting recovery values (Table 1) ranged from 89 to 95% in all cases, indicating that the sample treatment procedure selected was adequate and efficient. The results were good not only in terms of extraction efficiency, but also as regards simplicity and solvent consumption, as the overall sample treatment consisted of three stages (extraction, centrifugation, and concentration), and 31 mL of solvent was used per sample.

3.2 Chromatographic conditions

We decided to start separation optimization by using the chromatographic conditions that were proposed in our previous study [9] in which stilbenes were analyzed in honey. The analytical column was a Gemini C_{18} (150 × 4.6 mm), and the mobile phase was a mixture of formic acid in water (1%, v/v; pH 2.1) and acetonitrile at at a flow rate of 1 mL/min in isocratic elution mode. It was necessary to include a guard cartridge (Gemini C_{18}, 4 × 3.0 mm) in the present study to preserve the lifetime and performance of the column. In the previously reported LC conditions and with other different mobile phase compositions, it was not possible to achieve a complete separation of all the analytes from certain matrix components, especially *cis*-resveratrol. We decided to use ammonium formate (0.03 M; pH 6.5) instead of formic acid, as it has been proven to be effective in previous tests, maintaining acetonitrile as organic solvent. Several experiments were conducted by means of different mobile phases to separate all the analytes in the shortest time as possible, and the best results were obtained with the gradient elution conditions described in Section 2.4. It was necessary to use 100% of acetonitrile (gradient steps iv and v) to elute certain remaining matrix components subsequent to elution of the last analyte (*cis*-resveratrol), and for this reason it was also necessary to introduce an equilibration time step to obtain reproducible chromatograms and to return to the baseline (gradient steps vi, vii, and post-time). In addition, the ionic strength of the mobile phase was modified through the concentration of ammonium formate (0.01, 0.03, or 0.05 M). It was found that the best performance in terms of retention time and symmetry of the peaks were obtained when using ammonium formate 0.03 M. A study was also made of the influence of pH on analyte separation. Several pH values were assayed (pH 4.0–9.0), and although chromatographic performance was quite similar in all cases, slightly better peak symmetries and resolutions were obtained at pH 8.0. Consequently, pH 8.0 was selected for the continuation of the experiments.

Tests were carried out to study the influence of the column temperature (between 25 and 50°C at 5°C intervals) and the injection volume (between 5 and 30 μL) on the S/N ratio. The results showed an increase in the S/N when up to 20 μL was injected, and a significant loss of symmetry, which did not compensate for the slight increase in the S/N ratio, was observed at temperatures over 35°C. Therefore, it was decided that 35°C and 20 μL, respectively, should be the optimal temperature and injection volume. With the chromatographic conditions described above, all the compounds were separated in less than 20 min, and the overall run time was 32 min (see Fig. 2). In comparison with our previous proposal in honey [9], the four analytes eluted earlier, the overall run time was longer because of the required cleaning and equilibration steps.

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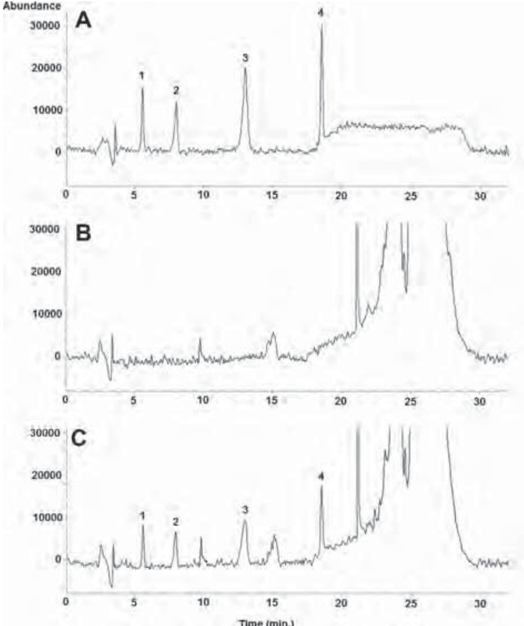

Figure 2. Representative LC–ESI-MS chromatograms (selected-ion monitoring mode) obtained from (A) an LOQ level standard solution of (1) *trans*-piceid, (2) *cis*-piceid, (3) *trans*-resveratrol, and (4) *cis*-resveratrol; (B) a blank beeswax sample; and (C) a blank beeswax sample spiked at LOQ level in each analyte. The LC–ESI-MS conditions are described in Section 2.4, while LOQ values are listed in Table 2.

3.3 MS conditions

3.3.1 MS optimization

Resveratrol and piceid isomers have previously been analyzed in negative ESI mode [9, 14, 15, 25, 26]. A few experiments (FIA) were conducted to choose the optimum ESI-MS (negative mode) parameters by using the infusion mode (5 μL/min) of standard and matrix-matched solutions (1000 μg/L) of the analytes. The best results were found with the values described in Section 2.4, which were different from the values used in our previous research [9]. The stilbenes studied showed a major ion m/z (100% of relative abundance) on their full-scan ESI-MS spectra (see Supporting Information Fig. S2), corresponding to the deprotonated molecular ions $[M–H]^-$, which was used as a quantification ion with the selected-ion monitoring mode (227 resveratrol isomers; 389 piceid isomers). Meanwhile, two ions that displayed relative intensities higher than 5% in the mass spectra were selected as confirmation ions (resveratrol isomers, 161 and 212; piceid isomers, 227 and 435).

3.3.2 Matrix effect

To observe how the matrix influenced ESI ionization, the peak areas of the resveratrol and piceid isomers in standard working solutions were compared with those acquired with blank beeswax samples spiked at three different concentrations (low, medium, and high QC levels) after sample treatment (B samples). The responses of all compounds at the three concentrations assayed were below 75% in all cases, as can be seen in Table 1. Moreover, if a comparison is made between the stilbene peaks in standard (Fig. 2A) and matrix-matched solutions (Fig. 2C) at the same concentration levels, a decrease in the analyte signals can be clearly observed due to the matrix effect. Hence, it was concluded that the matrix (beeswax) affected the ESI of all the compounds, generating ion suppression.

3.4 Method validation

Validation was carried out following different international guidelines [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm073384.pdf (accessed October 20, 2014), 29], determining selectivity, LOD, and LOQ, as well as linearity, precision, and accuracy.

3.4.1 Selectivity

To assess the selectivity of the method, extracts from blank beeswax samples ($n = 6$) along with blank beeswax samples spiked with the compounds under study were injected (see Fig. 2B and C). No matrix interference was observed at the retention time of each of the analytes and, as can be seen in the mass spectra (Supporting Information Fig. S2), the relative intensities of the selected diagnostic ions in the spiked samples matched the corresponding calibration standard within ±10%. Therefore, it can be concluded that the method was selective for determining resveratrol and piceid isomers in beeswax.

3.4.2 LOD and LOQ

The LODs and LOQs were experimentally determined by injecting a number of blank beeswax samples ($n = 6$), in which the absence of stilbene residues was previously confirmed, and measuring the magnitude of background analytical response at the elution time of the resveratrol and piceid isomers. The LODs and LOQs were estimated to be three and ten times the S/N ratio, respectively (Table 2). As may be observed, the LOD and LOQ values were 1.0–1.7 and 3.5–5.5 μg/kg, respectively. Since no study has been published in which resveratrol and piceid isomers were determined in beeswax, it was not possible to make a proper comparison; moreover, it should also be taken into account that the matrix usually plays an important role in MS detection. However, the LOD and LOQ values obtained were slightly lower than

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Table 2. Calibration curve data ($n = 6$) and LOD and LOQ values

Compound	Calibration curve	Analytical range (μg/kg)	Slope confidence intervals	R^2	LOD[a] (μg/kg)	LOQ[a] (μg/kg)
trans-Piceid (1)[b]	Standard	5.2–5000 (μg/kg)	$2.9 \times 10^6 \pm 1.8 \times 10^5$	0.997	1.6	5.2
	Matrix-matched		$1.8 \times 10^6 \pm 1.4 \times 10^5$	0.995		
cis-Piceid (2)[b]	Standard	5.5–5000 (μg/kg)	$2.6 \times 10^6 \pm 2.9 \times 10^5$	0.994	1.7	5.5
	Matrix-matched		$1.8 \times 10^6 \pm 2.1 \times 10^5$	0.993		
trans-Resveratrol (3)[b]	Standard	4.6–5000 (μg/kg)	$9.9 \times 10^6 \pm 4.7 \times 10^5$	0.998	1.4	4.6
	Matrix-matched		$5.3 \times 10^6 \pm 6.2 \times 10^5$	0.994		
cis-Resveratrol (4)[b]	Standard	3.5–5000 (μg/kg)	$1.0 \times 10^7 \pm 5.9 \times 10^5$	0.997	1.0	3.5
	Matrix-matched		$6.0 \times 10^6 \pm 6.5 \times 10^5$	0.993		

The stilbenes concentrations were same in the standard and spiked beeswax samples according to the proposed sample treatment and the unit conversion.
a) LOD and LOQ values were calculated in matrix (beeswax).
b) Compounds were numbered according to Fig. 2.

those obtained in the only study where these compounds were analyzed in a bee matrix (honey; LOD < 4 μg/kg; LOQ < 11 μg/kg) [9].

3.4.3 Linearity studies

Matrix-matched standard calibration curves were used to quantify the resveratrol and piceid isomers in the beeswax, as a matrix effect that affected analyte ionization was observed (Section 3.3.2). This finding was corroborated by the fact that the slopes of the standard and matrix-matched calibration curves did not overlap at the confidence intervals (Table 2). Blank beeswax was treated accordingly with the proposed procedure and spiked with variable amounts of the resveratrol and piceid isomers over an analytical range between LOQ and 5000 μg/kg (calibration levels of LOQ, 10, 50, 150, 300, 625, 1250, 2500, and 5000 μg/kg). Calibration curves ($n = 6$) were constructed by plotting the signal on the y-axis (analyte peak areas for ESI-MS) against analyte concentration on the x-axis. The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied, and the coefficient of the determination values (R^2) was above 0.99 in all cases (Table 2). Moreover, the lack of bias was confirmed by a t-test and a study of the distribution of residuals.

3.4.4 Precision and accuracy studies

Intraday precision and accuracy experiments were performed concurrently by repeated sample analysis using blank beeswax samples spiked at three different concentrations of resveratrol and piceid isomers (low, medium, and high QC levels) on the same day of ($n = 6$). Interday precision and accuracy were assessed by an analysis of blank beeswax samples spiked at three concentrations of the compounds studied (low, medium, and high QC levels) over three consecutive days ($n = 6$). Precision was expressed as the %RSD at three concentration levels for each analyte. Accuracy was calculated by means of relative error (%RE). Intra- and interday precision (%RSD values) for the compounds under study were in all cases lower than or equal to 8% (Supporting Information Table S3). Accuracy (%RE values) for resveratrol and piceid isomers ranged from 4 to 7% for the intraday readings, and from 5 to 8% for the interday values (Supporting Information Table S3). Such results indicate that the present method is both precise and accurate, and it should be added that although an internal standard is recommended in most validation guidelines, it was not necessary in the present study because of this degree of precision and accuracy.

3.5 Application of the method

Beeswax samples were obtained from experimental (E1–E10) and organic (O1–O6) apiaries and analyzed with the proposed method to search for residues of resveratrol and piceid isomers. All beeswax samples were examined in triplicate. Only two of the analytes, namely, *cis*- and *trans*-resveratrol, were found in concentrations above the LOQs in the beeswax sampled from resveratrol-treated apiaries (E1–E10, see Table 3). *trans*-Resveratrol was detected in six of these beeswax samples (10–300 μg/kg), while *cis*-resveratrol was determined in only two of these samples (15 and 30 μg/kg). Meanwhile, no residues of the stilbenes under study were detected in any of the beeswax samples used as blanks (O1–O6; Table 3). Thus, it can be postulated that feeding with resveratrol is directly related with the residues of stilbenes detected in some of the beeswax obtained from experimental apiaries, as the stilbenes were not detected in samples from organic apiaries. Moreover, it has been demonstrated that there is a need to develop an analytical method to determine such compounds in beeswax; such a method should, as we have proposed, be very sensitive and have a suitable linear range.

4 Concluding remarks

A new analytical approach has been developed to simultaneously identify and quantify resveratrol and piceid isomers in

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Table 3. Results of the investigation of resveratrol and piceid isomers in beeswax samples (means of triplicate analyses, microgram per kilogram)[a)]

Sample	*trans*-Resveratrol	*cis*-Resveratrol
E1	10	<LOD
E2	250	30
E3	<LOD	<LOD
E4	300	<LOD
E5	<LOD	<LOD
E6	150	<LOD
E7	<LOD	<LOD
E8	90	15
E9	<LOD	<LOD
E10	200	<LOD
O1	<LOD	<LOD
O2	<LOD	<LOD
O3	<LOD	<LOD
O4	<LOD	<LOD
O5	<LOD	<LOD
O6	<LOD	<LOD

a) All other analytes were less than LOD for all samples.
E, beeswax samples from experimental apiaries; O, beeswax samples from organic apiaries.

beeswax. A simple, efficient, and relatively cheap (a low consumption of solvents) sample treatment based on three steps (extraction, centrifugation, and concentration) was proposed. Moreover, the LC–ESI-MS method was fully validated, and the data demonstrated that it is consistent and reliable, with a wide linear range of applicability, and that it was necessary to employ matrix-matched standards to perform correct ESI-MS quantification of the stilbenes in beeswax, as ion suppression was observed. The usefulness of the method developed was verified by an analysis of several beeswax samples of different origins. *trans*-Resveratrol residues were detected in six of the samples from experimental apiaries over a wide concentration range, while *cis*-resveratrol was also found in two of these samples. Moreover, no residues of the stilbenes under study were detected in any of the beeswax samples from organic apiaries.

In summary, the proposed method presents an innovative potential for analyzing resveratrol and piceid isomers at trace levels in a complex matrix, such as beeswax. In addition, the detection of stilbenes in beeswax from experimental apiaries has demonstrated that residues of these compounds can be found in a bee product (beeswax) after the stilbenes are supplied to the beehive.

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V.2. PAPER II. Determination of resveratrol and piceid isomers in bee pollen by liquid chromatography coupled to electrospray ionization-mass spectrometry

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Determination of Resveratrol and Piceid Isomers in Bee Pollen by Liquid Chromatography Coupled to Electrospray Ionization-Mass Spectrometry

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Abstract A new method has been developed to determine resveratrol and piceid isomers in bee pollen using liquid chromatography (LC) coupled to electrospray ionization-mass spectrometry (ESI-MS). An efficient extraction procedure has also been proposed (average analyte recoveries were between 89 and 96 %); this involved a cleaning step with hexane, a solid–liquid extraction using a mixture of ethanol and water (80:20, *v*/*v*), and a concentration step in a rotary evaporator. The separation of all the compounds was achieved using a C_{18} column (150×4.6 mm) and a mobile phase composed of 1 % (*v*/*v*) formic acid in water and acetonitrile at a flow rate of 0.8 mL/min. The method was fully validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, precision, and accuracy. The LOD and LOQ values ranged from 10 to 25 and 35 to 80 μg/kg, respectively. Finally, the proposed method was applied to analyze bee pollen samples from different origins.

Keywords Bee pollen · LC-ESI-MS · Piceid isomers · Resveratrol isomers · Solid–liquid extraction

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Introduction

Resveratrol (3,4′,5-trihydroxystilbene) is the most representative member of the stilbenes, which are polyphenolic phytoalexins produced by plants as defense substances against biotic or abiotic stress (Cacho et al. 2013). In nature, it can be found in two isomeric forms (*cis* and *trans*; see Fig. 1), which may be transformed into the corresponding *cis* or *trans* piceids (resveratrol-3-*O*-β-D-glucopyranoside), respectively (Liu et al. 2013). Resveratrol and its related compounds have all been positively linked to health benefits, including anticancer, anti-inflammatory, antioxidant, antimicrobial, and antiaging properties (Cacho et al. 2013; Fernández-Mar et al. 2012; Leiro et al. 2010; Liu et al. 2013; Shukitt-Hale et al. 2006; Weng et al. 2010). It must be specified that the biological activity of *trans*-resveratrol has been more widely researched than the other isomers (*cis*-resveratrol, *trans*-piceid, and *cis*-piceid), but it appears that all of them may also have health benefits (Liu et al. 2013). What is more, the supposed benefits of these natural compounds have been investigated not only in humans, but also in animals such as honeybees. Some studies, for example, have focused their attention on observing in laboratory conditions the potential of resveratrol for controlling *Nosema* infection in honeybees (*Apis mellifera*) (Costa et al. 2010; Maistrello et al. 2008; Porrini et al. 2011), while two further studies have examined whether treatment with resveratrol affected the longevity of honeybees (Costa et al. 2010) or lengthened the average lifespan in wild-type honeybees (Rascón et al. 2012). Consequently, it may be surmised that residues of these compounds can be found in bee products, such as honey, beeswax, or bee pollen, after the stilbenes are supplied to the beehive. Moreover, an analysis of resveratrol and its related compounds could be of significant interest to verify that the stilbenes have been adequately administered, and whether these compounds could be present in bee products that would be consumed by

Fig. 1 Chemical structures of resveratrol and piceid isomers (*MW* molecular weight)

humans, which would be positively affected by their beneficial health properties. We have decided to investigate the potential presence of these compounds in bee pollen, a widely consumed natural food supplement due to its essential nutritional elements (carotenoids, flavonoids, phytosterols, polyphenols, and other beneficial compounds) (Kačániová et al. 2013); the decision has been taken not only for the reasons above-mentioned, but because, to the best of our knowledge, no research has been published in which resveratrol or its related compounds have been analyzed in bee pollen. In fact, these compounds have been studied in only one bee matrix, namely, honey (Soto et al. 2012). The methodology involved a solid-phase extraction (SPE) of the analytes prior to their analysis by liquid chromatography (LC) coupled to several detectors (diode array (DAD), fluorescence, electrospray ionization-mass spectrometry, ESI-MS). As has been previously mentioned, SPE was employed as sample treatment in the only method for analyzing resveratrol and piceid isomers in a bee product (Soto et al. 2012); yet, SPE was also used in a few cases to perform this task in other matrices, mostly in wines and grapes (Hashim et al. 2013; Rodríguez-Cabo et al. 2014; Viñas et al. 2008). However, several other sample treatments have been also employed to analyze resveratrol as liquid–liquid extraction (Counet et al. 2006) or solid–liquid extraction (Di Lecce et al. 2014; Liu et al. 2013; Piñeiro et al. 2013); in certain cases, these were assisted by ultrasound or microwave (Ballard et al. 2010), stir bar sorptive extraction (Cacho et al. 2013), supercritical fluid extraction (Casas et al. 2010), or pressurized liquid extraction (Piñeiro et al. 2006) in order to improve such processes. The determination of resveratrol and its related compounds has usually been achieved by means of LC in reverse phase mode (C_{18} columns) with different detectors like ultraviolet or DAD (Cvejic et al. 2010; Grippi et al. 2008; Kong et al. 2011; Piñeiro et al. 2013; Nicoletti et al. 2007; Soto et al. 2012), fluorescence (Grippi et al. 2008; Soto et al. 2012), or MS (Counet et al. 2006; Di Lecce et al. 2014; Hashim et al. 2013; Jaitz et al. 2010; Ji et al. 2014; Kong et al. 2011; Montsko et al. 2008; Moss et al. 2013; Nicoletti et al. 2007; Soto et al. 2012). As an alternative to LC, gas chromatography (Cacho et al. 2013; Dias et al. 2013) and capillary electrophoresis (Franquet-Griell et al. 2012) have likewise been employed. Our aim therefore was to develop a new LC-ESI-MS method to separate and determine resveratrol and piceid isomers (*cis* and *trans*) in bee pollen; to the best of our knowledge, this is the first study in this field to date. Moreover, we wish to propose and optimize a specific, efficient, and simple extraction procedure of these compounds. Accordingly, the proposed method was validated in an applied analysis of resveratrol and piceid isomers in bee pollen from different origins (commercial bee pollen and bee pollen samples obtained from experimental apiaries where resveratrol was used).

Materials and Methods

Reagents and Materials

Trans-resveratrol and *trans*-piceid standards, as well as formic acid, were supplied by Sigma-Aldrich Chemie (Steinheim, Germany). Hexane (Pestiscan grade), acetonitrile ethyl acetate, and methanol (LC grade) were obtained from Lab Scan Ltd. (Dublin, Ireland), while ethanol (LC grade) was purchased from Panreac (Barcelona, Spain). Ultrapure water was obtained in a Milli-RO plus system together with a Milli-Q system from Millipore (Bedford, MA, USA). A vibromatic mechanical shaker, a drying oven, and an ultrasonic bath (Ultrasons) were supplied by J.P. Selecta S.A.

(Barcelona, Spain), a 5810 R refrigerated bench-top Eppendorf centrifuge (Hamburg, Germany), a Moulinette chopper device from Moulinex (Paris, France), and a R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were also employed for all the extractions. A B100AP UV lamp from Cole-Parmer (Vernon Hills, IL, USA) and nylon syringe filters (0.45 μm) from Membrane Solutions (Ohio, USA) were also used.

Standard Solutions

Stock standard solutions of each *trans*-compound, at a concentration of 1000 mg/L, were prepared in a mixture of water and methanol (50:50, *v*/*v*). *Cis*-isomers were obtained from *trans*-isomers with the same procedure developed in a previous publication (Soto et al. 2012). Briefly, mixtures of *trans*-compounds were irradiated for 24 h with a Cole-Palmer B100APUV lamp at 254 nm. The *cis*-isomer ratios were higher than 90 % in both cases (93 % for *cis*-resveratrol and 94 % *cis*-piceid), and subsequently, the concentration values for the *cis*-isomer standards were assigned on the basis of the disappearance of *trans*-isomers following irradiation. Once conversion had been carried out, a mixture of the four analytes was prepared by diluting with methanol, at a concentration of 50 mg/L for each compound. This was tested on a daily basis and diluted with methanol to produce a set of working standards. Bee pollen samples (1.0 g) were spiked before (*A* samples) or after (*B* samples) sample treatment, as described in subsection "Sample Preparation," with different amounts of the standards in order to prepare the matrix-matched standards for validation (quality control (QC) samples and calibration curves), matrix effect, and sample treatment studies. Each QC sample was prepared with 1.0 g of bee pollen samples spiked with three different concentrations of the compounds within the linear range: low QC level—100 μg/kg; medium QC level—1000 μg/kg; and high QC level—10,000 μg/kg. All standard solutions were stored in amber glass containers and kept in the dark at −20 °C; they were determined stable for more than 3 weeks.

Sample Procurement and Treatment

Samples

Corbicular bee pollen samples (*n*=10, **E1–E10**) from experimental apiaries in which resveratrol was used to feed beehives under controlled conditions were supplied by the Centro Apícola Regional (CAR) of Marchamalo (Castilla la Mancha, Spain). Commercial bee pollen samples (*n*=6, **C1–C6**) were purchased in local markets (Valladolid, Spain) and were from different Spanish regions. Corbicular bee pollen samples (*n*=6, **O1–O6**) collected from organic apiaries where no resveratrol treatment had ever been applied, and also supplied by the CAR of Marchamalo, were the object of a preliminary analysis by LC-ESI-MS in order to verify the presence of resveratrol and piceid isomers. Once the absence of residues from these compounds was confirmed, subsamples of the corresponding pollen pools were used as blank samples to prepare matrix-matched samples for validation (QC and calibration curves), matrix effect, and sample treatment studies. Finally, the pollen samples collected were mixed and dried at 45 °C in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in the dark at −20 °C until analysis.

Sample Preparation

Briefly, 1 g of pollen sample and 10 mL of hexane were transferred to a centrifuge tube. The mixture was shaken mechanically for 10 min at 960 oscillations per minute in a Vibromatic and then centrifuged for 5 min at 10 °C and 10,414 g. Following this, the supernatant was discarded, and 20 mL of an ethanol and water mixture (80:20, *v*/*v*) were added to extract the analytes; the resulting mixture was shaken and centrifuged as described above. Finally, the supernatant was transferred to a 25-mL conical flask and evaporated to dryness in a rotary evaporator at 45 °C; the dry residue was reconstituted with 1 mL of methanol, filtered through a nylon filter, and injected into the LC-ESI-MS system. Figure 2 illustrates the scheme of the entire analytical method used during the present study.

LC-ESI-MS System

An Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MS system was used, consisting of a vacuum degasser, a quaternary solvent pump, a programmed autosampler, a column oven, and a single quadrupole MS analyzer with an electrospray (ESI) interface, all of which were controlled by Agilent Chemstation software (Rev.A.10.02, 1757). The analytical column was a Gemini 5 μm C_{18} 110 Å (150×4.6 mm I.D.) protected by a Gemini C_{18} security guard cartridge (4×3.0 mm), both from Phenomenex (Torrance, CA, USA). Subsequent to the optimization study, the mobile phase selected was a mixture of (A) formic acid in water (1 %, *v*/*v*) and (B) acetonitrile applied at a flow rate of 0.8 mL/min in an isocratic elution mode (72 %(A)/28 %(B), *v*/*v*). However, it is necessary to introduce some gradient steps after the isocratic separation of the analytes in order to elute from the column certain matrix components which remained subsequent to elution of the last analyte (*cis*-resveratrol), and because it was also necessary to introduce a further equilibration time step to obtain reproducible chromatograms. Thus, the final mobile phase conditions were as follows: (i) 0 min (A-B, 71:29, *v*/*v*); (ii) 21 min (A-B, 71:29, *v*/*v*); (iii) 24 min (A-B, 0:100, *v*/*v*); (iv) 27 min (A-B, 0:100, *v*/*v*); (v) 30 min (A-B, 71:29, *v*/*v*);

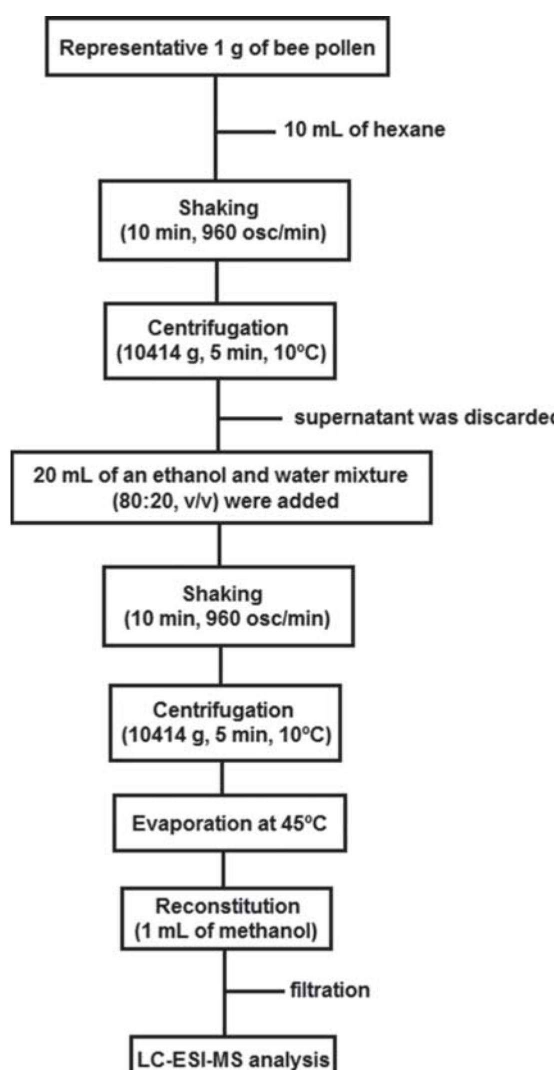


Fig. 2 Analytical procedure work-up flow chart

(vi) 33 min (A-B, 71:29, *v*/*v*). The injection volume and column temperature were set at 20 μL and 30 °C, respectively. Operating conditions of the ESI interface in negative mode that provided the greatest sensitivity for the stilbenes, which were obtained after conducting flow injection analysis tests of the MS parameters, were as follows: fragmentor voltage, 140 V (resveratrol isomers) and 180 V (piceid isomers); drying gas (N_2) temperature, 350 °C; drying gas flow (N_2), 12 L/min; nebulizer gas (N_2) pressure, 50 psi; capillary voltage, 4000 V; gain, 20. Full-scan spectra were obtained by scanning from *m/z* 100 to 600. The selected ion monitoring mode (SIM) for the most abundant ion of the compounds was used for quantifying resveratrol and piceid isomers, *m/z* 227 and *m/z* 389, respectively; two other ions (resveratrol isomers, *m/z* 161 and *m/z* 212; piceid isomers, *m/z* 227 and *m/z* 435) were employed to confirm the presence of those compounds. Chromatograms were treated with the Data Analysis program included in the ChemStation (Rev.A.10.02 (1757)) software from Agilent Technologies.

Method Validation

Validation was carried out following different international guidelines (International Conference on Harmonization 2005; Thompson et al. 2002) determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, precision, and accuracy. As previously stated in subsection "Samples," blank pollen samples were employed for the validation studies.

Selectivity

To determine the selectivity of the proposed method, a set of unspiked bee pollen samples (n=6) was injected onto the chromatographic system, and the results were compared with those obtained for spiked bee pollen samples.

Limits of Detection and Quantification

The LODs and LOQs were experimentally determined by injecting a number of blank pollen samples (n=6), in which the absence of stilbene residues was previously confirmed, and measuring the magnitude of background analytical response at the elution time of the resveratrol and piceid isomers. The LODs and LOQs were estimated to be three and ten times the signal to noise (S/N) ratio, respectively.

Linearity Studies

Matrix-matched standard calibration curves were used to quantify the resveratrol and piceid isomers in the bee pollen, as a matrix effect that affected analyte ionization was observed (see subsection "Matrix Effect"). Blank pollen was treated accordingly with the proposed procedure and spiked with variable amounts of the resveratrol and piceid isomers over an analytical range between LOQ and 10,000 μg/kg (calibration levels of LOQ, 100, 250, 500, 1000, 2000, 5000, and 10,000 μg/kg). Calibration curves (n=6) were constructed by plotting the signal on the y-axis (analyte peak areas for ESI-MS) against analyte concentration on the x-axis.

Precision and Accuracy Studies

Intraday precision and accuracy experiments were performed concurrently by repeated sample analysis using blank bee pollen samples spiked at three different concentrations of

resveratrol and piceid isomers (low, medium, and high QC levels) on the same day of $n=6$). Interday precision and accuracy were assessed by an analysis of blank bee pollen samples spiked at three concentrations of the compounds studied (low, medium, and high QC levels) over three consecutive days ($n=6$). Precision was expressed as the percentage of relative standard deviation (% RSD) at three concentration levels for each analyte. Accuracy was calculated by means of relative error (%RE).

Results and Discussion

Optimization of the Extraction from Bee Pollen

Selection of the Extraction Procedure

In view of the absence of specific procedures in which resveratrol and piceid isomers were determined in bee pollen, it was decided that a solid–liquid extraction should be developed and optimized as the sample treatment as a result of its simplicity. The extraction procedure was optimized on blank bee pollen samples spiked with different amounts of the stilbenes. First, a study was made of the amount of bee pollen that would be used in the experiments (0.5–2.0 g). It was found that 1.0 g of sample was the maximum weight that could be used, as recoveries were adequate and S/N ratios showed improvement in relation to lower weights, despite being quite similar to those obtained for greater amounts of pollen (data not shown). Different solvents, some of which had been previously used to extract resveratrol and related compounds in other matrices (Di Lecce et al. 2014; Liu et al. 2013; Piñeiro et al. 2013), were tested, namely, methanol, ethanol, ethyl acetate, and mobile phase (0.1 % formic acid in water and acetonitrile—72:28, *v*/*v*). After several preliminary experiments had been performed, it was observed that the best recoveries (~70 %) were obtained with ethanol or methanol. Different agitation sources were also checked (Vibromatic, magnetic agitation at 45 °C or sonication), and the best results in terms of recoveries (data not shown) were obtained with the Vibromatic at maximum speed (960 oscillations per minute). In order to increase extraction efficiency and consideration being given to the existing scientific literature (Di Lecce et al. 2014; Liu et al. 2013; Piñeiro et al. 2013), it was decided that the different mixtures of methanol and ethanol should be tested with water and that the influence of the pH on the extraction of the analytes be ascertained by using acid (HCl) or basic (NaOH) media (see Fig. 3). The best results (higher recovery percentages) were achieved with ethanol and water mixtures (80:20 and 70:30 *v*/*v*), while the recoveries were worse for the other solutions assayed (data not shown). However, as the recoveries were slightly better for the 80:20 (*v*/*v*), this mixture was selected to continue the experiments. In addition, the volume of extractant solution (10–30 mL), extraction time (5–30 min), and number of extractions (1 or 2) were also studied in order to check their influence on extraction recoveries. The results showed that 20 mL of the ethanol and water mixture chosen (80:20, *v*/*v*), 10 min of extraction time, and one extraction step provided adequate recovery percentages (>90 %); conversely, larger volumes, longer extraction times, or a second extraction did not significantly affect those values (data not shown). In addition, a centrifugation step (5 min at 10 °C and 10,414 g) was included in order to facilitate separation between the solid and liquid phases. Afterward, the supernatant was collected, transferred to a conical flask, and gently evaporated to dryness in a rotary evaporator at 45 °C. Next, reconstitution was deemed appropriate so as to improve extraction efficacy. Different solvents (methanol, ethanol, mobile phase (1 % formic acid and acetonitrile, 72:28, *v*/*v*), and a (80:20, *v*/*v*) ethanol and water mixture) and volumes (0.5–2.0 mL) were assayed in order to obtain the best results. It was observed that the recoveries were similar in all cases and that solvent volumes higher than 1 mL did not improve the recovery percentages. Moreover, the peak shapes (width and height) were enhanced when methanol was used (data not shown). Thus, it was decided that 1 mL of methanol should be employed to reconstitute the dry residue. It should be also pointed out that no matrix interferences coeluted with the stilbenes when injected bee pollen samples were treated with the proposed

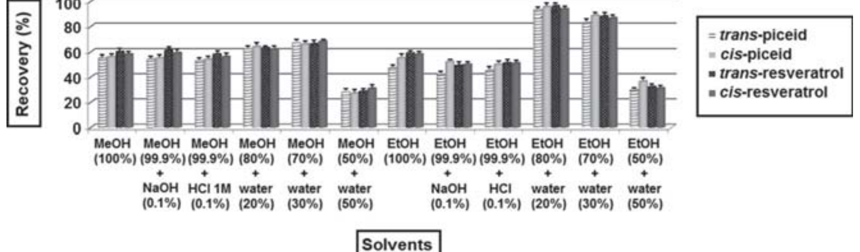


Fig. 3 Recovery (%) obtained after testing with 20 mL of different solvents to extract the stilbenes from spiked bee pollen samples at the medium quality control (QC) level (1000 μg/kg), treated with the proposed sample treatment (described in subsection "Sample Preparation"). Data represent the mean of three replicates ± the standard deviation of the mean (*narrow bars*)

extraction procedure, and the recovery percentages were acceptable for all the compounds (>85 %). However, several matrix components eluted at lower and higher retention times, which implied the need for longer run times in order to elute these compounds and avoid problems with the column. Therefore, the decision was taken to introduce a previous cleaning stage so as to remove as many matrix compounds as possible yet without affecting the analytes. Certain solvents (hexane, ethyl ether, and dichloromethane), which are less polar than the selected extraction mixture, were tested. After several tests had been performed, it was found that the cleanest chromatograms were obtained when using hexane, while stilbene recoveries remained unaffected. Different amounts of hexane were tested (5–20 mL), and no significant improvement in the cleanliness of the chromatograms was observed for volumes higher than 10 mL (data not shown). The shaking (10 min at 960 oscillations per minute) and centrifuging (5 min at 10 °C and 10,414 g) conditions were the same as in the extraction procedure. Finally, it was not possible to remove all the matrix components, especially those which eluted at earlier retention times or following elution of the last analyte (*cis*-resveratrol) (see Fig. 4). However, this did not affect analyte separation, as all the stilbenes were baseline separated for matrix interferences, and it was possible to remove the compounds that elute at higher retention times by using the proposed chromatographic conditions.

Extraction Efficiency

To assess the efficiency of the proposed sample, a comparison was made of the results (peak areas) obtained for blank bee pollen samples spiked at three different stilbene concentrations (low, medium, and high QC levels), either prior to (*A* samples) or following (*B* samples) sample treatment. The resulting recovery values (Table 1) ranged from 89 to 96 % in all cases, indicating that the sample treatment procedure selected was adequate and efficient. The results were good not only in terms of extraction efficiency, but also as regards simplicity and solvent consumption, as the overall sample treatment consisted of three stages (cleaning, extraction, and concentration), and 31 mL of solvent was used per sample.

LC Optimization

As mentioned previously, no method has been published in which resveratrol and piceid isomers have been determined in bee pollen. Consequently, we decided to start separation optimization by using the chromatographic conditions that were proposed in our previous study (Soto et al. 2012), in which stilbenes were analyzed in honey. The analytical column was a Gemini C_{18} (150×4.6 mm), and the mobile phase a mixture of formic acid in water (1 %, *v*/*v*) and acetonitrile. It was necessary to include a security guard cartridge (Gemini C_{18}, 4× 3.0 mm) in the present study in order to preserve the lifetime and performance of the column, since it prevents harmful contaminants from building up at the head of the column. In the previously reported LC conditions, it was not possible to effect complete separation of the analytes from certain matrix components. In addition, some matrix-related compounds were strongly retained, and it was necessary to use long analysis times in order to elute them from the column. Several experiments were conducted by means of different mobile phases comprising mixtures of formic acid in water (1 %, *v*/*v*) and acetonitrile, in order to separate all the analytes and elute the highly retained matrix compounds in the shortest time. The best results were obtained with the chromatographic conditions described in subsection "LC-ESI-MS System." As can be seen, separation of the analytes was achieved with an isocratic mobile phase composed of formic acid in water (1 %, *v*/*v*) and acetonitrile (71:29, *v*/*v*) at a flow rate of 0.8 mL/min.

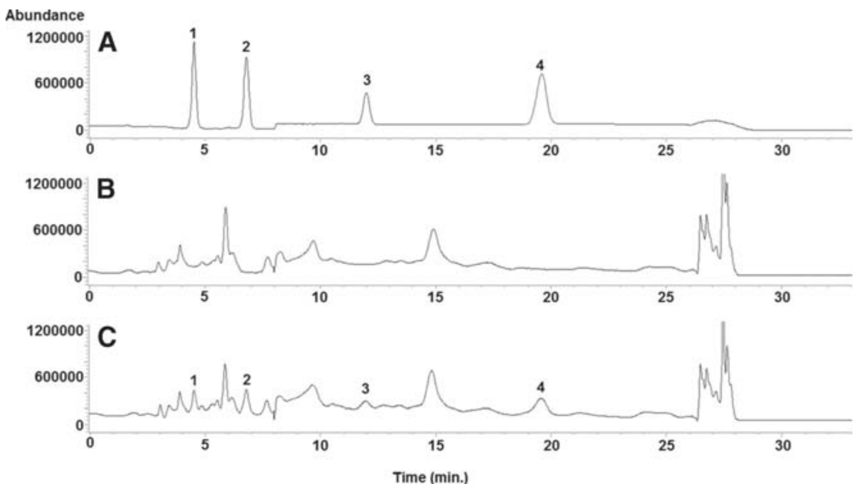


Fig. 4 Representative LC-ESI-MS chromatograms (selected ion monitoring, SIM, mode) obtained from **a** a 1000-μg/L standard solution of *trans*-piceid (**1**), *cis*-piceid (**2**), *trans*-resveratrol (**3**), and *cis*-resveratrol (**4**), **b** a blank bee pollen sample, and **c** a blank bee pollen sample spiked at 1000 μg/kg in each analyte. It must be pointed out that the stilbene concentrations were the same in the standard and spiked bee pollen samples according to the proposed sample treatment and the unit conversion. The LC-ESI-MS conditions are described in subsection "LC-ESI-MS System"

Table 1 Evaluation of the efficiency of the sample treatment and the matrix effect

Quality control (QC) sample	Evaluation of the recovery			Evaluation of the matrix effect		
	Mean (%)±RSD (%)			Mean (%)±RSD (%)		
	Low	Medium	High	Low	Medium	High
trans-piceid	93±4	91±4	90±5	26±6	23±5	28±5
cis-piceid	92±4	93±5	95±4	30±5	34±6	32±5
trans-resveratrol	96±5	94±4	95±5	20±6	22±6	24±6
cis-resveratrol	90±4	92±3	89±4	31±6	33±5	36±6

Data obtained as described in "Extraction Efficiency" and "Matrix Effect" subsections (n=6)

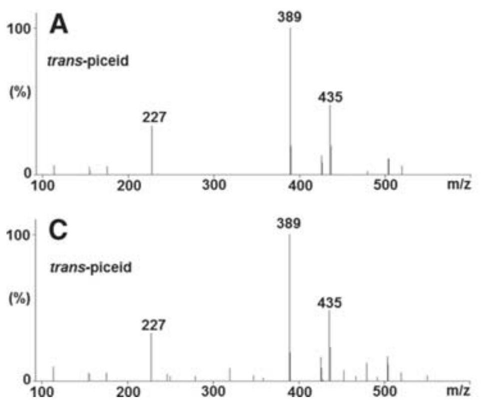
low QC-100 μg/kg; medium QC-1000 μg/kg; high QC-10,000 μg/kg

It was necessary to introduce several gradient steps (iii to vi) in order to elute certain remaining matrix components subsequent to elution of the last analyte (*cis*-resveratrol), and for this reason, it was also necessary to introduce an equilibration time step to obtain reproducible chromatograms. Tests were carried out to study the influence of the column temperature (between 25 and 45 °C at 5 °C intervals), which produced different retention times and peak symmetries. As expected, the former decreased slightly, and the S/N ratio was also enhanced as the temperature increased, but a significant loss of symmetry, which did not compensate for the decrease in analysis time or improvement in the S/N ratio, was observed at temperatures over 30 °C. As a result, 30 °C was the working temperature chosen. The possibility of enhancing the sensitivity of the method by injecting higher sample volumes was also considered; this meant testing the injection of blank bee pollen samples spiked with the stilbenes in amounts ranging from 5 to 30 μL. The results showed an increase in the S/N when up to 20 μL was injected, above which this ratio did not significantly improve and the chromatographic peaks began to appear somewhat deformed. Therefore, it was decided that 20 μL would be the injection volume. With the chromatography conditions described above, all the compounds were separated in less than 20 min (see Fig. 4), and the overall run time was 33 min.

MS Optimization

MS Conditions

Resveratrol and piceid isomers have previously been analyzed in negative ESI mode (Di Lecce et al. 2014; Moss et al. 2013; Soto et al. 2012), as the best results were obtained in terms of peak area and peak height (data not shown). A few experiments (flow injection analysis) were conducted in order to choose the optimum ESI-MS (negative mode) parameters by using the infusion mode (5 μL/min) of standard and matrix-matched solutions (1000 μg/L) of the analytes. The best results (data not shown) were found with the values described in subsection "LC-ESI-MS System," which were different from the values used in our previous research into honey (Soto et al. 2012). The stilbenes studied showed a major ion m/z



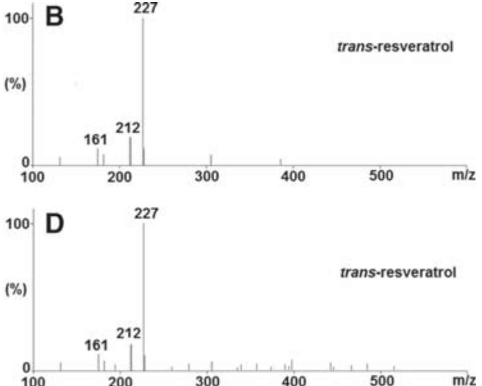


Fig. 5 Full-scan ESI-MS spectra of the trans-piceid and trans-resveratrol peaks in **a**, **b** standard solutions (1000 μg/L) and **c**, **d** a spiked bee pollen sample (1000 μg/kg). It must be pointed out that the stilbene concentrations were same in the standard and spiked bee pollen samples according to the proposed sample treatment and the unit conversion. The ESI-MS conditions are described in subsection "LC-ESI-MS System"

Table 2 Calibration curve data (n=6) and LOD and LOQ values

Compound	Calibration curve	Analytical range (μg/kg)	Slope confidence intervals	R^2	LOD[a] (μg/kg)	LOQ[a] (μg/kg)
trans-piceid	Standard	45 - 10000	$1.4\times10^7\pm9.9\times10^5$	0.996		
	Matrix-matched		$3.7\times10^6\pm9.3\times10^4$	0.999	15	45
cis-piceid	Standard	35 - 10000	$1.9\times10^7\pm8.6\times10^5$	0.998		
	Matrix-matched		$6.2\times10^6\pm7.8\times10^4$	0.999	10	35
trans-resveratrol	Standard	80 - 10000	$1.0\times10^7\pm2.2\times10^5$	0.999		
	Matrix-matched		$2.3\times10^6\pm3.7\times10^4$	0.999	25	80
cis-resveratrol	Standard	40 - 10000	$1.6\times10^7\pm1.6\times10^5$	0.999		
	Matrix-matched		$5.0\times10^6\pm3.8\times10^4$	0.999	13	40

[a] LOD and LOQ values were calculated in matrix (bee pollen)

(100 % of relative abundance) on their full-scan ESI-MS spectra (Fig. 5), corresponding to the deprotonated molecular ion $[M\text{-}H]^-$, which was used as a quantification ion with the SIM mode (*m/z* 227 resveratrol isomers; *m/z* 389 piceid isomers). Meanwhile, two ions that displayed relative intensities higher than 5 % in the mass spectra were selected as confirmation ions (resveratrol isomers: *m/z* 161, ~12 %-*trans*; ~15 %-*cis* and *m/z* 212, ~19 %-*trans*; ~16 %-*cis* %; piceid isomers: *m/z* 227, ~33 %-*trans*; ~38 %-*cis* and *m/z* 435, ~46 %-*trans*; ~42 %-*cis*).

Matrix Effect

To observe how the matrix influenced ESI ionization, the peak areas of the resveratrol and piceid isomers in standard working solutions were compared with those acquired with blank pollen samples spiked at three different concentrations (low, medium, and high QC levels) after sample treatment (*B* samples). The responses of all compounds at the three concentrations assayed were below 40 % in all cases, as can be seen in Table 1. Moreover, if a comparison is made between the stilbene peaks in standard (Fig. 4a) and matrix-matched solutions (Fig. 4c) at the same concentration levels, a marked decrease in the analyte signals can be clearly observed due to the matrix effect. Hence, it was concluded that the matrix (bee pollen) affected electrospray ionization of all the compounds, generating ion suppression.

Validation of the LC-ESI-MS Method

Selectivity

To assess the selectivity of the method, extracts from blank pollen samples (resveratrol and piceid isomer-free pollen samples) along with pollen samples spiked with the compounds under study were injected (see Fig. 4b, c). No matrix interference was observed at the retention time of each of the analytes, and as can be seen in the mass spectra (see Fig. 5), the relative intensities of the selected diagnostic ions in the spiked samples matched the corresponding calibration standard within ±10 %. Therefore, it can be concluded that the method was selective for determining resveratrol and piceid isomers in bee pollen.

Table 3 Summary of precision and accuracy studies for the stilbene determination in spiked bee pollen samples (n=6)

Validation parameter		*trans*-piceid	*cis*-piceid	*trans*-resveratrol	*cis*-resveratrol
Intraday precision (%RSD)	Low QC	6	6	5	4
	Medium QC	6	5	6	6
	High QC	5	7	6	5
Interday precision (%RSD)	Low QC	7	7	7	6
	Medium QC	7	6	8	7
	High QC	6	8	7	7
Intraday accuracy (%RE)	Low QC	5	4	6	5
	Medium QC	−6	−4	8	−4
	High QC	6	−6	−5	−6
Interday accuracy (%RE)	Low QC	6	−7	6	5
	Medium QC	6	−6	−6	−6
	High QC	5	7	9	8

QC quality control; low QC-100 μg/kg; medium QC-1000 μg/kg; high QC-10,000 μg/kg

Table 4 Results of the investigation of resveratrol and piceid isomers in bee pollen samples (means of triplicate analyses, μg/kg)

Sample	*trans*-piceid	*cis*-piceid	*trans*-resveratrol	*cis*-resveratrol
# E1	<LOD	<LOD	440	<LOD
# E2	<LOD	<LOD	90	<LOD
# E3	<LOD	<LOD	5380	115
# E4	<LOD	<LOD	910	<LOD
# E5	<LOD	<LOD	9100	100
# E6	<LOD	<LOD	430	<LOD
# E7	<LOD	<LOD	220	75
# E8	<LOD	<LOD	2450	<LOD
# E9	<LOD	<LOD	1910	80
# E10	<LOD	<LOD	1820	90
# O1	<LOD	<LOD	<LOD	<LOD
# O2	<LOD	<LOD	<LOD	<LOD
# O3	<LOD	<LOD	<LOD	<LOD
# O4	<LOD	<LOD	<LOD	<LOD
# O5	<LOD	<LOD	<LOD	<LOD
# O6	<LOD	<LOD	<LOD	<LOD
# C1	<LOD	<LOD	95	<LOD
# C2	<LOD	<LOD	150	<LOD
# C3	<LOD	70	<LOD	<LOD
# C4	<LOD	<LOD	730	<LOD
# C5	<LOD	<LOD	1450	<LOD
# C6	<LOD	<LOD	875	<LOD

E bee pollen samples from experimental apiaries, *O* blank bee pollen samples, *C* commercial bee pollen samples

Limits of Detection and Quantification

The LODs and LOQs were determined experimentally for each compound in bee pollen, as indicated in subsection "Method Validation" (Table 2). As may be observed, the LOD and LOQ values for *trans*-isomers were slightly higher. To reiterate what was said previously, no study has been published in which resveratrol and piceid isomers were determined in bee pollen; so, it was not possible to make a proper comparison; moreover, it should also be taken into account that the matrix usually plays an important role in MS detection. However, the LOD and LOQ values obtained were higher than those obtained in the only study where these compounds were analyzed in a bee matrix (honey; LOD <3 μg/kg; LOQ <11 μg/kg; Soto et al. 2012). This finding could be related with the strong matrix effect detected in bee pollen.

Linearity Studies

Matrix-matched standard calibration curves were used to quantify the stilbenes examined in bee pollen as it was observed in a strong matrix effect; this affected analyte ionization, and a suppression of the MS signal (>60 %) was observed (Table 1 and Fig. 4a, c). This finding was corroborated by the fact that the slopes of the standard and matrix-matched calibration curves did not overlap at the confidence intervals (Table 2). The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied, and the coefficient of the determination values (R^2) was above 0.99 in all cases (Table 2). Moreover, the lack of bias was confirmed by a *t* test and a study of the distribution of residuals.

Precision and Accuracy Studies

Intraday and interday precision (% RSD values) for the compounds under study were in all cases lower than or equal to 8 % (Table 3). Accuracy (% RE values) for resveratrol and piceid isomers ranged from 4 to 8 % for the intraday readings, and from 5 to 9 % for the interday values (Table 3). Such

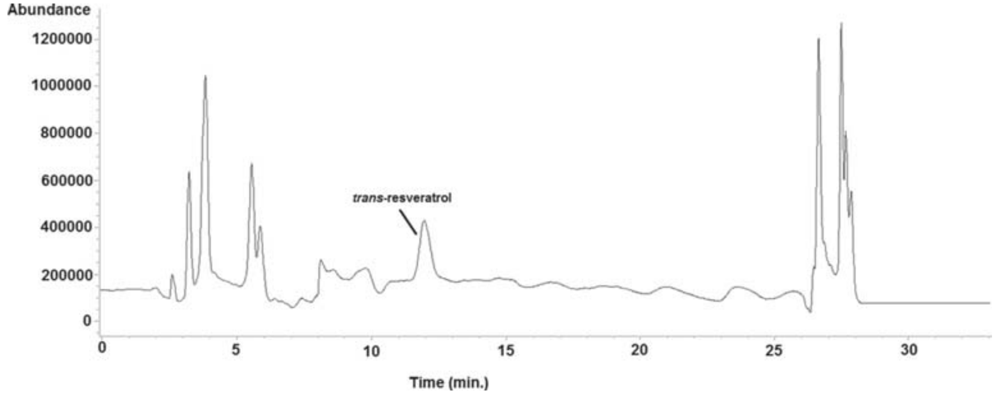


Fig. 6 Representative LC-ESI-MS chromatograms (selected ion monitoring, SIM, mode) obtained from a bee pollen sample obtained from an experimental apiary with a *trans*-resveratrol content of 2450 μg/kg. The LC-ESI-MS conditions are described in subsection "LC-ESI-MS System"

results indicate that the present method is both precise and accurate, and it should be added that although an internal standard is recommended in most validation guidelines, it was not necessary in the present study because of this degree of precision and accuracy.

Application of the Method

Corbicular bee pollen samples from experimental apiaries and commercial bee pollen samples were analyzed with the proposed method to search for residues of resveratrol and piceid isomers. All bee pollen samples were examined in triplicate. Only two of the analytes, namely, *cis* and *trans*-resveratrol, were found in concentrations above the LOQs in the bee pollen samples from resveratrol-treated apiaries (**E1–E10**, Table 4). *Trans*-resveratrol was detected in all these bee pollen samples (90–9100 μg/kg; see Fig. 6), while *cis*-resveratrol was determined in only five of these samples, and in much lower amounts (75–115 μg/kg). In addition, *cis*-piceid and *trans*-resveratrol were found in some of the commercial bee pollen samples analyzed (**C1–C6**, Table 4). *Cis*-piceid was detected in only one of these samples at a low concentration level (70 μg/kg), and *trans*-resveratrol was found in five of the samples, albeit in larger quantities (95–1450 μg/kg). Finally, no residues of the stilbenes under study were detected in any of the bee pollen samples used as blanks (**O1–O6**, Table 4). Certain conclusions could be postulated after an examination of the results described above: (i) *trans*-resveratrol was detected in 80 % of the commercial bee samples analyzed, and in three cases at relatively high concentrations (>700 μg/kg). Meanwhile, *cis*-piceid was detected in one sample at a low concentration. These are quite interesting results, as *trans*-resveratrol and *cis*-piceid have never been reported previously in commercial bee pollen and, due to the health benefits related to *trans*-resveratrol, the positive nutritional and health effects associated with the consumption of bee pollen would be greater. The presence of these compounds in the commercial bee pollen samples could be explained by the activity of honey bees, collecting the pollen from plants containing such compounds; (ii) the detection of *trans*-resveratrol in all the bee pollen samples from treated beehives in much greater amounts than in commercial bee pollen samples implies that feeding with resveratrol is directly related with the number of residues in this product. In addition, *cis*-resveratrol has also been encountered in several of these samples in smaller quantities, while piceid isomers were not detected in any of the samples. Thus, it has been demonstrated that there is a need to develop an analytical method to determine such compounds in bee pollen; such a method should, as we have proposed, be very sensitive and have a suitable linear range.

Conclusions

This is the first time that an analytical approach has been developed to simultaneously identify and quantify resveratrol and piceid isomers in bee pollen. The proposed extraction method based on three steps (cleaning, extraction, and concentration) has proven to be efficient, simple, and relatively cheap (a low consumption of solvents). Moreover, it was necessary to employ matrix-matched standards to perform correct ESI-MS quantification of the stilbenes in bee pollen, as ion suppression was observed. *Trans*-resveratrol residues were detected in all the samples from experimental apiaries over a wide concentration range, while *cis*-resveratrol was also found in certain samples. Moreover, *trans*-resveratrol was found in most of the commercial bee pollen samples tested, and in some cases at relatively high concentrations, while *cis*-piceid was found in one of these samples but at a much lower concentration. Finally, the observed presence in commercial bee pollen of resveratrol and piceid isomers, especially *trans*-resveratrol, is an interesting finding as this has hitherto never been reported, and it would increase the nutritional/functional value of this natural food supplement.

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Conflict of Interest Ana M. Ares declares that she has no conflict of interest.

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This article does not contain any studies with human or animal subjects.

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V.3. PAPER III. Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds

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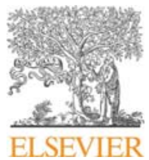

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Review

Extraction, chemical characterization and biological activity determination of broccoli health promoting compounds

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ABSTRACT

Broccoli (*Brassica oleracea* L. var. *Italica*) contains substantial amount of health-promoting compounds such as vitamins, glucosinolates, phenolic compounds, and dietary essential minerals; thus, it benefits health beyond providing just basic nutrition, and consumption of broccoli has been increasing over the years. This review gives an overview on the extraction and separation techniques, as well as the biological activity of some of the above mentioned compounds which have been published in the period January 2008 to January 2013. The work has been distributed according to the different families of health promoting compounds discussing the extraction procedures and the analytical techniques employed for their characterization. Finally, information about the different biological activities of these compounds has been also provided.

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Abbreviations: AA, ascorbic acid or vitamin C; AAS, atomic absorption spectroscopy; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ACN, acetonitrile; AES, atomic emission spectroscopy; $AlCl_3$, aluminium chloride; $C_6H_8O_7$, citric acid; Cl_3CH, chloroform; $C_6H_4(SH)_2$, 1,2-benzenedithiol; DAD, diode array detector; DCM, dichloromethane or methylene chloride; DHAA, dehydroascorbic acid; DTT, dithiothreitol; ECD, electrochemical detector; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; EtOAc, ethyl acetate; EtOH, ethanol; FA, formic acid; FAMEs, fatty acids methyl esters; FCR, folin-ciocalteu reagent; FES, flame emission spectroscopy; FID, flame ionization detector; FLD, fluorescence detector; FMOC-Cl, fluorenylmethyloxycarbonyl chloride; FRAP, ferric reducing antioxidant power; FTIR, Fourier transform infrared spectroscopy; FTICR, Fourier transform ion cyclotron resonance; GC × GC, comprehensive two-dimensional gas chromatography; GLSs, clucosinolates; GRA, glucoraphanin; HAc, acetic acid; H_3BO_3, boric acid; HCl, hydrochloric acid; $HClO_4$, perchloric acid; $H_2C_2O_4$, oxalic acid; HNO_3, nitric acid; H_2O_2, hydrogen peroxide; HSCCC, high speed counter current chromatography; IC, ion chromatography; ICP, inductively coupled plasma; IEC, ion-exchange chromatography; ITCs, isothiocyanates; KCl, potassium chloride; KOH, potassium hydroxide; LC, liquid chromatographic; MAE, microwave assisted extraction; MECK, micellar electrokinetic chromatography; β-MET, mercaptoethanol; MeOH, methanol; MES, 2-(N-morpholino)ethanesulfonic acid); MPA, meta-phosphoric acid; MS/MS, tandem mass spectrometry; NAA, neutron activation analysis; $Na_2B_4O_7$, sodium borate; NADPH, nicotinamide adenine dinucleotide phosphate; NaF, sodium fluoride; NaH_2PO_4, sodium dihydrogen phosphate; NaOH, sodium hydroxide; NMR, nuclear magnetic resonance; $NaC_2H_3O_2$, sodium acetate; $NaNO_2$, sodium nitrite; $NH_4C_2H_3O_2$, ammonium acetate; NS, not specified; OES, optical emission spectroscopy; ORAC, oxygen radical absorbance capacity; PBS, phosphate buffer saline; RPLC, reverse phase liquid chromatography; RT-PCR, reverse transcription polymerase chain reaction; SEC, size exclusion chromatography; SDS, sodium dodecyl sulfate; SF, sulforaphane; SFE, supercritical fluid extraction; SPE, solid phase extraction; TEAC, trolox equivalent antioxidant capacity; TCA, trichloroacetic acid; TCD, thermal conductivity detector; TCEP, tris(2-carboxyethyl) phosphine; THF, tetrahydrofuran; TLC, thin layer chromatography; TOC, tocopherol; Tris–HCl, tris–hydrochloride; UPLC, ultra performance liquid chromatography; USDA, United States Department of Agriculture; XRF, X-ray fluorescence.

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1. Introduction

The genus *Brassica* (Brassicaceae or Cruciferae family) includes a large number of vegetables comprising, amongst others, broccoli, cauliflower, Brussels sprouts, kohlrabi, cabbage, mustard, *etc.* [1]. Those vegetables are a good source of many health promoting compounds and potentially protective phytochemicals including phenolics, carotenoids, selenium, glucosinolates or vitamins [2]. Broccoli (*Brassica oleracea* L. var. *Italica*), which is thought to have originally come from the eastern Mediterranean area and to have been introduced to Europe a long time ago (in mediaeval times) [1,3], is nowadays consumed worldwide and is highly valued by large groups of the population due to its flavour, but also due to some health promoting effects, such as anticancer or antioxidant properties, which have been mainly attributed to glucosinolates and their degradation products as well as phenolic compounds, respectively [1]. Consequently, incorporating some of these broccoli health promoting compounds directly or added to pharmaceutical products (nutraceutical) or other foods (functional foods) once they have been isolated and extracted from this vegetable, is a safe and effective way to guard against many of today's most common diseases [2]. Over the past 5 years, the rising interest in the extraction, isolation, characterization and determination of the biological activity of these beneficial broccoli compounds has been demonstrated by the large number of published research papers dealing with this issue (Fig. 1). As can be observed in Fig. 2, several of such compounds have been investigated in this matrix during this period of time, most of the studies being devoted to the analysis of glucosinolates and related compounds (33% of the publications), while phenolic compounds (28%) have also been widely studied. It is also interesting to mention that many extraction, separation and determination techniques have been employed to obtain and characterize these compounds from broccoli (see Figs. 3 and 4). As can be seen in Fig. 3, solvent extraction has been predominantly chosen as sample treatment. This finding could be explained because this extraction procedure is usually the cheapest and simplest. However, solid phase extraction (SPE) has been employed in some other cases, especially when analyzing glucosinolates (GLSs), sulforaphane (SF) and vitamins, as it is an effective procedure to obtain cleaner and purer extracts, and at the same time it is possible to concentrate the sample. Hydrolisis has been mainly employed to analyze proteins, GLSs and SF, while an acid digestion was recommended to determine proteins and essential elements. Other sample treatments as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), or Soxhlet extraction have been selected in some specific cases. Meanwhile, liquid chromatography (LC) coupled to several detectors has mainly been used as characterization technique due to the physico-chemical characteristics of the broccoli health promoting compounds, and because it is possible to analyze the content in broccoli of single compounds

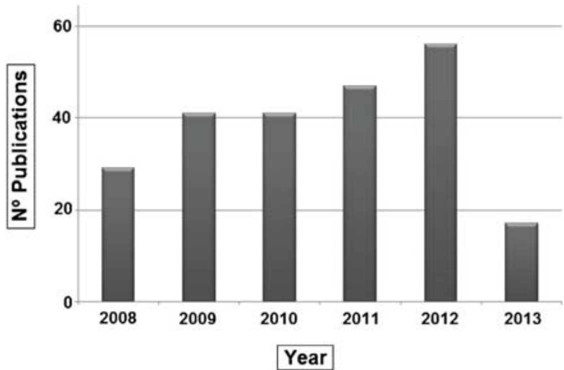

Fig. 1. Evolution of the published works in the last five years related to extraction, chemical characterization and biological activity determination of broccoli health promoting compounds (data up to January 2013). The sources of information were the databases: ISI-Web of Knowledge, Scirus, Scopus and Science Direct. The search has been done using as keywords [(Broccoli) or (*Brassica oleracea* L. var. *Italica*) or (Brassicaceae) or (Brassicaceae or Cruciferae)] and [(phytochemicals) or (glucosinolates) or (lipids) or (vitamins) or (proteins) or (phenolic) or (essential elements) or (amino acids) or (isothiocyanates) or (extraction) or (isolation) or (quantification) or (separation) or (determination) or (chromatography) or (biological activity)] among several others.

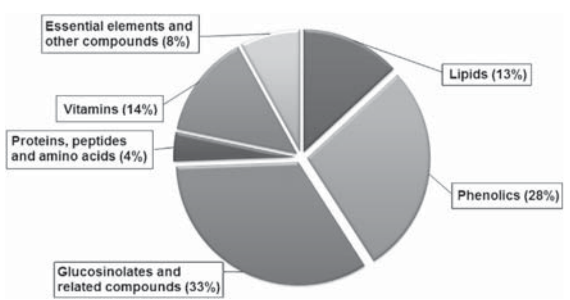

Fig. 2. Summary of the health promoting compounds analyzed of broccoli in the last five years.

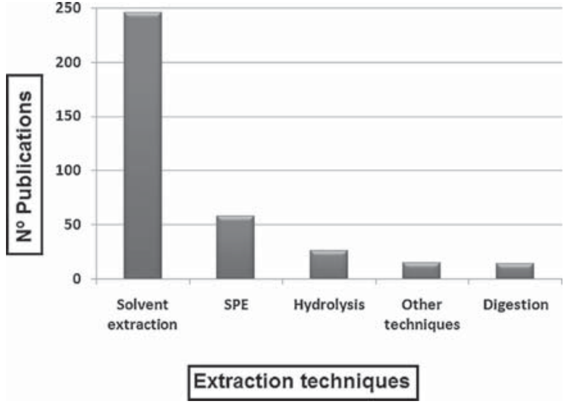

Fig. 3. Summary of the extraction techniques used to analyze health promoting compounds of broccoli in the last five years.

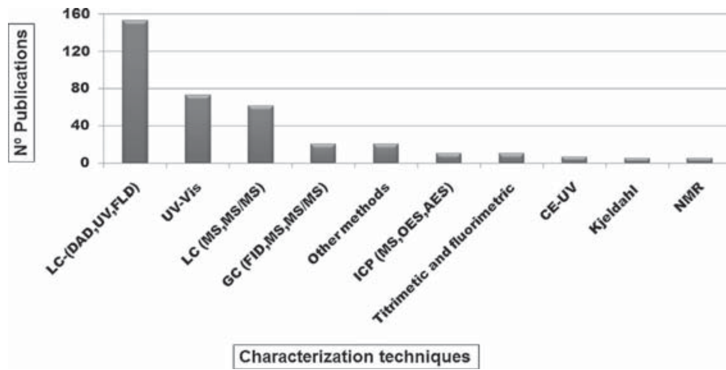

Fig. 4. Summary of the characterization techniques used to analyze health promoting compounds of broccoli in the last five years (AES, atomic emission spectroscopy; DAD, diode array detector; FID, flame ionization detector; FLD, fluorescence detector; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; OES, optical emission spectroscopy).

(see Fig. 4). It should be also commented that MS detectors have been employed in most cases to confirm the presence of specific compounds when the standards were not available, rather than to quantify those compounds as it was not required a great sensitivity because they are usually found at high concentrations. It should be also commented that spectrophotometric methods (UV–vis) have been preferred to determine the total content or to study the profile of a group of phytochemicals, as they are simpler and cheaper than other characterization techniques. Moreover, gas chromatography (GC) has been chosen to analyze lipids, proteins, SF and some essential elements, although it should be pointed out that its use has been more limited than LC. In relation to other characterization techniques, it should be commented that their use have been focused on specific groups of phytochemicals. For example inductively coupled plasma (ICP) has been mainly selected to analyze essential elements, while titrimetric and fluorimetric methods were predominantly used to determine vitamins.

Several interesting reviews and research studies focusing on the general health benefits of broccoli and its related compounds have been published [4–9]. For example, an in-depth study of the chemical and biological characterization of some phytochemicals from broccoli was published in 2006 [4]. In this work, more than 120 research papers were summarized, and although most of them were related to the isolation and determination of glucosinolates and isothiocyanates, several other compounds such as selenium, organic anions and cations were also investigated. Moreover, the influence of processing on the phytochemical composition of broccoli and the bioavailability of these compounds were also discussed. In a more recent publication, a summary was made of some of the literature on bioactive compounds from edible crucifers, including broccoli, indicating their beneficial health promoting effects and their possible role in building up defence against diseases, especially cancer [2]; however, nothing was said about the isolation and characterization of these compounds. Some other studies focused on phytochemicals [5], health affecting compounds [6], and the dietary constituents [7] of broccoli, among other Brassicaceae vegetables, have been published, but, as previously stated, their attention was not focused on the extraction and characterization of those compounds. Mention should also be made of an interesting review studying the variation in the bioactive components (glucosinolates, vitamins, flavonoids, *etc.*) found in broccoli due to genetics, environment and post-harvest processing [8]. The influence of some of these factors on phytochemicals obtained from Brassicaceae vegetables has been also reported [5]. As has been previously seen, several studies have demonstrated that broccoli might be beneficial for human health by reducing the risk of developing certain types of cancer, or on account of its antioxidant activity. However, recent *in vitro* and experimental animal studies indicate that broccoli, its extracts and glucosinolate-derived degradation products might also have undesirable effects, especially genotoxic activities [1,9], although the relevance of these to human health is as yet unknown. However, in this review attention will be focused on the health promoting effects of bioactive compounds extracted from broccoli. Considering all these aspects, the aim of the study is to present and discuss the main extraction and analytical techniques used to obtain, identify, characterize and/or quantify broccoli health promoting compounds in the period January 2008 to January 2013. The review is structured according to the different families of bioactive compounds (lipids, vitamins, proteins, glycosides, phenolic compounds, *etc.*), whilst the last section of the manuscript discusses the biological activity of broccoli and its related compounds and how this was determined.

2. Health promoting compounds of broccoli

2.1. Lipids

Lipids constitute a group of naturally occurring molecules that include fats, fatty acids, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, phospholipids, carotenoids and others. Some of the biological functions of lipids are related with energy storage, composition of cell membranes, and molecular signalling [10]. Although humans and other mammals use various biosynthetic pathways to both break down and synthesize lipids, some essential lipids cannot be made in this way and must be obtained from diet, and broccoli is a potential source of some of these compounds. They have usually been extracted from broccoli by means of solvent extraction, but in two cases supercritical fluid extraction (SFE) was employed to analyze fatty acids in broccoli leaves (Table 1). Moreover, acetone and mixtures of this solvent with water have been predominantly used to extract lipids from broccoli (see Supplementary information, Fig. S1). It can be also observed in this table that gas chromatography (GC) was the technique of choice when determining fatty acids and sterols, although fatty acids should be converted into their corresponding methyl esters (FAMEs) prior to their detection, as more robust and reproducible chromatographic data are obtained in this way. Finally, spectrophotometric methods and reverse phase liquid chromatography (RPLC) coupled to diode array detector (DAD) were employed to determine the other groups of lipids in broccoli (Table 1). It should be commented that the use of separation techniques like GC and LC is necessary in order to facilitate the individual determination of

Table 1
Applications in the analysis of lipids in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
13 FAMEs [11]; 6 FAMEs and 5 sterols [12]	NS (cv. Grandeur) [11]; broccoli roots [12]	Solvent extraction (toluene/MeOH) and acetylation [12]	GC-FID	[11,12]
15 FAMEs	Flowers and stems	Soxhlet extraction (hexane)	GC-FID	[13]
22 FAMEs	Leaves (cv. Nubia, Naxos, Viola, Parthenon and Marathon)	SFE (using CO_2/15% MeOH) or Soxhlet extraction (hexane)	GC–MS	[14]
45 FAMEs [15]; 47 FAMEs [16]	Leaves (cv. Naxos, Nubia and Viola)	SFE (using CO_2/15% MeOH)	GC × GC-FID	[15,16]
Total carotenoid content	Heads (cv. Lvxiong) [18]; Kailan-hybrid [19]; florets (cv. Green Star) [20]; Edible parts (cv. Sebastian) [27]	Solvent extraction (acetone/petroleum ether) [18]; (hexane/MeOH/acetone) [19]; (water/acetone) [20]; (MeOH) [27]	UV–vis	[18–20,27]
Lutein [21]; Lutein and β-carotene [28]	Kailan-hybrid stems and florets [21]; Edible parts [28]	Solvent extraction (hexane/MeOH/acetone) [21]; (EtOH/DCM/hexane) [28]	RPLC-DAD	[21,28]
Lutein and β-carotene	Heads (cv. Parthenon)	Solvent extraction (acetone)	RPLC-DAD	[22]
Total chlorophyll and carotenoid content	Heads [23]; sprouts [24]	Solvent extraction (acetone) [24]; (acetone/water) [23]	UV–vis	[23,24]
Chlorophyll *a* and *b*, total chlorophyll and carotenoid content	Bimi® broccoli (NS)	Solvent extraction (MeOH/acetone)	UV–vis	[25]
Chlorophyll *a*, *b*; and 6 major carotenoids	Edible parts	Solvent extraction (acetone or MeOH)	RPLC-DAD	[26]
Chlorophyll *a*, *b*, Lutein and β-carotene	Heads (cv. Monaco [33] and Parthenon [29,33]); florets [34]	Solvent extraction (acetone)	RPLC-DAD [29,33,34]; UV–vis [34]	[29,33,34]
Chlorophyll *a*, *b* [31]; total chlorophyll content [30,32,35]	Florets [30,35] (cv. iron [31]); heads (cv. Cicco) [32]	Solvent extraction (acetone) [30]; (acetone/water) [31,32,35]	UV–vis	[30–32,35]
Chlorophyll *a*, *b*, and 6 carotenoids	Heads and florets (cv. VI-158, BNC, Pirate, purple-headed)	Solvent extraction (EtOH)	UPLC-DAD	[37]
Total chlorophyll content	Heads (cv. Chaoda) [36]; leaves and roots [38]	Solvent extraction (EtOH/water) [36]; (MeOH) [38]	UV–vis	[36,38]
Chlorophyll *a*, *b*, Lutein, β-carotene total chlorophyll and carotenoid content	NS	Solvent extraction (THF)	RPLC-DAD	[39]

DAD, diode array detector; DCM, dichloromethane; EtOH, ethanol; FAMEs, fatty acids methyl esters; FID, flame ionization detector; GC × GC, comprehensive two-dimensional gas chromatography; MeOH, methanol; NS, not specified; THF, tetrahydrofuran.

these compounds, but when the main goal of the study is to determine the total content (carotenoid or chlorophyll), it is acceptable to simply use UV–vis.

Fatty acids have been extracted in different ways from broccoli; solvent extraction followed by an acetylation process was successfully employed to extract FAMEs and sterols [11,12], Soxhlet extraction was also selected in two cases [13,14], while SFE was used in other publications [14–16]. It should be added that in one of these publications [14], the results obtained with SFE and Soxhlet were compared, and it was concluded that SFE is a promising alternative to the traditional Soxhlet methods for extracting lipids from broccoli leaves. Moreover, compared with Soxhlet extraction, supercritical fluid extracts presented higher percentages of unsaturated fatty acids, and SFE is also a more environmental friendly extraction technique. As seen previously, GC (non- or low polar columns) coupled to flame ionization (FID) or mass spectrometry (MS) detectors was used in all the studies in which fatty acids were analyzed in broccoli [11–16]. Moreover, in two of these publications [15,16], the usefulness of comprehensive two-dimensional gas chromatography (GC × GC) was demonstrated by separating and determining more than 40 FAMEs in a single chromatographic run. In these studies, they were employed highly polar columns in the first dimension and non-polar columns for the second dimension. Taking into account those publications, the best option to characterize fatty acids is the use of GC. In relation to the extraction methods, it could be said that the simplest and cheapest option is solvent extraction, although SFE has provided also promising results, which were comparable or even better than the obtained using Soxhlet extraction.

Carotenoids, which possess great importance because of their nutritional and physiological activities, cannot be synthesized by animals, so they should be acquired through the diet [17]. Total or individual (lutein, lycopene, β-carotene, *etc.*) carotenoid contents have also been studied in different broccoli parts such as heads, florets and sprouts (Table 1). In all cases, solvent extraction with different solvents or composition has been used to isolate these compounds, and in most cases acetone was predominantly employed as extractant (alone or in mixtures with other solvents) [18–26], while in other cases methanol [27] or mixtures of ethanol with other solvents [28] were also selected. Only spectrophotometric detectors (UV–vis, DAD) were employed to determine the compounds, although in certain cases prior separation was required, which was usually accomplished by RPLC with C_{18} [21,22,28,34,37,39] and C_{30} [26,29,33] stationary phases.

The last group of lipids that have been studied in broccoli were chlorophylls. Individual (chlorophyll *a* and *b*), as well as total chlorophyll content was determined in broccoli heads, florets and sprouts. As with carotenoids, solvent extraction has been employed in all the publications where these compounds were analyzed (Table 1). Acetone [23,24,26,29–35], ethanol [36,37], methanol [26,38], tetrahydrofuran [39] or acetone and methanol mixtures [25] were the solvents employed. Finally, spectrophotometric methods with or without previous separation (RPLC with C_{18} [37,39] and C_{30} [26,29,33] columns), depending on the goal of the study (total or individual content), were chosen to identify and quantify the compounds. So it can be postulated that to analyze carotenoids and chlorophylls in broccoli, acetone and a spectrophotometric detector (coupled to RPLC if it is necessary to

determine single compounds) are recommended as extractant and characterization method, respectively.

2.2. Phenolic compounds

More than 4000 compounds divided into 12 subclasses belong to this group of natural compounds, and several of them can be found in broccoli. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. The number and arrangement of their carbon atoms are classified in flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamates, stilbenes and others), and are commonly found conjugated to sugars and organic acids [40]. The main activity reported for phenolic compounds has been as antioxidants, although they are also associated with other health promoting effects such as anti-carcinogenic, anti-inflammatory, anti-ageing, and anti-thrombotic activity [41].

Several sample treatments have been published in order to extract these compounds from broccoli, the majority being solvent extractions or microwave assisted extraction (MAE). As can be observed (see Supplementary information, Fig. S2) methanol a water mixtures have been predominantly used to extract those compounds from broccoli, while methanol, ethanol and water have been also widely employed. Moreover, spectrophotometric techniques have been successfully used to determine the total phenolic content in broccoli (see Table 2). However, in some cases, single phenolic compounds were specifically analyzed by using other techniques like LC, GC and CE. It should also be mentioned that LC with C_{18} analytical columns has currently become the technique of choice to perform this task [25,29,41–47,49–57,60], while GC–MS with a low polar column [48] and capillary electrophoresis (CE, fused silica capillaries) with UV [49] were scarcely employed.

One of the classic methods used to analyze total phenolic content is the Folin Ciocalteu reagent (FCR), in which the measured colour change is associated with the reduction of a molybdate–tungstate reagent induced by the phenols in the sample [41]. This reagent does not only react with phenols, but with any reducing substance of the broccoli sample. As can be observed in Table 2, FCR has been mainly employed to determine the total phenolic content in broccoli. Solvent extraction is the most commonly used sample treatment when analyzing phenolics, with several extractants, such as water [24,61–63], methanol [20,27,41,43,64,65,76], ethanol [30,32,66,67], mixtures of water and methanol [25,34,36,44–46,73,74], water with other solvents [19,22,42–48,68], or mixtures of ethanol with hexane [63], being employed. Accordingly, it can be concluded that a mixture of methanol and water is recommended to perform the solvent extraction in this case. Moreover, the effect of independent variables of MAE (extraction temperature, solvent concentration and extraction time) on total phenolic and flavonoid contents of broccoli extracts was also investigated [74]. After that, the samples were directly measured spectrophotometrically by UV–vis to determine the total phenolic content, as it was the fastest and simplest procedure when it was not required to analyze a specific phenolic compound. All types of broccoli parts are analyzed in these studies (florets, heads, sprouts, *etc.*).

Flavonoids are a family of phenolic compounds that have been investigated in broccoli. Quercetin and kaempferol are the predominant flavonoids in broccoli. In general, their levels depend on several factors like environmental pressures, cultivar, post-harvest transport or genotype [44]. Several research papers have been published in order to determine their total content in broccoli samples [45–47,62,71,74–77]. As with total phenolic content, solvent extraction was mainly used to isolate flavonoids, although an additional step of mixing the broccoli extract with sodium nitrite, aluminium chloride and sodium hydroxide was required [45,47,62,71,74]. However, other extractants have been employed in order to analyze total flavone [75], flavonoid [25] and anthocyanin [47,76–78] content, such as methylene chloride [75], mixtures of methanol and water [25], methanol and hydrochloric acid [76,77] and hydrochloric acid with potassium chloride, ammonium or sodium acetate [47,78]. Spectrophotometric (UV–vis) detection was used in most of the research, and in some of the studies devoted to analyzing anthocyanins [47,78] an extra pH differential method was needed due to the fact that they had different colours depending on the pH value. Some special cases are related to the analysis of acylated anthocyanins in broccoli sprouts [59,60] or in stems and florets [42,43]. It has been necessary to perform chromatographic (RPLC) separation subsequent to solvent extraction of these compounds with water, methanol and formic acid [59,60] or with water and methanol [42,43] mixtures, respectively. Identification and quantification were performed by DAD [42,43] or DAD with electrospray ionization tandem mass spectrometry (ESI-MS/MS) [59,60]. A different group of phenolic compounds, flavonoid glycosides, have been also determined in broccoli. It should be pointed out that most of these compounds have been analyzed in conjunction with total flavonoid or phenolic content, so the preferred sample treatment, as can be expected, is solvent extraction (see Table 2). Similar solvents to those mentioned previously have been employed to analyze flavonoid glycosides. Methanol [79], water [52,58] or solvent mixtures such as ethanol and phosphate buffer (PBS) [80], methanol and water [39,48–51,53–57,59,81,82], or hydrochloric acid with ethanol [29] and methanol [33], have been chosen to perform the extractions in these studies. Meanwhile, it should be added that in one case the use of SPE [49] was required. The separation, identification and quantification of these compounds were largely conducted by RPLC (C_{18} columns) coupled to DAD [25,29,41–44,46,49–59,79,81], UV–vis [82,83], or MS [29,33,50–59] detectors. Moreover, CE with UV–vis detector [49] and GC–MS [48] were also employed to determine flavonoid glycosides. It should be also mentioned that in one case [49], flavonols and phenolic acids were isolated and concentrated by SPE with C_{18} cartridges.

After studying the scientific literature related to the analysis of phenolic compounds in broccoli, it can be recommended a solvent extraction with a mixture of methanol and water as sample treatment, while spectrophotometric detection (coupled to RPLC in order to analyze single or several compounds) is the best choice to characterize those compounds.

2.3. Proteins, peptides and amino acids

The consumption of proteins, peptides and/or amino acids offers several benefits for human health, as they are involved in anti-bacterial, antioxidant, immuno-stimulating, anti-thrombotic and anti-inflammatory activities, among other positive effects in the organism. Such compounds can be found in broccoli, and their extraction and identification requires the use of different analytical techniques, such as SPE, SFE, acid digestion, hydrolysis or solvent extraction, due to the complexity of these compounds (see Table 3). However, solvent extraction was selected in several studies, because it is usually simpler, faster and cheaper than other alternatives as SPE or SFE. Although with the latter techniques it was possible to obtain cleaner and purer extracts. Moreover, RPLC (C_{18} columns), GC (Zebron™ ZB-AAA column), UV–vis, and amino acid analyzers have been commonly used to study them (Table 3).

Proteins (soluble and total) have been extracted from broccoli florets using different solvents like trichloroacetic acid (TCA) [66] or buffer solutions [30,32,35], but in this case it could not be recommended one specific solvent, as all of them were equally employed (see Supplementary information, Fig. S3). The protein

Table 2
Applications in the analysis of phenolic compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Total phenolic content	NS [61,68]; Kalian-hybrid broccoli [19]; sprouts [24]; edible parts [65,70]; florets (cv. Volta F1) [73]; heads [72] (cv. Parthenon [22], Chaoda [36], Cicco [32]); florets [69] (cv. iron [30], Monaco [64], Cicco [66], Green Star [20], Parthenon [34]); seeds [67]; NS (cv. Sebastian) [84]	Solvent extraction (water) [24,61]; (EtOH/water/HCl) [22]; (acetone/water) [68]; (acetone/FA) [19,69,70,73]; (MeOH/water) [34,36,73]; (EtOH) [30,32,66,67]; (MeOH) [20,27,64,65]; (acetone/water/HAc) [72]	UV–vis (FCR)	[19,20,22,24, 27,30,32,34,36, 61,64–70,72,73]
Total phenolic content[a,b] and flavonoid compounds[b]	Bimi® broccoli (NS) [25]; sprouts [41]	Solvent extraction (MeOH/water) [25]; (MeOH) [41]	UV–vis (FCR)[a]; RPLC-DAD[b]	[25,41]
Phenolic and flavonoid compounds	Heads (cv. Nubia and Naxos [56], Parthenon [29]); heads, stalks and leaves (cv. Marathon) [50]; leaves and stalks (cv. Nubia, Marathon and Viola) [51]; broccoli beverages from leaves and stalks (cv. Nubia) [52]; plants (cv. Parthenon and Naxos) [53]; sprouts [55] (cv. Nubia, Marathon and Viola) [54]; edible sprouts and seeds [57]	Solvent extraction (EtOH/water/HCl) [29]; (MeOH/water) [50,51,53–57]; (water) [52]	RPLC-DAD-ESI-MS/MS	[29,50–57]
Phenolic and flavonoid compounds	Heads (cv. Parthenon and Monaco)	Solvent extraction (MeOH/HCl)	RPLC-ESI-MS/MS	[33]
Phenolics and flavonoids compounds	NS [39] (cv. Sebastian) [79]; sprouts (cv. Cezar) [80]; leaves and florets [82], roots and sprouts (cv. Marathon) [81], brocoletto heads [83]	Hydrolysis enzymatic and solvent extraction (MeOH/water) [39,81–83]; (MeOH) [79]; solvent extraction (EtOH/PBS) [80]	RPLC-DAD [39,79–81]; RPLC-UV [82,83]	[39,79–83]
Total phenolic content[a,b], anthocyanin[b], flavone[b] and flavonoid[b] compounds	Irish florets [42]; stems (cv. Monaco) [43]	Solvent extraction (EtOH or acetone with water) [42]; (MeOH) [43]	UV–vis (FCR)[a]; RPLC-DAD[b]	[42,43]
Total phenolic[a,b] and flavonoid[b] content	Florets	Solvent extraction (acetone/water)[a]; (MeOH/water)[b]	UV–vis (FCR)[a]; RPLC-DAD[a,b]	[44]
Total phenolic[a] and flavonoid[b] content	Heads (cv. Calabrese and Shouthern star) [45]; sprouts (cv. Wiarus) [71]; inflorescences [62]; heads (cv. Green Comet) [63]; NS (cv. Marathon) [74]	Solvent extraction (MeOH/water) [45][a] (water) [62][a]; (hexane/EtOH) [63][a,b]; (acetone/FA) [71][a,b]; ($NaNO_2$/$AlCl_3$/NaOH) [45,62,63,71,74][b]; MAE and solvent extraction (MeOH/water) [74][a,b]	UV–vis (FCR)[a]; UV–vis[b]	[45,62,63,71,74]
Total phenolic[a,b] and flavonoid[b] content	Heads (cv. Monaco)	Solvent extraction (MeOH/water)[a]; (MeOH or acetone)[b]	UV–vis (FCR)[a]; RPLC-DAD[a]; UV–vis[b]	[46]
Phenolic[a], flavonoid[b], and anthocyanin[c] compounds	Florets and stems (green and purple-sprouting)	Solvent extraction (MeOH/water)[a]; ($NaNO_2$/$AlCl_3$/NaOH)[b]; (KCl/HCl/$NaC_2H_3O_2$)[c]	UV–vis (FCR)[a]; UV–vis[b]; UV pH differential method[c]	[47]
Phenolic and flavonoid compounds	Florets	Solvent extraction (MeOH/water)	UV–vis (FCR); GC–MS	[48]
Phenolic and flavonoid compounds	NS	Solvent extraction (MeOH/water) and SPE	CE-UV; RPLC-DAD	[49]
Acylated anthocyanin compounds	Sprouts (cv. Marathon, Nubia, Viola)	Solvent extraction (MeOH/FA/water)	RPLC-DAD-ESI-MS/MS	[60]
Total flavone content	Heads and stalks	Solvent extraction (DCM)	UV–vis	[75]
Total phenolic[a] and anthocyanin[b] content	Florets and leaves (cv. Windsor) [76], sprouts (cv. Youxiu) [77]	Solvent extraction (MeOH) [76][a]; (EtOH/water) [77][a]; (MeOH/HCl) [76,77][b]	UV–vis (FCR)[a]; UV–vis[b]	[76,77]
Anthocyanin compounds	Sprouts (cv. Youxiu)	Solvent extraction (KCl, HCl and $NH_4C_2H_3O_2$)	UV pH differential method	[78]

$AlCl_3$, aluminium chloride; DCM, dichloromethane; DAD, diode array detector; ESI-MS/MS, electrospray ionization coupled to tandem mass spectrometry; EtOH, ethanol; FA, formic acid; FCR, Folin-Ciocalteu reagent; HAc, acetic acid; HCl, hydrochloric acid; KCl, potassium chloride; MeOH, methanol; MAE, microwave-assisted extraction; $NaC_2H_3O_2$, sodium acetate; $NaNO_2$, sodium nitrite; $NH_4C_2H_3O_2$, ammonium acetate; NS, not specified; PBS, phosphate buffer saline; SPE, solid phase extraction.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

Table 3
Applications in the analysis of proteins, peptides and amino acids in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Soluble[a] and total protein[b] content	Florets (cv. iron [30], Cicco [32])	Solvent extraction (buffer solution tris–HCl/β-MET/EDTA)[a]; (NaOH/SDS)[b]	UV–vis (FCR)[b]	[30,32]
Soluble protein content	Florets (variety non-specified)	Solvent extraction (buffer solution tris–HCl/DTT/EDTA)	UV–vis	[35]
Total protein content	Florets (cv. Cicco)	Solvent extraction (TCA)	UV–vis	[66]
Crude protein content[a], glutathione [84][b]	Heads (cv. Monaco [84], Shogun F1 [85], Pirate F1 [85], Sultan F1 [85], Marathon F1 [85])	Digestion (H_2SO_4) with selenium mixture as catalyst [84,85][a]; solvent extraction (HPO_3) [84][b]	Kjeldahl method[a]; RPLC-DAD [84][b]	[84,85]
Crude protein content[a], 15 amino acids[b]	Seeds (cv. Plenk)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl)[b]	Kjeldahl method[a]; IEC[b], photometrically[b] (amino acid analyzer)	[86]
Crude protein content[a], 17 amino acids[b]	Florets (cv. Lord F1)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl)[b]	Kjeldahl method[a], photometrically[b] (amino acid analyzer)	[87]
Crude protein content[a], 15 amino acids[b]	NS (cv. Grandeur)	Digestion (H_2SO_4) with selenium mixture as catalyst[a]; hydrolysis (HCl) and derivatization (FMOC-Cl)[b]	Kjeldahl method[a]; RPLC-FLD[b]	[88]
Proline	Leaves (cv. Parthenon and Naxos)	SPE	UV–vis	[53]
20 amino acids	Leaves (cv. Nubia, Naxos, Marathon, Parthenon and Viola)	SFE (MeOH as organic modifier) or solvent extraction (MeOH/water or water)	GC–MS	[91]
Cysteine and methionine	Leaves (cv. Monaco)	Solvent extraction (EtOH)	RPLC-DAD-ESI-MS	[92]

DAD, diode array detector; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization coupled to mass spectrometry; EtOH, ethanol; FCR, Folin-Ciocalteu reagent; FLD, fluorescence detector; FMOC-Cl, fluorenylmethyloxycarbonyl chloride; IEC, Ion-exchange chromatography; MeOH, methanol; NS, not specified; SDS, sodium dodecyl sulfate; SPE, solid phase extraction; β-MET, β-mercaptoethanol; TCA, trichloroacetic acid; Tris–HCl, tris–hydrochloride.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

content was determined using UV–vis, but in one case the FCR method was employed [30,32]. Meanwhile, crude proteins were obtained from broccoli [84–88] using acid digestion catalyzed with a selenium mixture. Thus, it must be noted that crude protein content was determined in broccoli heads following the procedures of the Association of Official Analytical Chemists [89] or the European recommendations [90], where it was stated that the crude protein content was calculated by multiplying the nitrogen content by 6.25. Total nitrogen was determined by the Kjeldahl method.

Several amino acids have also been found in different broccoli parts such as florets, sprouts and leaves. Acid hydrolysis with hydrochloric acid was selected as the sample treatment in three cases [86–88], while SFE [91], solvent extraction [91,92] and SPE [53] were also successfully employed. These compounds were determined photometrically by means of amino acid analyzers [86,87], an UV–vis spectrophotometer [53], a DAD [92] or a fluorescence detector (FLD), subsequent to conversion of the amino acids with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl), or MS [91,92]. It should be made clear that occasionally the amino acids were previously separated by ion-exchange chromatography [86], GC [91] and RPLC [88,92], in order to perform a single compound determination.

Glutathione has been extracted from broccoli heads by the use of ice-cold meta-phosphoric acid [84]. It was quantified using a homemade electrochemical detector (ECD), and the purity and identification of the peak was confirmed with a DAD. In this case, it was also necessary to perform a previous RPLC separation, in order to facilitate the isolation of glutathione from other matrix compounds.

Finally, it could not be recommended any specific analytical methodology to determine proteins, peptides and amino acids, as they have been employed several and quite different extraction and characterization methods. However, the advantages and disadvantages of some of them have been indicated, which could help potential readers to select the most adequate procedure depending on the analyte, and the equipment of their laboratories.

2.4. *Vitamins*

Brassica vegetables such as broccoli contain high levels of vitamins, which are organic compounds essential in trace amounts for the normal growth and maintenance of life. These compounds have diverse biochemical roles as regulators of mineral metabolism or of cell and tissue growth and differentiation, antioxidant activity and some are precursors of enzyme cofactors. Reducing equivalents for biochemical reactions is one of the most important physiological functions of ascorbic acid (vitamin C), and in some vegetables it is responsible for 35–95% of antioxidant capacity [4]. Several sample treatments based on solvent extraction have been proposed in order to isolate and obtain ascorbic acid (AA) from broccoli (see Table 4 and Supplementary information, Fig. S4). After examining these data, it can be recommended the use of MPA or a solvent mixture ($C_6H_8O_7$/EDTA/NaF/methanol/water) to perform this task. It should be mentioned that common deficiencies have been found in the existing methods used to determine AA in foods such as lack of specificity, not efficient extraction or stabilization of AA during analysis, and incomplete separation of AA from food-specific interferences in chromatographic analyses [93]. For example, titrimetric and fluorimetric methods are simple and, therefore, popular, but they are not chemically specific for AA. The titrimetric method relies on reduction of the blue dye 2,6-dichloroindophenol by AA to a colourless solution. Meanwhile, the fluorimetric method is based on oxidation of AA to dehydroascorbic acid (DHAA), followed by reaction with o-phenylenediamine to produce a fluorescent quinoxaline derivative [93]. As can be seen in Table 4, RPLC and spectrophotometric methods have additionally been employed to perform the determination of AA in order to solve some of the problems related to the titrimetric and fluorimetric methods, as specificity and separation from matrix compounds.

Use has been made of different solvents to extract AA from broccoli prior to carrying out titrimetic [13,23,24,27,85,94,95] and fluorimetric [69] determination. Methanol [24,27], meta-phosphoric acid (MPA) [13,69], or mixtures of MPA and acetic

Table 4
Applications in the analysis of vitamins in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
AA [13,23,85,94,95] and L-AA [24,27]	Flowers and stems [13]; heads [23]; sprouts [24,27]; heads (cv. Shogun F1, Pirate F1, Marathon F1 and Sultan F1) [85]; florets (cv. Marathon) [94]; florets (cv. Sultan F1, Majestic F1 and Marathon F1) [95]	Solvent extraction (MPA) [13]; (MeOH) [24,27]; (MPA/HAc) [94,23,85,95]	Titrimetric method	[13,23,24,27, 85,94,95]
Total Vitamin C (AA and DHAA) content	Kailan-hybrid florets [19]; florets (cv. Marathon) [34]; Inflorescences (cv. Marathon) [50]; florets [51], leaves [6] and stalks [6] (cv. Marathon, Nubia and Viola) [54]; sprouts [55]; sprouts (cv. Nubia, Marathon and Viola) [54] purple-sprouts [56]; heads (cv. Naxos and Nubia) [59]; sprouts (cv. Marathon) [81]; leaves and florets (cv. Marathon) [82]	Solvent extraction ($C_6H_8O_7$/EDTA/NaF/MeOH/water), SPE and DHAA after pre-column derivatization	RPLC-DAD [19]; RPLC-UV [34,50,51,54–56,59,81,82]	[19,34,50,51, 54–56,59,81,82]
AA	Florets and stems (cv. British green and purple-sprouting)	Solvent extraction (MeOH)	(UV–vis) FCR	[47]
L-AA	Florets (cv. Marathon)	Solvent extraction (MPA)	UV–vis	[62]
AA and DHAA	Florets (cv. Cicco)	Solvent extraction (TCA)	UV–vis	[66]
Total vitamin C (AA and DHAA)	Florets (cv. Volta F1)	Solvent extraction (MPA) and DHAA after pre-column derivatization	FLD	[69]
Total vitamin C (AA and DHAA)[a], L-AA[b]	NS	Solvent extraction (MPA/HAc/EDTA)	UPLC-DAD[a], iodometric titration[b]	[96]
AA	Heads (cv. Parthenon [22,29] and Marathon [33]); stems and leaves [93]	Solvent extraction (MPA/EDTA) [22,29,33]; (MPA/EDTA/TCEP) [93]	RPLC-DAD	[22,29,33,93]
Total vitamin C (L-AA and L-DHAA)	NS [39]; florets [44,97,98]; stalks [97,98]; heads [97,98]	Solvent extraction (MPA) [44]; ($H_2C_2O_4$) [39]; (NaH_2PO_4/EDTA) [98,97] and DHAA after pre-column reduction	RPLC-UV [97]; RPLC-DAD [39,44,98]	[39,44,97,98]
AA	Heads (cv. Lvxiong) [18]; florets (cv. Monaco) [64]; florets and stems (cv. Youxiu [77,99] and Lvling [99])	Solvent extraction ($H_2C_2O_4$) [18,77]; (MPA) [64]; (water) [99]	RPLC-UV [18,99]; RPLC-DAD [64,77]	[18,64,77,99]
Vitamins B (B1, B2, B3, B5, B6, B9, B12) and C	NS	Solvent extraction (water/NaOH/PBS)	RPLC-DAD	[100]
AA[a] and 5-methyl-tetrahydrofolate[b]	Heads (cv. Green Star)	Solvent extraction (MPA)[a]; ($NH_4C_2H_3O_2$/TCA)[b]	RPLC-UV[a], RPLC-FLD[b]	[20]
α,γ-Tocopherol and vitamin A	Edible parts	Solvent extraction (DCM/hexane)	RPLC-DAD	[28]
α,γ-Tocopherol	Florets (cv. VI-158, BNC, Brocolette Neri E. Cespuglio and Violet Queen)	Solvent extraction (MeOH)	UPLC-DAD	[37]
Total tocopherol (α,β,δ,γ-tocopherol)	Leaves	Saponification (KOH)	RPLC-FLD	[38]
Ascorbate and α-tocopherol	Heads (cv. Monaco)	Solvent extraction (MPA)	RPLC-DAD	[84]
5-Methyl-tetrahydrofolate	Florets [101] (cv. Belstar [102])	Solvent extraction (PBS) and chemical derivation	RPLC-FLD	[101,102]

AA, ascorbic acid; $C_6H_8O_7$, citric acid; DAD, diode array detector; DHAA, dehydroascorbic acid; DCM, dichloromethane; EDTA, ethylenediaminetetraacetate disodium salt; EtOH, ethanol; FCR, Folin-Ciocalteu reagent; FLD, fluorescence detector; HAc, acetic acid; $H_2C_2O_4$, oxalic acid; KOH, potassium hydroxide; MeOH, metanol; MPA, meta-phosphoric acid; NaH_2PO_4, sodium dihydrogen phosphate; NaF, sodium fluoride; NaOH, sodium hydroxide; NS, not specified; PBS, phosphate buffer saline; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl) phosphine.
Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method

acid [23,85,94,95], have been used to extract AA or L-AA from different broccoli parts such as florets, heads or sprouts. Furthermore, the differences between LC and titrimetic methods have been investigated in one study aimed at determining AA, DHAA and L-AA in broccoli extracts [96]. In this research, different experiments were carried out, which involved the use of solvent extractions with mixtures of MPA, acetic acid and ethylenediaminetetraacetic acid (EDTA), and quantification and identification by ultra performance liquid chromatography (UPLC) or iodometric titration. It was concluded that L-AA should be determined by using a titrimetic method, while L-AA, DHAA were determined with UPLC-DAD. Several other studies based on LC

methods with C_{18} based stationary phases to determine simultaneously AA, L-AA or DHAA have been published in the last years (see Table 4). In some of them [19,50,51,54–56,59,81,82] the same sample treatment was employed, involving solvent extraction with mixtures of $C_6H_8O_7$/EDTA/NaF/methanol/water) after which a SPE with C_{18} cartridges was performed. The compounds were determined using a DAD [19] or UV–vis detectors [34,50–54,59,81,82]. It must be specified that in some of the above-mentioned publications, DHAA was converted into a fluorophore [34,50–54,59,81,82] whilst in one conversion occurred by mixing AA and 1,2-phenylenediamine [19]. DHAA can also be determined indirectly in broccoli (sprouts, leaves, stalks, heads or florets) after its conversion to L-AA by RPLC-UV [20,97] and RPLC-DAD [39,44,98]. In two of these analyses [97,98], pre-column conversion of DHAA to L-AA was carried out using tris(2-carboxy-ethyl) phosphine solution in hydrochloric acid following extraction with mixtures of sodium dihydrogenophosphate and EDTA. Meanwhile, DL-1,4-dithiothreitol (DTT) was used in other studies to convert DHAA into L-AA after solvent extraction with oxalic acid [39] or MPA extractions [20,44]. Several publications were focused on determining AA or L-AA individually (see Table 4). Different solvents were employed as extractants such as MPA [64,84], oxalic acid [18,77], water [99] and mixtures [22,33,93,100]. The determination of those phytochemicals in broccoli florets [64], heads [22,29,33], sprouts [77], stems or leaves [93] have been mainly achieved by RPLC (C_{18} columns) coupled to DAD [22,29,33,64,77,84,93,100] or UV–vis [18,99]. Finally, spectrophotometric detection (UV–vis) was employed for the analysis of AA and related compounds after performing a solvent extraction with methanol (florets and stems) [47], MPA (inflorescences) [62] or TCA (florets) [66].

B-group vitamins, which are water-soluble vitamins related to metabolism, were also investigated in broccoli samples [100]. In this research, vitamins B1, B2, B3, B5, B6, B9 and B12 were extracted from broccoli samples using a composite formed by water, sodium hydroxide and PBS, and were determined by RPLC-DAD (C_{18} stationary phases). Folate, also known as vitamin M or vitamin B9, has been associated with neural reduction defects and with certain beneficial effects against some types of cancer and other diseases. RPLC-FLD (C_{18} stationary phases) was the preferred analytical technique to determine folate and related compounds in broccoli florets [101,102] and heads [20]. In two studies, chemical conversion to 5-methyl-tetrahydrofolate was conducted prior to its extraction with PBS [101,102], while solvent extraction with TCA and ammonium acetate was sufficient in the other study [20].

Tocopherols (TOC), which are a class of chemical compounds many of which have vitamin E activity, are involved in the protection of membrane lipids from oxidative damage, acting as anti-inflammatory agents. These compounds have been determined in broccoli by RPLC with C_{18} stationary phases (DAD [28,84]; FLD [38]) or UPLC-DAD [37]. Solvent extraction with MPA (heads) [84], methanol (florets) [37] and a mixture of DCM and hexane (edible parts) [28] was used in some cases, or saponification with potassium hydroxide [38] was employed as the sample treatment.

It can be concluded that a sample treatment based on solvent extraction is required in order to obtain vitamins from broccoli. However, the selection of the solvent should be done accordingly to the vitamin group. For example, MPA or a solvent mixture ($C_6H_8O_7$/EDTA/NaF/methanol/water) is recommended to analyze AA, L-AA or DHAA, while other solvents like PBS, MPA, methanol or different solvent mixtures are usually employed to extract B-group vitamins and tocopherols. Moreover, the use of SPE is necessary in some cases in order to obtain cleaner and eliminate some matrix compounds that could interfere with the determination of vitamins. Finally, RPLC coupled to several detectors (UV, DAD, and FLD) is the best option to determine this group of compounds as it solves most of the problems related to the titrimetric and fluorimetric methods. Although, it should be also mentioned that it is more complicated and expensive than these latter methods.

2.5. Glucosinolates and related compounds

Glucosinolates (GLSs) are thioglucosides containing a cyano group and a sulphate group [50]. Because GLSs coexist with myrosinase in the plant, fresh plant material processing in the presence of water (grinding, cutting) will initiate a rapid hydrolysis of the parent compounds. For this reason, analytical approaches could be divided into methods for total GLSs, individual GLSs and breakdown products (thiocyanates, isothiocyanates (ITCs), and oxazolidine-2-thiones) [103]. GLSs and/or their breakdown products have long been known for their fungicidal, bactericidal, nematocidal and allelopathic properties, and they have recently gained research interest because of their anticancer activity. The potential beneficial effects of GLSs and related compounds, such as ITCs, in relation to several diseases (cancer, cardiovascular and neurological diseases) have been previously discussed in some review articles [104,105]. Meanwhile, another study sought to provide evidence for and against ITCs as chemopreventive agents [106]. In relation to determining GLSs in broccoli, it should be specified that these compounds are usually established according to the presence (intact) or absence (desulfo-derivatives, desulfo-GLSs) of the sulphate group in their structure. A common approach, which is widely used [107], is to convert the intact GLSs into desulfo-derivatives, as they can be more easily determined by RPLC. Identification of the GLSs by means of this method is based solely on comparing retention times and UV spectra with reference standards, but due to the lack of availability of many GLSs standards it is difficult or even impossible to determine unequivocally unknown GLSs. For that reason, the use of MS detectors appears to be a solution for a more trustworthy identification of GLSs.

An on-column enzymatic desulfation treatment has been adopted in order to determine by RPLC desulfo-GLSs in most of the broccoli parts such as seeds, florets, heads, shoots, roots or leaves (see Table 5). These studies largely made use of a preliminary solvent extraction (see Supplementary information, Fig. S5) with heated solvents such as mixtures of methanol and water at different ratios (70:30, v/v) [69,84,108–124], (80:20, v/v) [125,126], (90:10, v/v) [62], although water alone has also been employed [18,45,78,99,127,128] followed in most cases by a SPE desulfation. In one study, however, desulfo-GLSs were extracted using a microwave digestion procedure [100]. It can be concluded after studying the summarized data (Table 5 and Supplementary information, Fig. S5) that the best choice to extract desulfo-GLSs is the use of a mixture of water and methanol (70:30, v/v). Furthermore, RPLC (C_{18} analytical columns) is the technique of choice to analyze these compounds, and this has been coupled to several detectors like spectrophotometric (UV–vis [77,99,108,117,118,122,124,125,127,128] or DAD [78,84,111,112,115,116,119,121,123,126]), and MS detectors such as ESI-MS [18,121,123] or ESI-MS/MS [69,110–113], in order to perform identification and quantification. It should be pointed out that MS detectors have been predominantly used with identification and confirmation purposes, as it is not necessary a great sensitivity to determine those compounds because they are present in high concentrations in broccoli. Several aliphatic, indole and aromatic desulfo-GLSs were analyzed in the above-mentioned publications. Other methodologies have been developed, not based on RPLC, to determine desulfo-GLSs [45,120]. For example, a CE-DAD method was employed to determine several desulfo-GLSs, subsequent to boiling solvent extraction with a mixture of methanol and water (70:30, v/v) [120]. Meanwhile, total desulfo-GLSs content was measured spectrophotometrically (UV–vis) in broccoli heads [45]. In this study, solvent extraction with boiling

Table 5
Applications in the analysis of glucosinolates and related compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Desulfo-GLSs	Heads (cv. Lvxiong) [18]; florets [127,109,79] and stems (cv. Youxiu [127,109,99], Lvling [99] and Ironman [112], Volta F1 [69]; seeds (*cymosa* Duch. Monopoly [125], F1 Hybrid [125], Syngenta Enkhuizen [125], Netherlands [125], Marathon [113]); edible parts [108]; roots and shoots (cv. Marathon) [110]; shoots (38 cultivars) [126]; heads (cv. Youxiu [115], Lvxiong [115], Sijilv [115], Shenglvand [115], Yangguang [115], '1997' [117], Monaco [84]); spouts [116,27] (cv. Youxiu [78]); inflorescences (cv. Marathon [62,118] and Parthenon [118]); leaves (cv. GDDH33) [114]	Solvent extraction (water) [18,78,99,127]; MeOH [39]; (90/10 MeOH/water, v/v) [62]; (80/20 MeOH/water, v/v) [125,126]; (70/30 MeOH/water, v/v) [69,84,108–118] and SPE desulfation	RPLC-MS [18]; RPLC-UV [39,99,108,109,117,118,125,127]; RPLC-DAD [78,84,115,116]; UPLC-DAD [126]; RPLC-DAD-ESI-MS/MS [69,110–113]; NS [62,114]	[18,39,62,69, 78,84,99,108–118,125,126,127]
Total desulfo-GLSs content	Heads (cv. Calabrese and Southern Star)	Boiling solvent extraction (water), SPE desulfation, column isolation and tubes caption	UV–vis	[45]
Desulfo-GLSs[a] and SF[b]	Sprouts (cv. Youxiu [77,128]); boiled broccoli (NS) [119]	Boiling solvent extraction (H_2O) [77,128][a]; (70/30 MeOH/H_2O, v/v) [119][a] and SPE desulfation; solvent incubation (MES buffer solution) and extraction (DCM) [77,128][b]; conjugation with 2-mercaptoethanol [119][b]	RPLC-UV [77,128][a]; RPLC-DAD [119][a,b]; GC-FID [77,128][b]	[77,119,128]
Desulfo-GLSs	Steamed, raw and cooked broccoli (NS)	MAE digestion	RPLC-MS/MS	[100]
Desulfo-GLSs[a] and ITCs[b]	Heads (cv. Monaco) [120]; florets (cv. Monaco), sprouts (cv. Calabrese), Se and indole-GLS enrichment broccoli [124]	Boiling solvent extraction (70/30 MeOH/water, v/v) [120,124][a] and SPE desulfation; GLSs hydrolisis by water and solvent extraction (EtOAc) [120][b]; (DCM) [124][b]	RPLC-UV [124][a,b]; CE-DAD [120][a]; GC–MS [120][b]	[120,124]
Desulfo-GLSs[a] and ITCs[b]	Sprouts (cv. Calabrese [121]); heads [122]; NS (cv. Cezar) [123]	Boiling solvent extraction (70/30 MeOH/water, v/v) and SPE desulfation [121–123][a]; GRA hydrolysis by acidic water and solvent extraction (DCM) [121,122][b]; (EtOAc) [123][b]	RPLC-UV [122][a]; RPLC-DAD-ESI-MS [121,123][a]; RPLC-DAD [123][b]; GC–MS [122][b]; GC-FID [121][b]	[121–123]
Intact-GLSs[a] and SF[b]	Kailan-hybrid stems and florets [21]; Florets (cv. Marathon and Blooster™) [138]	Boiling solvent extraction (water) [138][a]; (70/30 MeOH/water, v/v) [21][a]; GRA hydrolysis by acidic water and solvent extraction (DCM) [21,138][b] and SPE [21][b]	RPLC-ESI-MS/MS [138][a,b]; RPLC-DAD-ESI-MS/MS [21][a]; RPLC-DAD [21][b]	[21,138]
Intact-GLSs	Heads/florets and controlled samples (cv. Parthenon) [22,29,34]; commercial broccoli [39]; leaves [51], stalks [51], sprouts [54] and seeds [54] (cv. Nubia, Viola and Marathon); green-tea infusions of inflorescences (cv. Nubia) [52,68]; inflorescences (cv. Marathon) [50]; shoots (cv. Parthenon and Naxos) [53]; sprouts [55] (cv. Marathon) [81,129]; leaves and florets (cv. Marathon) [82]; heads (cv. Parthenon [33], Monaco [68], Nubia [56] and Naxos [56]); leaves (cv. Monaco) [90]; florets and stems (cv. purple-sprouting) [59]; edible sprouts and seeds [57]; NS [130,92,132]	Boiling solvent extraction (70/30 MeOH/water, v/v) [22,29,33,34,50,51,55–57,59,68,81,92,129,82,130]; (MeOH) [39,52]; (80/20 MeOH/water, v/v) [53,54]; (water) [132]	RPLC-DAD-ESI-MS/MS [33,39,50–57,59,68,81,92,129,82]; RPLC-ESI-MS/MS [22,29,34]; RPLC-ESI-FTICR-MS/MS [131]; UPLC-ESI-MS/MS [132]	[22,29,33,34,39, 50,57,59,68,81, 92,129,82, 130,132]
Total intact-GLSs[a] content and SF[b]	Heads (cv. Chaoda)	Boiling solvent extraction (95/5 EtOH/water, v/v)[a]; (acetonitrile)[b] and myrosinase hydrolysis	Phenol–sulfuric acid method (UV–vis)[a]; RPLC-UV[b]	[36]

Table 5 (*Continued*)

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Intact-GLSs	Leaves	Boiling solvent extraction (20/80, 0.1%FA/MeOH v/v) and SPE	RPLC-ESI-MS	[131]
Novel intact-GLSs	Florets	Boiling solvent extraction (water) and SPE	RPLC-UV-ESI-MS/MS [134,135]; NMR [134,135]; FTIR [135]	[134,135]
Intact-GLSs	NS (cv. Rubra)	Cold maceration (80/20 MeOH/water, v/v) and SPE	TLC, paper chromatography and RPLC-UV	[140]
GRA	Florets	Boiling solvent extraction (water)	MECK-DAD, RPLC-DAD	[133]
GRA[a] and SF[b]	Seeds and sprouts (ZS ZaoSheng, XB XueBai, RF RuiFan N732, YX YinXing 100, TY TaiYou, LLX LuLingxiang and XMYH XiaMenYinhua) [136]; seeds (cv. Calabrese, DeCiccoand Romanesco) [137]	Boiling solvent extraction (water) [136,137][a] and SPE [136][a], GRA hydrolysis by acidic water and solvent extraction (EtAcO) [136][b]; (DCM) [137][b]	RPLC-UV [136][a,b]; RPLC-UV-MS [137][a]; GC-FID [137][b]	[136,137]
Intact-GRA[a] and SF[b]	Sprouts	Boiling solvent extraction (water or solvent)	HILIC-DAD[a]; RPLC-DAD[b]	[139]
GRA configuration	Seeds	NS	RPLC-UV-MS/MS and NMR	[141]
SF and related compounds	Heads and stalks [75]; sprouts [103,151]	Hydrolysis with water and solvent extraction (Cl_3CH) [151]; (DCM) [75,103]	Titrimetric method [75]; GC-FID–MS [103,151]	[75,103,151]
SF	NS [142,143]; heads (cv. Grandeur) [144]	GRA hydrolysis with acidic water and solvent extraction (DCM) and SPE	RPLC-UV	[142,143,144]
SF[a] and total ITCs[b]	NS	Solvent extraction (DCM) and SPE[a]; incubation with $Na_2B_4O_7$ and $C_6H_4(SH)_2$ and $C_6H_4(SH)_2$ following solvent extraction (hexane)[b]	RPLC-DAD	[149]
SF	Seeds	GRA hydrolysis by water, solvent extraction (EtAcO), SPE	HSCCC-UV, MS, NMR	[150]
Se-GLS[a] and Se-ITCs[b]	Florets	Boiling solvent extraction (80/20 EtOH/water, v/v)[a]; (50/50 diethyl ether/pentane, v/v)[b]	RPLC-MS/MS[a]; GC–MS/MS[b]	[152]
SF	Seeds	Boiling water, hydrolysis by acidic water (HCl)	RPLC-ESI-MS and NMR	[153]

Cl_3CH, chloroform; $C_6H_4(SH)_2$, 1,2-benzenedithiol; DAD, diode array detector; DCM, dichloromethane; ESI-MS/MS, electrospray ionization coupled to tandem mass spectrometry; EtOAc, ethyl acetate; EtOH, ethanol; FA, formic acid; FID, flame ionization detector; FTIR, Fourier transform infrared spectroscopy; FTICR, Fourier transform ion cyclotron resonance; GLSs, glucosinolates; GRA, glucoraphanin; HSCCC, high speed counter current chromatography; ITCs, isothiocyanates; MECK, micellar electrokinetic chromatography; MeOH, methanol; MES, 2-(N-morpholino)ethanesulfonic acid); MAE, microwave-assisted extraction; $Na_2B_4O_7$, sodium borate; NS, not specified; NMR, nuclear magnetic resonance; SF, sulforaphane; SPE, solid phase extraction.

Superscript letters (a,b) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

water was performed, desulfation being carried out in a SPE cartridge, and finally the desulfo-GLSs were isolated and purified on small ion-exchange columns.

Otherwise, most of the treatments proposed to analyze intact-GLSs included a heating process, as it is necessary to deactivate the enzyme myrosinase in order to achieve a better extraction of the compounds. As can be seen (Table 5 and Supplementary information, Fig. S5), several studies have been published dealing with this issue, and the preferred choice to extract intact-GLSs from broccoli extracts was the use of heated mixtures of methanol and water at different ratios (70:30, v/v) [21,22,29,33,34,50,51,55–57,59,68,81,92,129,82,130] (80:20, v/v) [54], although different mixtures, for instance, methanol and formic acid 1% in water (80:20, v/v) [131] or ethanol and water (95:5, v/v) [36] have been also employed. Moreover, other proposals exist in which heated methanol [39,52] and water [132–139] were used individually to carry out extraction. Prior to identifying and quantifying the intact GLSs, it was necessary to include a cold maceration with methanol and water [140] or SPE procedures [131,133–136] in order to obtain a better purification of the extracts. As occurred when analyzing desulfo-GLSs, several analytical techniques have been chosen to determine the intact-GLSs in broccoli extracts, which were obtained from all its parts, and RPLC (C_{18} columns) coupled to several detectors (UV–vis [36,134–136,140], DAD [21,33,39,50–57,59,68,81,92,129,82,139], MS [131,137] and MS/MS [21,33,34,39,50–57,59,68,81,92,129,82,132,134,135,138,140]) was selected in most of the studies (see Table 5). It must be pointed out that micellar electrokinetic chromatography [133] and hydrophilic interaction liquid chromatography [139] were also chosen to study intact glucoraphanin (GRA) in broccoli florets [133] and sprouts [139]. Meanwhile, GLSs fractions were also separated using thin layer chromatography or paper chromatography. Taking into these data, it is recommended the use of RPLC with UV or DAD detectors when it is possible to obtain the intact-GLSs standards, while MS detectors should be employed if they are not available.

Not only chromatographic methods have been employed to analyze intact GLSs. Fourier transform ion cyclotron resonance MS was chosen to identify 24-intact GLSs identified in broccoli [130], while nuclear magnetic resonance (NMR) has been also selected to carry out other studies in which some new GLSs were investigated [134,135] or the GRA configuration was specified [141]. Moreover, Fourier transform infrared spectroscopy (FTIR) was also used in one study to determine novel intact GLSs in broccoli florets [135]. The quality and quantity of data obtained with these latter analytical techniques are really high, but it should be pointed out that they are more complex and expensive than the LC or spectrophotometric methods.

After examining the scientific literature related to the analysis of GLSs in broccoli, it can be recommended a solvent extraction with a heated mixture of methanol and water, which is followed by a SPE desulfation when analyzing desulfo-GLSs, as sample treatment. Meanwhile, RPLC with UV or DAD detectors is the best choice to determine those compounds when it is possible to obtain individual standards, and MS detectors are recommended if they are not available.

The other huge sulphur-containing phytochemical group that can be found in broccoli are ITCs. Their separation and identification have been typically accomplished by RPLC. However, the application of this technique to investigate certain glucosinolate breakdown products may be limited due to the volatility of many compounds. For this reason, some studies opted for using GC. One of the most extensively studied ITCs is sulforaphane (SF), because of its potential health benefits (see Section 3). Most of the studies in which this compound was analyzed were based on the hydrolysis of its precursor (GRA) by using acidic water at different pHs, and a further extraction step with an organic solvent such as DCM [21,75,77,103,121,122,124,128,137,138,142–147,149], ethyl acetate [120,136,150], or chloroform [146,151] (see Supplementary information, Fig. S5). In some cases, instead of acidic water, SF was incubated with 2-(N-morpholino) ethanesulfonic acid [77,128], or it was conjugated with 2-mercaptoethanol [119]. It should also be mentioned that in some studies SPE with silica cartridges was performed to purify the broccoli extracts [21,142–144,149,150]. Meanwhile, in other study, the myrosinase enzyme was used to perform SF hydrolysis before extracting with acetonitrile [36]. Most of the above-mentioned research studies employed RPLC with C_{18} based stationary phases (DAD or UV–vis [21,36,119,133,139,142–145,149], MS [138]), and GC with non- or low polar capillary columns (FID [77,103,121,128,137,147,148,151] and MS [103,120,122,146,151]). However, it has been postulated that in some cases SF was thermally degraded in the injection ports of GC equipment [143], and usually the GC analysis times were longer than those of LC. As happened with GLSs, MS was not usually employed because of the high SF content in broccoli. It must be added that in one study [150] separation was carried out by high-speed counter-current chromatography, while SF presence was confirmed by NMR and MS. NMR has also served to analyze SF in broccoli seeds [153]. Moreover, a titrimetic method was applied to determine SF and related compounds in broccoli heads and stems by using hexahydropyridine as a reagent [75]. So, in order to determine SF in broccoli, it is recommended to carry out a hydrolysis with acidic water, a further solvent extraction with DCM, and a SPE procedure with silica cartridges before injecting the extracts in a RPLC-UV or DAD system.

Finally, research has been published in which ITCs other than SF were analyzed [120,123,124,149,152]. As can be seen in Table 5, the extraction and characterization methodologies employed in those cases were quite similar to the ones selected when analyzing SF.

2.6. Essential elements and other compounds

Broccoli is a good vegetable source of essential elements like Se, minerals for human nutrition (Ca and Mg), main (Na, K, Cl and P) or trace (Fe, Zn, Cu, Mn, I, F, Se, Cr, Mo, Co, and Ni) elements and sugars. Selenium is an essential nutritional element that has attracted interest due to its potential anticancer activity [154]. Moreover, it is known that Broccoli has the ability to accumulate high levels of Se with most of the seleno-amino acids in the form of Se-methylselenocysteine and seleniomethionine. Several papers have been published in last 5 years where selenium was investigated in this matrix (see Table 6 and Supplementary information, Fig. S6). For example, the selenium profile in florets and sprouts was directly determined by means of neutron activation analysis (NAA) [124], whilst total selenium content in florets, leaves and stems was calculated using inductively coupled plasma with mass spectrometry (ICP-MS) [38,112] or FLD [145], although in one case [38] it was necessary to perform acid digestion as a sample treatment. The Se speciation was subjected to study in leaves, roots and sprouts [38,145] by ICP-MS. In both cases it was necessary to conduct a sample treatment (acid digestion [38] and sonication [145]) and a previous separation with ion [38] or size exclusion [145] chromatography in order to obtain satisfactory results. Se-methylselenocysteine and seleniomethionine were also determined in broccoli florets and leaves, after performing acid digestion [73] or solvent extraction [126] and a further liquid chromatographic separation and detection by FLD [73] and DAD [126]. Finally, it should be said that other selenium compounds (organoselenides and volatiles) were identified by GC–MS in broccoli sprouts after performing solvent extraction [152]. As can be seen, different analytical techniques have been employed to determine Se and related compounds, some of them allowed the separation and identification of single analytes (LC and GC), while the other

Table 6
Applications in the analysis of essential elements and other compounds in broccoli samples.

Compounds	Broccoli part	Sample treatment	Characterization method	Refs.
Total Se and Cd content[a] and Se speciation[b]	Leaves and roots	Digestion (HNO_3/H_2O_2)	ICP-MS[a]; IC and ICP-MS[b]	[38]
Se-methylselenocysteine	Florets	Solvent extraction (HCl)	RPLC-FLD	[73]
Total Se content	Florets (cv. Ironman)	NS	ICP-MS	[112]
Se profile	Florets (cv. Monaco), sprouts (cv. Calabrese)	NS	NAA	[124]
Cations (Fe, Zn, Cu, and Mn)[a], Total selenium content[b], Se-methylselenocysteine[b] and selenomethionine[b]	Florets and leaves (38 varieties)	Digestion ($HNO_3/HClO_4$)[a]; Solvent extraction (HCl)[b]	ICP-MS[a]; UPLC-DAD[b]	[126]
Total Se content[a] and speciation[b]	Sprouts	NS[a]; Sonication (deionized water)[b]	FLD[a]; SEC and ICP-MS[b]	[145]
Organoselenides and Se volatiles	Florets (cv. Triathlon)	Solvent extraction (pentane/diethyl ether)	GC–MS	[152]
Anions (Cl and P) and cations (Na, K, Ca, Mg, and Fe)	Stem and flower ashes	Solvent extraction (H_3BO_3)	XRF	[13]
(P, Ca, Mg, K, Na, Fe, Mn, and Zn)[a] and total (C and N)[b] content	Leaves and stalks (cv. Nubia, Marathon and Viola)	Digestion ($HNO_3/HClO_4$)	ICP-OES[a]; TCD[b]	[51]
Anions (Cl^-, NO_3^-, SO_4^{2-}, and PO_4^{3-})[a], cations (Ca^{2+}, K^+, Mg^{2+}, and Na^+)[b] and soluble sugar content[c]	Shoots, roots[a,b] and leaves[c] (cv. Parthenon and Naxos)	Digestion (HNO_3/H_2O_2)[a]; SPE[b]	IC-ECD[a]; ICP-AES[b]; UV–vis[c]	[53]
Anions (P, S, and B) and cations (Na, K, Ca, Mg, B, Cu, Fe, Mn and Zn)	Heads (cv. Nubia and Naxos)	Digestion (HNO_3/H_2O_2)	ICP-OES	[56]
Anions (Cl^-, NO^{3-}, PO_4^{3-}, and SO_4^{2-})[a] and cations (Na^+, Ca^{2+}, K^+, and Mg^{2+})[b]	Leaves and florets (cv. Marathon)	NS	IC-UV–vis[a]; ICP-OES[b]	[82]
Anions (N[a] and P[b]) and cations (K[c], Ca[c], Mg[d], Fe[d], Mn[d], Cu[d], and Zn[d])	Heads (cv. Shogun F1, Sultan F1, Marathon F1 and Pirate F1)	NS	Kjeldahl method[a]; Vanadat-Molibdat method[b]; FES[c], AAS[d]	[85]
Anions (N[a], NO_3^- [b]) and cations (K[c] and Ca[d])	Inflorescences (cv. Parthenon and Marathon)	NS[a,b]; Digestion (HNO_3)[c,d]	Dry combustion[a]; IC-ECD[b]; FES[c]; AAS[d]	[118]
Anions (Cl^-[a], SO_4^{2-}[b], and B[b]), cations (Ca, Mg, Na, and K)[c], total S and P content[b]	Leaves, stems and heads (cv. Seminis PX511018)	Digestion ($HNO_3/HClO_4$)	Coulometric–amperometric titration NS[b]; ICP-OES[c]	[156,157]
Anions (Cl^- and NO_3^-)[a] and cation (Na^+)[b]	Leaves (cv. Lord)	NS	CE-UV[a]; ICP-OES[b]	[158]
K	NS	Digestion (HNO_3/H_2O_2)	FES	[160]
Heavy metals (Cd, Pb, Ni, Cu)	Roots	NS	Phytotoxicity scales (agar and filter paper)	[161]
Total [32] and reducing sugar content	Florets (cv. iron)	Solvent extraction (EtOH)	UV–vis	[30–32]
8 sugars	Roots and shoots (cv. Marathon)	Solvent extraction (MeOH)	LC-ECD	[110]
3 soluble sugars	Leaves and heads (cv. Marathon)	Reduction (NADPH)	UV–vis	[155]

AAS, atomic absorption spectroscopy; AES, atomic emission spectroscopy; ECD, electrochemical detector; EtOH, ethanol; FES, flame emission spectroscopy; HNO_3, nitric acid; H_3BO_3, boric acid; H_2O_2, hydrogen peroxide; HCl, hydrochloric acid; $HClO_4$, perchloric acid; ICP, inductively coupled plasma; MeOH, methanol; NAA, neutron activation analysis; NADPH, nicotinamide adenine dinucleotide phosphate; NS, not specified; OES, optical emission spectroscopy; TCD, thermal conductivity detector; IC, ion chromatography; SEC, size exclusion chromatography; SPE, solid phase extraction; UPLC, ultraperformance liquid chromatography; XRF, X-ray fluorescence spectroscopy. Superscript letters (a,b,c,d) are used to relate an specific analyte with the corresponding sample treatment and characterization method.

techniques (NAA, ICP, and FLD) are more recommended to investigate the analyte profiles and total content.

Moreover, some studies have been published in relation to the argument that broccoli could be an alternative source of Ca or Mg in sectors of the population that consume limited amounts of dairy products [155]. As can be seen in Table 6, they were determined in almost all broccoli parts such as leaves, stalks, stems, heads, roots and inflorescences. Most of the proposed sample treatments consisted of acid digestion with nitric acid [118] or mixtures of nitric acid with perchloric acid [51,156,157] or hydrogen peroxide [53,56], yet in one case a solvent extraction with boric acid was used [13]. The characterization mode of choice adopted for simultaneously determining these compounds was ICP coupled to either optical emission spectroscopy (OES) [51,56,156–158] and atomic emission spectroscopy (AES) [53], although X-ray fluorescence (XRF) [13] and flame emission spectroscopy (FES) [118] have been also employed. These compounds were detected in a different way in one publication referring to broccoli heads [85]. In this case Ca was quantified by FES, while Mg was measured by atomic absorption spectroscopy (AAS). Taking into account those findings, it can be recommended an analytical methodology to determine Ca and Mg in which an acid digestion of the broccoli sample should be done prior to analyze the extract by ICP-OES.

In relation to main and trace elements, it should be stated that they have varied functions with regard to humans, as they act as electrolytes, enzyme constituents, or building materials (*e.g.*, in

bones and teeth). However, not all their potential health effects are positive, as is the case with their nitrate content. Human dietary nitrate and nitrite exposure should be controlled as they may be considered a health risk factor. This could be explained by the conversion of nitrates, which are relatively harmless to humans, to nitrites or other N-nitrose compounds perhaps producing toxic products [159]. The composition of these main and trace elements in broccoli has not been examined in depth, and as a consequence not many studies have been published relating to this topic. Different sample treatments have been proposed regarding their determination in several broccoli parts such as florets, leaves, roots, stems, heads and inflorescences (see Table 6). As occurred with Ca and Mg, acid digestion with HNO_3 or mixtures with other compounds [51,53,56,118,156,157] and ICP coupled to OES or AES [51,53,56,156–158] are recommended as sample treatment and determination method, respectively. Other methodologies have been less often employed to perform this task. For example, several of these compounds were extracted with boric acid and measured by XRF in broccoli stems and flower ashes [13], while in another publication [118], different determination methods were proposed according to the compound studied: (i) a dry combustion method was used to determine total nitrogen content; (ii) a digestion method with concentrated nitric acid followed by FES was employed to measure potassium; (iii) ion chromatography separation with ECD was applied to quantify nitrates. Potassium and nitrate have been determined in a similar way in other researches [160] and [82], respectively, but to measure nitrates it was employed UV–vis. Moreover, nitrate and chloride were analyzed in a different study by using CE-UV [158]. Total C and N contents have also been determined in broccoli leaves and stalks with a thermal conductivity detector (TCD) following acid digestion [51]. It is of interest to note that, as special cases, a Kjeldahl method was also employed to determine total N content and a colorimetric method was used to measure total P content, both of them in broccoli heads [85]. Moreover, chloride was determined in several broccoli parts by coulometric–amperometric titration [156,157]. Not only beneficial elements were found in broccoli. In one analysis [161] broccoli roots were used to test the toxicity of four heavy metals (Cd, Pb, Ni and Cu).

Several studies related to investigating sugars in broccoli have been published in the last years (see Table 6). Total and reducing sugar content have been determined in florets. It was necessary to perform a solvent extraction with ethanol prior to quantifying them with a UV–vis spectrophotometer [30–32]. The same detection method was used to measure the content of several soluble sugars in leaves and heads [159] or in shoots, roots and leaves [53], but in these cases the sample treatment consisted of a reduction with NADPH [159] or a SPE procedure [53]. Finally, a solvent extraction method with methanol, followed by LC-ECD with an anion-exchange column, has been proposed in order to determine eight sugars in broccoli roots and shoots [110].

3. Biological activity determination

As can be deduced from the previous sections of this manuscript, broccoli is an excellent dietary source of phytochemicals, including lipids, phenolic compounds, proteins, peptides, amino acids, vitamins, glucosinolates and their breakdown products, and certain minerals and essential elements. The potential health promoting roles of these compounds have been extensively studied in the last years. Beneficial effects such as antioxidant, anticancer, antimicrobial, amongst several others (Fig. 5), have been reported in many publications. Most of the research that focussed attention on the biological activity of broccoli was related to the antioxidant and anticancer properties of this vegetable due to the action of several of the above-mentioned phytochemicals. In this section the different biological activities of broccoli will be discussed, including many of the recent publications related to this issue, and how these beneficial effects were determined in some specific cases.

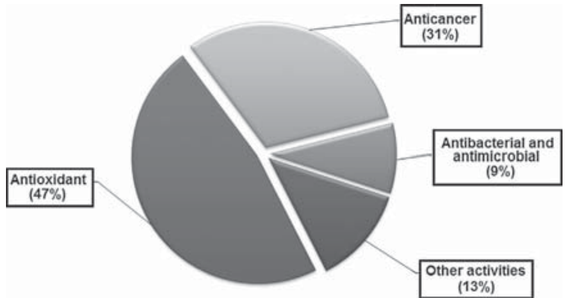

Fig. 5. Summary of the different biological activities attributed to the health promoting compounds of broccoli in the last five years.

3.1. Antioxidant

Broccoli is renowned for its vast range of non-enzymatic bioactive compounds, being rich in both nutritional antioxidants like vitamins, and non-nutritional antioxidants as are carotenoids, and phenolic compounds, particularly flavonoids [47]. It should be added that the antioxidant properties of glucosinolates and related compounds has been also reported [103].

The antioxidant properties of broccoli extracts were studied in many of the publications by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method [13,19,20,25,31,32,34,36, 42,43,45–48,51,52,54,56,57,61,62,64–67,69,72,73,75,76,83,126, 146,162–164]. DPPH is a dark crystalline powder composed of stable free-radical molecules, which is used to monitor chemical reactions involving radicals, and most notably it is a common antioxidant assay. Moreover, in some cases this was undertaken in combination with other antioxidant assays with the aim of obtaining a more detailed understanding of the antioxidant properties of the samples. Thus, DPPH was used together with several other assays as ferric reducing antioxidant power (FRAP) [42,43,46,56,57,61,83,126], ferrous ion chelating capacity [13,45,46], hydroxyl radical-scavenging activity [69,75,163], oxygen radical absorbance capacity (ORAC) [72], 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical method or Trolox equivalent antioxidant capacity (TEAC) [54,56,64]. Finally, it should also be mentioned that the majority of these research studies were performed *in vitro* [31,32,34,47,51,52,54,56,75,76].

A different way of studying this positive health effect is by using the FRAP assay. In this procedure, antioxidants are used as reducing agents in a redox-linked colorimetric method, employing an easily reduced oxidant system present in stoichiometric excess. These assays have usually been carried out in combination with the DPPH method to determine the antioxidant properties of broccoli extracts [42,43,46,56,57,61,83,126]. In one interesting publication, there was a comparison of three different assays (FRAP, TEAC and total radical-trapping antioxidant parameter (TRAP)) to study the antioxidant characteristics of raw and frozen broccoli florets and stems [39]. Meanwhile, FRAP was exclusively used in one study, in which it was concluded that microwave cooking and boiling cause losses of antioxidants and phenolics in vegetables as broccoli [165].

ABTS assays were also employed to check the antioxidant properties of different broccoli extracts (heads, sprouts and seeds) [22,27,29,33,41,54,56,80,163]. In this procedure, ABTS is converted to its radical cation by the addition of sodium persulfate. This radical cation is reactive towards most antioxidants including phenolics, thiols and vitamins. This assay is often referred to as the TEAC assay.

The ORAC assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators, which produce a radical that damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are considered to protect the fluorescent molecule from oxidative degeneration. However, it must be added that in 2012 this was considered biologically invalid by the United States Department of Agriculture (USDA) [166], because no physiological proof *in vivo* existed in support of the free-radical theory. Therefore, this assay was no longer deemed relevant to human diets or biology by the USDA. However, different broccoli extracts have been tested using this method in the last years [63,68,70,72,167,168].

Finally, it should be said that several other assays have also been employed to analyze the antioxidant characteristics of different broccoli extracts, such as ferrous ion chelating capacity [13,45,46], hydroxyl radical-scavenging activity [69,75,163,169], TRAP [39,70] and different methods based on reactive oxygen species, for instance, superoxide anion, peroxyl, and alkoxyl radicals [42,46,63,71,75,163].

3.2. Anticancer

In the last few years, cancer prevention by natural products has received considerable attention. The potentially protective role of cruciferous vegetables, including broccoli, and active components present in these vegetables, such as GLSs and related products (ITCs, especially SF), has been extensively studied in experimental *in vitro* and *in vivo* carcinogenesis models [103]. Recent studies have demonstrated that in humans SF is rapidly absorbed following consumption of liquidized broccoli, but repeated intake of the vegetable does not lead to higher plasma levels, and subsequently to an accumulation in the organism [170]. Several review articles have been published [171,172] which explain and discuss some of the experimental, clinical and epidemiological evidence of anticancer activity among other potential health benefits of SF. This compound has shown itself to be useful as a chemopreventive agent in colon cancer with inactivated or lost p53 [173], as an inhibitor of pancreatic cancer cell growth *in vitro* and *in vivo* tumour suppressor in mouse models [174], and has also shown some beneficial effects in relation to prostate [175] and breast [176] cancer. For all these reasons, it is not surprising that most of the publications devoted to investigating the anticancer activity of broccoli and derived products were related to SF [145,146,149,170,177–188]. In one of these studies [177], it has been demonstrated that there is a synergy between SF and gemcitabine which may enhance the therapeutic index of prevention and/or treatment of cervical cancer [177]. In this *in vitro* study, a colorimetric assay was used for detection of apoptosis in treatment, as well as a reverse transcription polymerase chain reaction (RT-PCR) for RNA isolation and expression analysis. Meanwhile, in order to obtain a better understanding of the temporal effects of SF and broccoli sprouts on gene expression in prostate cells, a comprehensive transcriptome analysis was conducted using cDNA microarrays [181].

Phenolic compounds have also been investigated for their potential anticancer activity [58,80,168,189]. For example, an analysis was undertaken of the effect of the bio-accessibility of phenolic compounds on the *in vitro* anticancer activity of broccoli sprouts [80]. Meanwhile, the compounds present in new broccoli-enriched green tea drinks and their potential antitumor activity *in vitro* were evaluated in a different study [58]. After performing RPLC-PAD-ESI-MS analyses, it was found that the compounds were mainly phenolics and glucosinolates.

Finally, it should be mentioned that one study has been published suggesting that the intake of cruciferous vegetables is inversely associated with lung cancer risk [190], while two different studies argued that is not sufficient to eat broccoli frequently to prevent the occurrence of cancer [191], and that no protective role had been found in a high intake of fruits or vegetables regarding the risk of endometrial cancer in older women [192]. Consequently, after a meticulous revision of existing literature related to this issue, it can be concluded that the consumption of broccoli provides the organism with several phytochemicals which have been shown to possess anticancer activity. However, it cannot be postulated that a high intake of broccoli could increase the above-mentioned anti-carcinogenic effects.

3.3. Other effects

The antibacterial and antimicrobial activities of broccoli extracts have been the subject of study in several publications [19,42,46,151,193–196]. In some of this research [42,46], a comparative study has been conducted of the antibacterial activities of broccoli (florets and stalks), Brussels sprouts, white and York (only in [46]) cabbage extracts against several bacteria. It was observed that broccoli [42] and York cabbage [46] displayed the highest rate of antibacterial activity against most of the tested bacteria in comparison with the other vegetables tested.

Broccoli extracts have also been investigated for their potentially beneficial effects for patients with diabetes, cholesterol, cardiovascular diseases and asthma [139,197–203]. Broccoli sprout powder was used as a supplementary treatment in type 2 diabetic patients [197,199]. It was observed that using broccoli sprout powder as a supplementary treatment in type 2 diabetes could have favourable effects on lipid profiles as risk factors for cardiovascular disease. Although, on the other hand, it has been also postulated that green leafy vegetables such as broccoli may inhibit warfarin, which is nowadays used for many patients on therapy for various cardiovascular diseases, due to the high content of vitamin K [202]. It should be also commented that several of the above-mentioned publications were related to broccoli affecting cholesterol metabolism in rodents [139,200,201]. Moreover, broccoli sprout homogenates with high SF content have been successfully employed to induce phase II enzyme expression in the human airway [203].

Broccoli has shown beneficial effects not only for humans but similarly for insects and fishes [204–207]. For example, the effect of broccoli in the diet on the enzyme activities of tilapia fish during pollutant exposure has been studied in two of these publications [206,207]. Several procedures were applied in order to check different enzyme activities, which included the use of HPLC-DAD and UV–vis spectrophotometers. The results showed that diets containing broccoli induce beneficial changes in the enzymatic systems involved in the detoxification metabolism of fish.

Finally, it is appropriate to mention two research studies referring to particular biological activities observed in plants [208] and eggs [209]. The first article contained a description of the isolation of a broccoli defensive gene and its effect on downy mildew resistance [208]. Meanwhile in the latter study an analysis (HPLC-DAD and GC-FID) was made of the effects of broccoli stem and leaf meal on the production performance and egg quality of laying hens [209].

4. Conclusions

In this paper we have presented an overview of broccoli health promoting compounds over the period January 2008 to January 2013, discussing the different bioactive compounds (lipids, vitamins, proteins, glucosinolates, phenolic compounds, *etc.*), the analytical techniques mainly employed for their extraction, as well as their characterization and determination of biological activity. Scientific interest is demonstrated by the number of research

papers (>200) published on this topic during the period reviewed. It may be concluded that the main group of phytochemicals analyzed in broccoli have been glucosinolates and related compounds, although the study of phenolic compounds in broccoli have also attracted the attention of many researchers. Solvent extraction has been the treatment of choice to isolate broccoli bioactive compounds, as this has been predominantly used for all of them. Moreover, SPE has also been widely employed, especially in the analysis of glucosinolates and related compounds. In relation to characterization techniques, liquid chromatography has mainly been employed, probably due to its versatility, generalized availability and simplicity. Other techniques such as UV–vis, GC, CE, MS, ICP, NMR, UV–vis, or FTIR have also given good results, although their use is not as widespread as LC. The health promoting compounds in broccoli possess a large variety of biological activities, as has been demonstrated by the large number of publications related to this issue. The antioxidant properties of broccoli extracts determined with different assays (DPPH, FRAP, ORAC, ABTS, *etc.*) was a predominant feature in these research papers, although the study of the potential anticancer activities of several broccoli compounds using several methodologies (RT-PCR, arrays, *etc.*), and especially that of sulforaphane, is currently gaining attention. Moreover, some research has also been published focussing on several other biological activities, such as antimicrobial and antibacterial, or the potentially beneficial effects of broccoli for patients with diabetes, cholesterol, cardiovascular diseases and asthma. All the information summarized in this manuscript should facilitate the identification of health promoting compounds in broccoli, their extraction and chemical characterization, as well as making the study of the different biological activities related to these compounds easier.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.07.051.

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V.4. PAPER IV. Optimized extraction, separation and quantification of twelve intact glucosinolates in broccoli leaves

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Analytical Methods

Optimized extraction, separation and quantification of twelve intact glucosinolates in broccoli leaves

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ABSTRACT

A new method has been developed and validated to determine twelve intact glucosinolates (glucoiberin, GIB; glucoraphanin, GRA; glucoerucin GER; gluconapin, GNA; glucotropaeolin, GTL; glucobrassicin, GBC; gluconasturtiin, GST; glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4-OH; 4-metoxyglucobrassicin, 4ME; neoglucobrassicin, NEO; sinigrin, SIN) in broccoli leaves using liquid chromatography (LC) coupled to diode array (DAD) and electrospray ionization mass spectrometry (ESI-MS) detection. An extraction procedure has also been proposed and optimized by means of statistical analysis (the Box–Behnken design and analysis of variance); this is based on the deactivation of myrosinase using a microwave and heated water. Low limits of detection and quantification were obtained, ranging from 10 to 72 μg/g with DAD and 0.01 to 0.23 μg/g with ESI-MS, and the resulting recovery values ranged from 87% to 106% in all cases. Finally, glucosinolates were analyzed in broccoli leaf samples from six different cultivars (*Ramoso calabrese Parthenon, Marathon, Nubia, Naxos* and *Viola*).

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1. Introduction

Numerous epidemiological studies indicate that Brassica vegetables in general, and broccoli (*Brassica oleracea* L. var. *italica*) in particular, protect humans against cancer, since they are rich sources of glucosinolates as well as of other phytochemicals (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006). Glucosinolates are β-thioglucoside *N*-hydroxysulfates, with a side chain (R) derived from amino acids and a sulfur-linked β-D-glucopyranose moiety (see Fig. 1 and Table 1), and some of these compounds appear to be protective against chemical carcinogens (Tolrà, Alonso, Poschenrieder, Barceló, & Barceló, 2000). Moreover, certain metabolic breakdown products of glucosinolates, particularly isothiocyanates and nitriles, have been shown to both modify xenobiotic metabolizing enzymes that protect DNA from damage and induce cell cycle arrest and apoptosis (Lund, 2003). A large body of research into anticarcinogens has focused on a single bioactive component within broccoli, namely, sulforaphane, which is formed by hydrolysis of a glucosinolate (glucoraphanin, GRA). For these reasons, it can be concluded that the chemoprotective effect of broccoli is related with glucosinolate content. It is, therefore, necessary to determine the vegetable's glucosinolate content and profile, as each of the glucosinolates from broccoli generates a specific breakdown product, which possesses different biological properties.

Intensive broccoli cultivars are associated with the production of considerable waste products, mainly leaves that are discarded, even though they may have a similar composition to the edible parts of the plant. These by-products could complement animal feed or be used as a source of nutraceuticals, which would reduce environmental impact and, at the same time, increase economic value. In the scant published scientific literature dealing with the nutritional content of broccoli leaves, as compared with the parts normally consumed, studies have been made of amino acids (Arnáiz et al., 2012), fatty acids (Manzano et al., 2011), vitamins, phenolic compounds, minerals (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010) and glucosinolates (Branca, Li, Goyal, & Quiros, 2002; Domínguez-Perles et al., 2010; Hennig, Verkerk, Bonnema, & Dekker, 2012; López-Berenguer, Martínez-Ballesta, García-Viguera, & Carvajal, 2008; Sasaki, Neyazaki, Shindo, Ogawa, & Momose, 2012).

Glucosinolates are usually determined according to the presence (intact or non-intact desulfo-derivatives) of a sulfate group. Desulfo-glucosinolates have been analyzed previously (Branca et al., 2002; Hennig et al., 2012) following extraction with heated methanol and a water mixture (70:30, v/v, Hennig et al., 2012) or with methanol (Branca et al., 2002). Extracts were subsequently absorbed on a solid support and subjected to enzymatic desulfation. The extraction methods employed with intact glucosinolates mainly involved the use of heated mixtures of methanol and water (70:30, v/v) for 30 min (Domínguez-Perles et al., 2010; López-Berenguer et al., 2008) or 0.1% formic acid in water and methanol (20:80 v/v), subsequent to autoclaving with boiling water, for

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Fig. 1. General chemical structure of glucosinolates.

10 min (Sasaki et al., 2012). All these treatments include heating, which is necessary to deactivate the myrosinase enzyme and obtain a better extraction of intact glucosinolates. In addition, intact glucosinolates have been extracted from edible parts (sprouts, florets, heads, stems and roots) using heated water (Jones, Frisina, Winkler, Imsic, & Tomkins, 2010), methanol (Song & Thornalley, 2007) or water and methanol mixtures (Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Fernández-León et al., 2013; Lelario, Bianco, Bufo, & Cataldi, 2012; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2011a; Tian, Rosselot, & Schwartz, 2005; Wang et al., 2011).

Determining intact glucosinolates has been achieved by means of liquid chromatography (LC) in reversed phase mode (C_{18} columns) with ultraviolet or diode array (LC-DAD) (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Jones et al., 2010; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2011b; Pérez-Balibrea et al., 2011a; Sasaki et al., 2012; Wang et al., 2011) and electrospray ionization-mass spectroscopy (LC–ESI-MS) (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Domínguez-Perles, Martínez-Ballesta, Riquelme, Carvajal, & García-Viguera, 2011; Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Domínguez-Perles, Moreno & García-Viguera, 2012; Fernández-León, Fernández-León, Lozano, Ayuso, & González-Gómez, 2012; Fernández-León, Fernández-León, Lozano, Ayuso, & González-Gómez, 2013; Fernández-León et al., 2013; Lelario, Bianco, Bufo, & Cataldi, 2012; López-Berenguer et al., 2008; Pérez-Balibrea, Moreno, & García-Viguera, 2010; Pérez-Balibrea et al., 2011a; Pérez-Balibrea et al., 2011b; Song & Thornalley, 2007; Tian et al., 2005; Wang et al., 2011).

Our aim, therefore, was to develop a new, robust LC-DAD–ESI-MS method, which was sensitive and as rapid as possible, to separate and determine twelve intact glucosinolates from broccoli leaves; to our knowledge this is the largest number of intact glucosinolates studied in broccoli leaves to date. Moreover, we wish to propose and optimize, by means of a statistical analysis (Box–Behnken design and analysis of variance), a specific, efficient, rapid, cheap and environmentally-friendly extraction procedure based on the deactivation of myrosinase by heating. For these reasons, two different extraction procedures involving the use of microwave (MW) or a conventional oven were tested in order to select the best choice for extracting intact glucosinolates from broccoli leaves. Moreover, this is the first study where an extraction procedure for intact glucosinolates has been developed and optimized in broccoli leaves. In addition, the use of an MW and statistical analysis to optimize the extraction procedure of intact glucosinolates in this matrix has not been previously described. Accordingly, the proposed method was validated and applied in an analysis of glucosinolates in broccoli leaf samples from six cultivars (*Ramoso calabrese, Parthenon, Marathon, Nubia, Naxos* and *Viola*) to determine differences in glucosinolate content and to corroborate the argument that broccoli leaves could be a potential source of glucosinolates.

2. Materials and methods

2.1. Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (GST) and sinigrin (SIN), which was used as the external standard (ES), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). SIN was used as an external standard to quantify the glucosinolates from broccoli where individual standards were not available (glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4-OH; neoglucobrassicin, NEO; 4-metoxyglucobrassicin, 4ME), as has normally been the case (Domínguez-Perles, Moreno, Carvajal, & García-Viguera, 2011; Pérez-Balibrea et al., 2011a). Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), composed of GNA, GBN, ALY, 4-OH, NEO, GST, GBC, SIN and another four glucosinolates (napoleiferin, brassicanapin, progoitrin and epiprogroitrin), largely absent in broccoli, was used to optimize separation in preliminary tests. This certified material was also employed to identify and confirm the presence of ALY, 4-OH, and NEO due to the absence of individual standards for these glucosinolates. LC grade methanol and acetonitrile were both supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst formic acid was purchased from Sigma Aldrich Chemie Gbmh. Syringe filters (17 mm, Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA), quantitative filter paper (Albet 140) was supplied by Albet-Hahnemuehle S.L. (Barcelona, Spain), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA,USA). An Eppendorf Centrifuge 5810R (Eppendorf-Netheler–Hinz GmbH, Hamburg, Germany), a vacuum filter system (Supelco, St. Louis, MO, USA), and an R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), were used for all the extractions. A drying oven from Selecta (Barcelona, Spain) was also employed, as well as a Vortex mechanical mixer from Heidolph (Schwabach, Germany). A microwave model MG-3924W from LG electronics (Madrid, Spain) was used for sample treatment. The samples were ground with a Moulinette chopper device from Moulinex (Paris, France).

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each glucosinolate in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Broccoli leaf samples (50 mg) were spiked with different amounts of the available glucosinolate standards for calibration at different concentrations (between LOQ and 400 μg/g (ESI-MS) or 4000 μg/g (DAD)). These matrix-matched standards were extracted by means of the same procedure as described for samples (Section 2.4). Each quality control (QC) sample was prepared with 50 mg of broccoli leaf samples spiked after sample treatment, with the same amounts of glucosinolates as those used in the calibration studies. The concentrations of different QC samples were as follows: low QC level – 0.1 μg/g for GNA, GTL, GER and GST or 0.3 μg/g for GIB, SIN, GRA and GBC (ESI-MS) and 80 μg/g (DAD); medium QC level – 20 μg/g (ESI-MS) and 500 μg/g (DAD); and high QC level – 400 μg/g (ESI-MS) and 4000 μg/g (DAD). Certified rapeseed material was also spiked and extracted using the same approach. All standard solutions were stored in glass containers and kept in the dark at +4 °C, where they were determined to be stable for more than two weeks (data not shown).

2.3. Plant material

Fresh broccoli leaf samples (*Ramoso calabrese*) were harvested after six months' growth from seed. They were dried at 60 °C in

Table 1
Chemical structure, quantification and confirmation ions selected for each of the isolated glucosinolates to perform the ESI-MS detection in negative EIC mode.

Compound name (abbreviation)	Molecular weight	Side chain (R)	Quantification ions	Confirmation ions	Confirmation transition
Aliphatic GLS					
Alkyl GLS					
Glucoiberin (GIB)	423		422	423, 424	422>97
Glucoraphanin (GRA)	437		436	437, 438	436>97
Glucoalyssin (ALY)	451		450	451, 452	450>97
Glucoerucin (GER)	421		420	421, 422	420>97
Alkenyl GLS					
Sinigrin (SIN)	359		358	359, 360	358>97
Gluconapin (GNA)	373		372	373, 374	372>97
Aromatic GLS					
Glucotropaeolin (GTL)	409		408	409, 410	408>97
Gluconasturtiin (GST)	423		422	423, 424	422>97
Indole GLS					
4-hydroxyglucobrassicin (4-OH)	464		463	464, 465	463>97
Glucobrassicin (GBC)	448		447	448, 449	447>97
4-metoxyglucobrassicin (4-ME)	478		477	478, 479	477>97
Neoglucobrassicin (NEO)	478		477	478, 479	477>97

an oven and ground to a powder before being stored at 4 °C. Freeze-dried broccoli leaf samples ($n = 25$) from five commercial cultivars (*Parthenon, Marathon, Nubia, Naxos* and *Viola*) were kindly donated by CEBAS-CSIC (Murcia, Spain). These samples were stored at 4 °C until analysis.

2.4. Sample preparation

Broccoli leaf extraction conditions were determined following optimization studies. Briefly, 50 mg of freeze-dried or dried ground broccoli leaf powder was put into a beaker and water (23 mL) was

added. The resulting mixture was heated for 3.5 min using a 70 W microwave (MW). The mixture was cooled in an ice-water bath for 30 s and centrifuged at 11 000 rpm for 8 min at 10 °C. The supernatant was collected, filtered under vacuum (Albet 140), and evaporated to dryness at 55 °C in a rotary evaporator; the dry residue was reconstituted with 1 mL of ultrapure water, filtered through a nylon 0.45 μm filter, and injected into the LC-DAD–ESI-MS system. Statgraphics Centurion XVI 16.1.03 (®Statpoint Technologies Inc., Warrenton, Virginia, USA) was used to perform the Box–Behnken design (BBD) statistical analyses.

2.5. LC-DAD–ESI-MS system

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-DAD/MSD Trap XCT instrument, which was used in conjunction with electrospray ionization (ESI) in negative ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment with a diode array detector. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data were analyzed using Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. The analytical column was a Gemini® 3 μm C_{18} 110 Å (150 × 4.60 mm) protected by a Gemini® C_{18} security guard cartridge (4 × 3.0 mm), both from Phenomenex (Torrance, CA, USA). Subsequent to the optimization study, the mobile phase selected was a mixture of (A) formic acid in water (0.5%, v/v) and (B) formic acid in acetonitrile (0.5%, v/v) applied at a flow rate of 1 mL/min in a gradient mode as follows: (i) 0 min (A–B, 100:0, v/v); (ii) 0–13 min (A–B, 100:0, v/v); (iii) 13–18 min (A–B, 40:60, v/v); (iv) 18–26 min (A–B, 40:60, v/v); (v) 26–30 min (A–B, 100:0, v/v); (vi) 30–35 min (A–B, 100:0, v/v). A linear gradient was used between each of the segments, except for i and ii (0–13 min) and iii and iv (18–26 min), where the mobile phase was isocratic. The injection volume and column temperature were set at 20 μL and 30 °C, respectively. The detection wavelength selected after examining the UV spectra collected was 227 nm as most of the glucosinolate absorption maxima were at this wavelength. The ESI interface was operated in negative mode after studies had been performed with standard solutions of the more relevant MS parameters. Full-scan LC–MS spectra were obtained by scanning from m/z 70 to 1000. Compounds were identified by comparing the DAD, MS and MS/MS data (retention time, spectra, ions, and confirmation transition) with those obtained from pure standard solutions when commercially available, or with data obtained from analyzing the certified rapeseed material. Otherwise, one glucosinolate (4ME) was tentatively assigned by comparing the information obtained for DAD and MS with available bibliographic data (Fernández-León et al., 2012; Pérez-Balibrea et al., 2011a; Tian et al., 2005). Glucosinolates found in high concentrations (>400 μg/g) were quantified with DAD, while MS in extracted ion chromatogram (EIC) mode was selected to quantify the less abundant glucosinolates (<80 μg/g). The other glucosinolates could be determined in either mode. MS/MS was also used to confirm the presence of each glucosinolate. All the transitions corresponded to fragmentation of the abundant precursors (ion m/z $[M-H]^-$ to ion m/z 97 ($[SO_3H]^-$) (Fernández-León et al., 2012; Fernández-León et al., 2013). The MS ions and MS/MS transitions monitored for each compound are summarized in Table 1. Optimal MS and MS/MS conditions were set as follows: capillary voltage, 3500 V; drying gas (N_2) flow, 10 L/min; drying gas (N_2) temperature, 350 °C; Nebulizer pressure, 25 psi; trap drive, 35; skimmer, 40 V: octopole RF amplitude, 130 V; capillary exit, 105.0 V; fragmentation scanning, 30–200%; isolation width (m/z), 4.0; fragmentation width (m/z), 10.0; max. accumulation time, 200 ms; ion charge control (ICC), 200,000; delay, 5 ms. To protect the ion source from matrix constituents, the MSD Trap system automatically controlled the run at different times: at t_{0-5min} to waste, $t_{5-28min}$ to mass and $t_{28-30min}$ to waste. During MS integration a Gauss function was used as a smoothing algorithm.

2.6. Method validation

Validation was carried out following different international guidelines (Tompson, Ellison, & Wood, 2002) determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, precision and accuracy. The validation and extraction studies were performed using broccoli leaves from the *Ramoso calabrese* variety, as there was an insufficient number of leaves from other varieties to carry out the required experiments. Moreover, validation was not conducted for ALY, 4-OH, NEO and 4ME due to the lack of individual standards.

2.6.1. Selectivity

To determine the selectivity of the proposed method, a set of unspiked broccoli leaf samples (n = 6) were injected onto the chromatographic system, and the results were compared with those obtained for standard glucosinolate solutions, certified rapeseed material and existing scientific literature.

2.6.2. Limits of detection and quantification

The LOD and LOQ were determined experimentally as, respectively, three and ten times the standard deviation of the intercept for the calibration curve (standard addition) divided by the slope. LOD and LOQ values for GNA when using DAD were calculated on the basis of standard calibration curves, as this compound coeluted with some matrix interference peaks.

2.6.3. Linearity studies

Standard calibration curves were used to quantify the glucosinolates in the samples as treatment did not affect DAD output. Working solutions used to construct the calibration curve were prepared using standard solutions containing the eight commercial glucosinolates over a concentration range of LOQ to 4000 μg/g (calibration levels of LOQ 80, 280, 500, 1000, 2000, 3000, 4000 μg/g). A 20 μL aliquot of these was injected onto the chromatographic system. The standard addition method, with matrix-matched standards, was used to quantify glucosinolates in broccoli leaves with MS. Accordingly, broccoli leaves were spiked with varying amounts of glucosinolates across the analytical ranges from LOQ to 400 μg/g (calibration levels of LOQ, 0.80, 2, 10, 20, 100, 200, 400 μg/g).The solutions obtained were considered standards to acquire the calibration curves (n = 6), which were constructed by plotting the signal on the y-axis (analyte peak areas for MS or DAD) against the analyte concentration on the x-axis.

2.6.4. Precision and accuracy studies

Intra-day precision and accuracy experiments were performed concurrently by repeated sample analysis using broccoli leaf samples spiked with three concentrations of glucosinolates (low, medium and high QC levels) on the same day of (n = 6). Inter-day precision and accuracy were assessed by an analysis of broccoli leaf samples spiked with three concentrations of the compounds studied (low, medium and high QC levels) over three consecutive days (n = 6). In each run a calibration curve was established and replicates (n = 6) of each spiked concentration level were analyzed. Precision was expressed as the percentage of relative standard deviation (%RSD) at the three concentrations for each analyte. Accuracy was calculated by means of relative error (%RE).

3. Results and discussion

3.1. Optimization of the glucosinolate extraction from broccoli leaves

3.1.1. Selection of the extraction parameters

Prior experiments were carried out to optimize a Box–Behnken design (BBD). Water, methanol and water: methanol mixes were tested as solvents. In these experiments, 50 mg of dried broccoli leaves were added to 3 mL of heated water, methanol or a methanol/water mix (70:30, v/v); the solution was kept in a water bath at 70 °C for 30 min and shaken every 5 min with a vortex mechanical shaker. The solution was cooled and evaporated to dryness in a rotary evaporator at 40 °C, and residue was re-dissolved in 1 mL of water. An increase in the peak areas was found only when water was used as the extractant. Thus, water was the solvent chosen to continue the experiments. The amount of water used to extract the glucosinolates varied between 5 and 30 mL to perform the BBD. This volume range was selected as in preliminary experiments it was found less was unsuitable for large amounts of sample (500 mg) whilst greater volumes did not significantly improve extraction. The number of broccoli leaves necessary was also determined. It was found that amounts greater than 500 mg required larger volumes of water, which increased evaporation time, but also caused bubbling, preventing efficient evaporation. Some extracts were obtained without heating, but these provided inconsistent results in relation to the glucosinolate content (%RSD >15%) when analyzing the same sample; this was probably due to the incomplete deactivation of the myrosinase enzyme. Our conclusion was that a heating process was necessary to deactivate the enzyme, and it was decided that two different heating devices, microwave and oven, should be tested. The heating time was also examined, and it was found that 3.5 min (MW) or 30 min (oven) were the longest periods that could be employed without solvent loss due to evaporation and subsequent incomplete enzyme deactivation. Finally, the temperature of the oven was also determined as a factor likely to affect extraction (60, 70 and 80 °C).

3.1.2. Box–Behnken design (BBD)

A BBD design is a type of response surface method, which provides detailed information about solution space, allowing better understanding of the forces affecting the output of the model. In this design, treatment combinations are at the midpoint of the edges and the center of the process space. BBD design is rotatable (or nearly rotatable) and is based on three-level incomplete factorial design (Ferreira et al., 2007). In this study, a BBD design was applied in order to select optimal conditions for the two different extraction procedures involving the deactivation of myrosinase by sample heating (microwave or oven). Subsequently, the data obtained were analyzed and compared to select the best extraction conditions. BBD was performed with three factors for MW treatment (water volume, time and amount of sample) and four in oven treatment (temperature, water volume, time and amount of sample). The lower MW power value (70 W) was used because of significant problems (rapid solvent loss by evaporation or even projections) detected in preliminary experiments with higher power (e.g. evaporation). Water volume and sample quantity were selected as optimizing factors in both procedures because each could have an influence on the number of glucosinolates extracted. Meanwhile, the effects of heating time and temperature (oven) on extraction were studied to determine if it was possible to reduce overall extraction time, as much as possible, without losing extraction efficiency. The design involved a total of 15 and 27 experiments for MW- and oven-based treatments, respectively, and these were completed in random order. Moreover, all the factors selected were interrelated. Data analysis was performed using regression analysis and the target response was taken as a function of the variables (xi) using polynomials. The model was evaluated and optimal values for each factor obtained. Optimal water volume and sample amount were practically the same for both procedures, but the heating time was much lower when MW was employed. Greater quantities of the 12 target glucosinolates were obtained with MW (optimal response value). Consequently, after performing BBD, the MW-based treatment was selected for the remaining experiments, as it provided better results in terms of quantity of glucosinolates obtained and overall extraction time (see Supplementary data, Table 1S).

3.1.3. Analysis of variance (ANOVA)

At the same time as BBD was performed, the software balanced global information for the model by means of an analysis of variance (ANOVA) relating to response surfaces, main effects and interaction among variables, so as to obtain the best extraction conditions for MW- and oven-based treatments. The effects were considered statistically significant when $p \leqslant 0.05$. It was found that the sample amount was the only significant parameter for MW treatment, whereas the sample amount, water volume and interaction with the sample amount were significant for oven treatment. Moreover, the adjustment of the models obtained (oven and MW) to the target variable (extraction of intact glucosinolates, μg/g) was good in terms of adjustment coefficient (R^2 >0.90) and capacity of prediction (over 95%); (see Supplementary data, Tables 2S and 3S). Finally, the results showed that the responses did not display lack of fit; the threshold was $p > 0.05$.

3.1.4. Extraction efficiency

To assess the efficiency of the proposed sample treatment based on MW heating, a comparison was made between the results obtained for broccoli leaf samples spiked at three glucosinolate concentrations (low, medium and high QC levels), before (A samples) or after (B samples) sample treatment. The resulting recovery values (Table 2) ranged from 87% to 106% in all cases, indicating that the sample treatment procedure selected was adequate and efficient. The results were good not only in terms of extraction efficiency, but in total sample treatment time and simplicity. The overall time taken for the proposed procedure was 20 min, which is much shorter, and the method simpler, than previous published research, where intact glucosinolates were determined in broccoli leaves (Hennig et al., 2012; López-Berenguer et al., 2008; Sasaki et al., 2012).

3.2. LC optimization

3.2.1. Column

Preliminary experiments to select the analytical column were performed using the certified rapeseed material. Initially, hydrophilic interaction liquid chromatography (HILIC) was tested because it had been previously employed to separate glucosinolates in broccoli seeds (Wade, Garrard, & Fahey, 2007). Two different HILIC stationary phases (Kinetex® 2.6 μm HILIC 100 Å (50 × 2.1 mm), and Luna® 3 μm HILIC 200 Å (50 × 2.0 mm), both from Phenomenex) were assayed. However, separation of the glucosinolates was unsatisfactory, and it was not possible to elute some of these from the analytical columns in a reasonable period of time. Several C_{18} stationary phases of different polarities and lengths were tested: Synergi® 4 μ Hydro-RP 80 Å (150 × 4.6 mm), Synergi® 4 μ Fusion-RP 80 Å (150 × 4.6 mm), Luna® C_{18} 5 μm 100 Å (250 × 4.6 mm), Gemini® 3 μ C_{18} 110 Å (150 × 4.6 mm), all of which were provided by Phenomenex. The best separation of the rapeseed glucosinolates was obtained with the Gemini® and Luna® analytical columns, although, as might be expected, a faster mobile phase flow rate was required with the longer column (Luna® C_{18})

Table 2
Recovery data obtained for blank leaf samples treated accordingly to the procedures described in Sections 2.2, 2.4, 3.1.4 and 3.4 ($n = 6$).

Quality control (QC) sample	Evaluation of the sample treatment						Evaluation of the matrix effect					
	DAD			MS			DAD			MS		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
Compounds	Mean recovery (%) ± RSD (%)											
GIB	96 ± 4	93 ± 3	91 ± 4	99 ± 5	95 ± 5	99 ± 5	100 ± 5	97 ± 4	99 ± 5	68 ± 7	65 ± 6	76 ± 6
SIN	105 ± 4	95 ± 3	98 ± 4	96 ± 5	99 ± 6	102 ± 5	98 ± 4	94 ± 5	97 ± 4	67 ± 6	75 ± 7	70 ± 6
GRA	102 ± 3	96 ± 3	93 ± 3	104 ± 6	103 ± 5	106 ± 6	95 ± 4	93 ± 5	94 ± 5	65 ± 7	60 ± 5	63 ± 5
GNA	100 ± 4	97 ± 5	93 ± 4	104 ± 5	98 ± 5	99 ± 6	102 ± 4	98 ± 5	100 ± 5	65 ± 5	60 ± 5	61 ± 5
GTL	101 ± 5	98 ± 5	104 ± 4	87 ± 7	92 ± 5	96 ± 5	97 ± 4	92 ± 4	95 ± 5	78 ± 6	75 ± 6	80 ± 6
GER	105 ± 5	100 ± 4	97 ± 4	106 ± 6	104 ± 5	97 ± 6	101 ± 6	96 ± 4	103 ± 4	73 ± 6	69 ± 6	77 ± 6
GBC	97 ± 5	101 ± 3	98 ± 4	98 ± 6	101 ± 6	102 ± 6	99 ± 5	95 ± 5	98 ± 5	68 ± 6	61 ± 6	62 ± 7
GST	90 ± 4	94 ± 3	88 ± 4	95 ± 6	89 ± 5	92 ± 6	96 ± 5	93 ± 6	95 ± 5	74 ± 6	79 ± 6	70 ± 6

DAD: low QC–80 μg/g; medium QC–500 μg/g; high QC–4000 μg/g. MS: low QC–0.1 μg/g (GNA, GTL, GER, GST), 0.3 μg/g (GIB, SIN, GRA, GBC); medium QC–20 μg/g; high QC–400 μg/g.

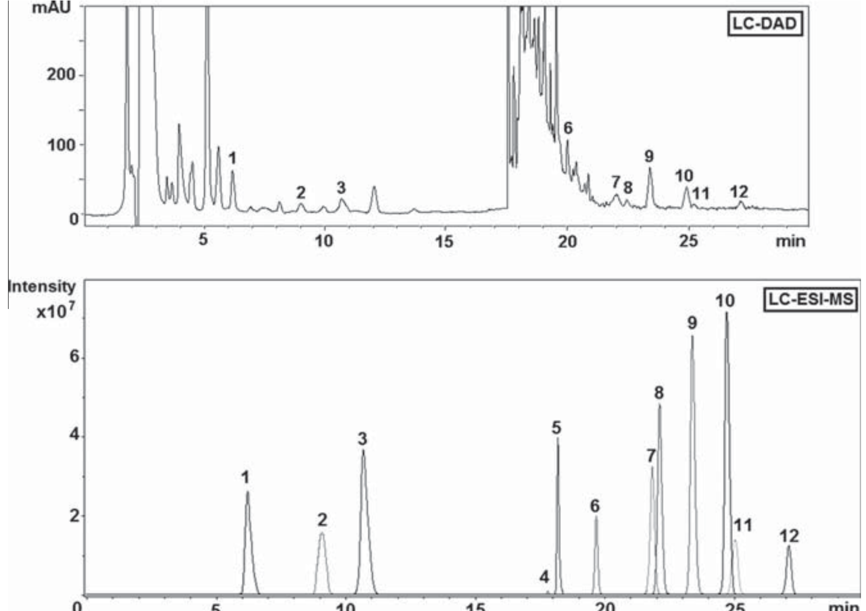

Fig. 2. LC-DAD (227 nm) and LC–ESI-MS (EIC) chromatograms obtained from a broccoli leaf sample spiked at 100 μg/g with the 8 individual glucosinolate standards. The chromatographic and MS conditions are described in detail in Section 2.5 and Table 1. (glucoiberin-1; sinigrin-2; glucoraphanin-3; glucoalyssin-4; gluconapin-5; 4-hydroxyglucobrassicin-6; glucotropaeolin-7; glucoerucin-8; glucobrassicin-9; gluconasturtiin-10; 4-metoxyglucobrassicin-11, neoglucobrassicin-12).

to obtain comparable analysis times. Consequently, as one of the goals of this study was to develop the quickest possible LC method using as few reagents as possible, the Gemini® column was chosen.

3.2.2. Mobile phase

Once the analytical column had been selected, a study was made of the mobile phase composition. Some experiments were conducted using different mobile phases in a gradient elution mode comprising mixtures of 0.02 mol/L ammonium formate in water or 0.1% formic acid in water, and methanol or acetonitrile as organic solvents. The best results, in terms of resolution and peak symmetries, were obtained by using formic acid in water and acetonitrile as mobile phase components. However, it was not possible to completely separate SIN and GRA at 20 °C so the temperature was increased step-wise to 50 °C; all the glucosinolates separated at 30 °C. Moreover, the peaks were narrow and the signal-to-noise ratio (S/N) was enhanced compared with lower temperatures, but the decrease in analysis time did not compensate for the loss of peak symmetry achieved at higher temperatures. Once separation had been optimized using the certified rapeseed material, the study continued with broccoli leaves using a mixture of individual standards. The gradient elution program and mobile phase flow rate were also examined, and the shortest analysis time with no loss of separation capacity was achieved with 1 mL/min; the gradient elution program is summarized in Section 2.5. Other parameters such as pH (2.4 to 8.0) and ionic strength (0.1%, 0.5% and 1% of formic acid) of the aqueous component of the mobile phase were also evaluated. The best results in terms of analysis time and S/N ratios were obtained with formic acid (0.5%) in water. It was decided that the lower pH value (2.4) should be selected as working pH since chromatographic separation was not improved at higher pH values. Furthermore, the

addition of 0.5% formic acid to acetonitrile was needed in order to decrease the variation in the DAD signal brought about by working in the gradient elution mode.

3.2.3. Analysis of the chromatographic performance

With the chromatography conditions described above, the twelve intact glucosinolates were separated in under 28 min (Fig. 2), and the overall run time was 35 min. Improved separation of the twelve compounds was achieved when using MS, as two of the glucosinolates (ALY and GNA) could not be detected in the DAD chromatograms due to matrix interference. It is also important to point out that, due to the relatively high concentration of glucosinolates for MS, some of the compounds were not completely separated at the baseline, but this did not affect their quantification because different quantification ions were selected.

Intact glucosinolates have been widely studied in broccoli, but very little research has been carried out on broccoli leaves. Nevertheless, if the chromatographic performance of the proposed method is compared with the few publications in which several glucosinolates were analyzed in broccoli leaves, it can be concluded that the overall run time is comparable (shorter – Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; López-Berenguer et al., 2008; longer – Sasaki et al., 2012). Yet it should be taken into account than in those studies the number of glucosinolates studied, when specified, was lower (<7) than in the present study. Consequently, the advantages of the proposed chromatographic conditions have been demonstrated, as it was possible to separate a large number of intact glucosinolates (twelve) from broccoli leaves in a similar period of time to that required in previous research for fewer compounds.

3.3. Mass spectrometry (MS)

Intact glucosinolates have previously been analyzed in the negative ESI mode (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Jones et al., 2010; Lelario, Bianco, Bufo, & Cataldi, 2012; López-Berenguer et al., 2008) in broccoli matrices. However, both ESI ionization modes were tested by direct introduction using the infusion mode (5 μL/min) standard and matrix-matched solutions (1 mg/L) of glucosinolates. The best results, in terms of peak area and peak height, were obtained for ESI in the negative mode. In ESI-MS, the glucosinolates showed an intense $[M-H]^-$ on their full-scan spectra. The EIC mode was used to obtain maximum sensitivity for quantitative analysis, and the mass-to-charge (*m*/*z*) values, which were chosen for EIC analysis, are summarized in Table 1. Moreover, the ions selected to quantify each glucosinolate were chosen as precursors in order to obtain product ions in multiple reaction monitoring (MRM) mode and confirm their presence in the broccoli leaf samples. A flow injection method was employed to ensure maximum sensitivity for glucosinolate detection. Accordingly, 20 μL of mixed standard and matrix-matched solutions of the eight glucosinolates at 1.0 mL/min was used to optimize the most relevant MS parameters (the optimal conditions defined are given in Section 2.5).

3.4. Study of the matrix effect

To check how the matrix influenced the detection of glucosinolates, the peak areas of the glucosinolates in standard solutions were compared with those obtained in B samples (broccoli leaf samples spiked after sample treatment). Because broccoli leaves contain endogenous glucosinolates, these must be taken into account when comparing results. Thus, to calculate the signal for the broccoli leaf samples, glucosinolate areas corresponding to endogenous levels have to be determined. These areas were subtracted from the total area obtained for the spiked samples and

Table 3
Method validation parameters for glucosinolate determination in broccoli leaves samples ($n = 6$).

Validation parameter	GIB		SIN		GRA		GNA*		GTL		GER		GBC		GST	
	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS	DAD	ESI-MS
LOD (μg/g)	10	0.04	11	0.05	13	0.07	13	0.02	14	0.01	22	0.02	23	0.03	14	0.01
LOQ (μg/g)	34	0.12	35	0.16	44	0.23	41	0.06	45	0.04	69	0.06	72	0.11	45	0.04
Linear range (μg/g)	34–4000	0.12–400	35–4000	0.16–400	44–4000	0.23–400	41–4000	0.06–400	45–4000	0.04–400	69–4000	0.06–400	72–4000	0.11–400	45–4000[A]	0.04–400
Coefficient of determination (R^2)	0.999	0.999	0.998	0.999	0.999	0.993	0.997	0.999	0.997	0.999	0.999	0.999	0.998	0.998	0.998	0.999

* Calculated with standard solutions for DAD.

the results compared with the standard solutions. The response of all compounds at the three concentrations assayed was less than 80% in all cases, as shown in Table 2; no matrix effect was observed for DAD. The responses were close to 100%. Thus, it was concluded that the matrix (broccoli leaves) affected ESI ionization of the analytes, causing, in this case, ion suppression.

3.5. Validation of the LC-DAD–ESI-MS method

3.5.1. Selectivity

To determine the selectivity of the proposed method, a set of non-spiked broccoli leaf samples were injected onto the LC-DAD–ESI-MS system. As it is impossible to obtain broccoli leaves free from glucosinolates, absorption and mass spectra of analyte standard solutions, certified rapeseed material and values in the scientific literature were compared with the matrix data. No matrix interference was detected at the elution time of any glucosinolate using MS, while, as previously stated, two glucosinolates (ALY and GNA) could not be separated because of matrix interference with DAD. In addition, both spectra (DAD and MS) were similar for the glucosinolates studied, and only minor differences were encountered in relation to ion intensity. Similar results were obtained when analyzing broccoli leaf samples from the different cultivars.

3.5.2. Limits of detection and quantification

The LODs and LOQs were determined experimentally for each glucosinolate in broccoli leaves in the manner indicated in Section 2.6 (Table 3). The LOD and LOQ values for DAD were worse than those obtained for ESI-MS, but the use of DAD could be considered a cheap alternative to determine major glucosinolates and in experiments where a high degree of sensitivity is unnecessary. As previously stated, only a few studies have been published describing intact glucosinolates in broccoli leaves, and LOD and LOQ values were not calculated, so no comparison can be made. However, LOD and LOQ values obtained with ESI-MS were generally better than those obtained in previous research in other broccoli matrices, while the limits obtained with DAD were similar.

3.5.3. Linearity studies

As mentioned in Section 2.6, different calibration curves were used to quantify the compounds of interest according to the detection mode used. For DAD analyses, standard calibration curves could be employed as no matrix effect was observed. Meanwhile, matrix interference affected analyte ionization, causing a suppression of the MS signal (>20%). Consequently, glucosinolates should only be quantified in broccoli leaf samples using matrix-matched calibration curves. All the calibration graphs were straight lines, and the coefficient of determination (R^2) was greater than 0.99 in all cases (Table 3). Moreover, the lack of bias was confirmed using a Student's *t* test and the distribution of residuals.

3.5.4. Precision and accuracy studies

Intra- and inter-day precision (% RSD values) were all less than or equal to 5% or 8% with DAD and ESI-MS, respectively. Accuracy (% RE values) for all compounds ranged from 2% to 8% for the intra-day readings, and from 3% to 8% for the inter-day values. The accuracy and precision measures are better for DAD than for ESI-MS (see Supplementary data, Table 4S). These results indicate that the method is precise and accurate and, although an internal standard is recommended in most validation guidelines, it was not necessary in this study with both detection modes because of this degree of precision and accuracy.

3.6. Application of the method

The validated method was applied to determine intact glucosinolate content in 30 broccoli leaf samples from 6 different cultivars (*Ramoso calabrese, Parthenon, Marathon, Nubia, Naxos* and *Viola*). The glucosinolate content of each broccoli variety is summarized in Table 4. As can be seen, nine glucosinolates were found in all the varieties, GER being determined in three of them (*Ramoso calabrese, Parthenon* and *Nubia*), albeit at very low concentrations (<9 μg/g), while GTL and SIN were not detected in any of the broccoli varieties investigated. This absence of SIN and GTL is in good agreement with most of the existing bibliography (Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; López-Berenguer et al., 2008; Pérez-Balibrea et al., 2011a). The highest total content of glucosinolates was found in broccoli leaf samples from the *Viola* cultivar, with the *Ramoso calabrese* cultivar being the poorest in glucosinolate content. GRA was the glucosinolate found in larger amounts in four of the broccoli varieties assayed (*Ramoso calabrese, Parthenon, Nubia* and *Naxos*), while GBC was the glucosinolate detected at the highest concentration in *Marathon* and *Viola* broccoli leaves. GER, ALY, GNA and 4-OH were found at only low concentrations. Furthermore, GRA and GBC occurred in higher concentrations in most of the broccoli varieties. This is particularly important, if we consider these compounds have been extensively studied in *Brassica* vegetables because they are precursors of sulforaphane and indole-3-carbinol, which have putative health benefits, specifically reduction of cancer risk (Elbarbry & Elrody, 2011). The differences observed in glucosinolate content depending on the cultivar are in agreement with

Table 4
Concentration (μg/g, dry weight) of glucosinolates in broccoli leaves (five samples per cultivar).

Cultivars Compound	Ramoso calabrese Min.–Max. values	Parthenon Min.–Max. values	Marathon Min.–Max. values	Nubia Min.–Max. values	Naxos Min.–Max. values	Viola Min.–Max. values
GIB[a,b]	34–36	65–76	22–29	38–44	4–6	860–1006
SIN	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
GRA[a,b]	150–163	690–793	207–230	475–491	620–740	112–127
ALY[*,b]	0.5–0.9	11–13	5–6	7–8	7–9	4–6
GNA[b]	<LOD	0.3–0.6	0.1–0.5	0.2–0.3	0.3–0.4	0.1–0.4
4-OH[*,b]	28–33	0.7–0.8	<LOD–0.6	1.3–1.4	0.6–0.7	0.2–0.4
GTL	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
GERB	2–4	6–9	<LOD	3–7	<LOD	<LOD
GBC[a,b]	25–34	113–127	400–430	227–247	380–450	915–1050
GST[b]	1–4	30–32	10–12	37–42	36–45	25–28
4ME[*,b]	55–67	40–46	22–24	32–38	68–85	45–52
NEO[*,a,b]	125–130	146–164	104–143	155–158	125–160	216–240

[*] Quantified with SIN calibration curves.
[a] Quantified with DAD.
[b] Quantified with ESI-MS.

the scientific data available for broccoli leaves (Pérez-Balibrea, Moreno, & García-Viguera, 2011a). Finally, we have demonstrated that broccoli leaves could be a potential source of glucosinolates. In fact, the content for some glucosinolates, such as GRA or GBC, was appreciable.

4. Conclusion

A new LC-DAD–ESI-MS method has been developed to separate and quantify 12 intact glucosinolates in broccoli leaves. The extraction of these compounds from broccoli leaves was optimized by statistically analyzing the data (Box–Behnken design and analysis of variance). The proposed extraction method based on MW heating has proven to be efficient, rapid, cheap and environmentally-friendly, due to the solvent used (water), the few steps taken and amounts of reagents required. Moreover, the LC-DAD–ESI-MS method was fully validated, and the data demonstrated that the proposed method is consistent and reliable, and that it is necessary to employ matrix-matched standards to perform correct ESI-MS quantification of the glucosinolates in broccoli leaves as ion suppression was observed. Finally, broccoli leaves from six different cultivars were analyzed, and differences in glucosinolate content depended on the cultivar. Most of the glucosinolates studied were identified in these samples, and the greatest total content of glucosinolates was found in broccoli leaf samples from the *Viola* cultivar.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.11.125.

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V.5. PAPER V. Optimized formation, extraction, and determination of sulforaphane in broccoli by liquid chromatography with diode array detection

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Optimized Formation, Extraction, and Determination of Sulforaphane in Broccoli by Liquid Chromatography with Diode Array Detection

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Abstract A new method has been developed to determine sulforaphane in broccoli florets, stems, and leaves using liquid chromatography coupled to diode array detection. The hydrolysis reaction, required to convert glucoraphanin to its breakdown product, sulforaphane, and the extraction procedure, based on solvent extraction with methyl *t*-butyl ether followed by solid phase extraction with silica cartridges, were optimized for the three broccoli matrices; the resulting recovery values ranged from 92 to 102 % in all cases. A fast separation was performed on a C_{18} analytical column with a mobile phase composed of ammonium formate (20 mM) in water and acetonitrile (55:45, *v*/*v*) in isocratic elution mode. This method was fully validated and was found to be selective, linear from 2.5 to 800 mg/kg, and precise (percent relative standard deviation values below 5 %). Moreover, the limits of detection and quantification were 0.8 and 2.5 mg/kg, respectively. Finally, the proposed method has been applied to the analysis of sulforaphane in broccoli samples of two different cultivars (Parthenon and Marathon).

Keywords Sulforaphane · Broccoli · LC-DAD · Silica cartridges · Methyl *t*-butyl ether

Abbreviations

DAD	Diode array detection
DCM	Dichloromethane
DW	Dry weight
ESI	Electrospray ionization
GRA	Glucoraphanin
HILIC	Hydrophilic interaction liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MTBE	Methyl *t*-butyl ether
QC	Quality control
RT	Retention time
SF	Sulforaphane
SPE	Solid phase extraction
RSD	Relative standard deviation

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Introduction

The potentially protective role of cruciferous vegetables including broccoli (*Brassica oleracea* L. var. *italica*) and the active components present in these vegetables, such as glucosinolates and their breakdown compounds (isothiocyanates), has been extensively studied (Chuanphongpanich et al. 2006). A large amount of research on functional foods like anticarcinogens has focused on broccoli and on a single bioactive component within broccoli, namely, sulforaphane (SF). Sulforaphane (4-methylsulfinylbutyl isothiocyanate; see Fig. 1) is formed by the hydrolysis of a glucosinolate (glucoraphanin, GRA) due to the action of the enzyme myrosinase, and its formation depends on several factors such as pH, hydrolysis time, volume, and temperature (Campas-Baypoli et al. 2010). Moreover, it has also been suggested in several in vivo and in vitro studies that SF has the potential to reduce the risk of various types of cancers, diabetes, atherosclerosis, respiratory diseases, neurodegenerative disorders, ocular disorders, and cardiovascular diseases (Elbarbry and Elrody 2011). Consequently, incorporating SF directly or adding to pharmaceutical products (nutraceutical) once it has been isolated and extracted from this vegetable is a safe and effective way to guard against many of today's most common diseases (Moreno et al. 2006).

Fig. 1 Molecular structure of sulforaphane ($C_6H_{11}NOS_2$; molecular weight, 177.29)

Sulforaphane (SF)

As can be expected, many methodologies have been reported concerning the extraction and determination of SF in broccoli (Campas-Baypoli et al. 2010; Shen et al. 2010; Li et al. 2012; Guo et al. 2011, 2013a, b; Han and Row 2011; Xu et al. 2012; Liang et al. 2005, 2006, 2008; Campas-Baypoli et al. 2009; Totušek et al. 2011; Azizi et al. 2011; Bertelli et al. 1998; Jones et al. 2010; Martínez-Hernández et al. 2013; Ghawi et al. 2013; Liu et al. 2009; Abdulah et al. 2009; Haina et al. 2010; Matusheski et al. 2001; Nakagawa et al. 2006; Gu et al. 2012a; Sivakumar et al. 2007; Moon et al. 2010), but to the best of our knowledge, no specific study has been published in which hydrolysis and extraction conditions were optimized and compared for different broccoli parts, as is described in the present study. This is an important issue, particularly when we consider that the number, variety, and amount of plant compounds in each broccoli part are different (including GRA and myrosinase). For this reason, the degree of conversion of GRA to SF could vary depending on the broccoli matrix analyzed and the optimal hydrolysis conditions. Most of the studies in which this compound was analyzed were based on the hydrolysis of its precursor (GRA) by using acidic water at different pH values (Campas-Baypoli et al. 2010; Shen et al. 2010; Li et al. 2012; Guo et al. 2011, 2013a, b; Xu et al. 2012; Liang et al. 2005, 2006, 2008; Campas-Baypoli et al. 2009; Totušek et al. 2011; Azizi et al. 2011; Bertelli et al. 1998; Jones et al. 2010; Martínez-Hernández et al. 2013; Ghawi et al. 2013; Liu et al. 2009; Abdulah et al. 2009; Haina et al. 2010; Matusheski et al. 2001; Nakagawa et al. 2006; Gu et al. 2012a; Sivakumar et al. 2007; Moon et al. 2010), but only in a few cases was hydrolysis optimization carried out (Campas-Baypoli et al. 2010; Shen et al. 2010; Li et al. 2012; Guo et al. 2013a; Han and Row 2011), and this was performed using one broccoli part (heads: Campas-Baypoli et al. 2010; seeds: Shen et al. 2010; Li et al. 2012; sprouts: Guo et al. 2013a) or not specified (Han and Row 2011). It should be noted that, to our knowledge, no study has been published in which the influence of these parameters was simultaneously researched in different broccoli parts. Moreover, it has never been studied in broccoli stems and leaves. A further extraction step with an organic solvent such as dichloromethane (DCM; Campas-Baypoli et al. 2010; Guo et al. 2011, 2013a, b; Han and Row 2011; Liang et al. 2006; Campas-Baypoli et al. 2009; Totušek et al. 2011; Azizi et al. 2011; Bertelli et al. 1998; Jones et al. 2010; Martínez-Hernández et al. 2013; Ghawi et al. 2013; Liu et al. 2009; Abdulah et al. 2009; Haina et al. 2010; Matusheski et al. 2001; Nakagawa et al. 2006; Sivakumar et al. 2007), ethyl acetate (Li et al. 2012; Liang et al. 2005; Gu et al. 2012a), or chloroform (Moon et al. 2010) was performed in all cases. As can be seen, chlorinated compounds (DCM or chloroform) have been commonly used to extract SF, but as the use of those reagents should be avoided, it was decided to test the suitability of other solvents such as heptane, 1-butanol, ethyl acetate, or methyl *t*-butyl ether (MTBE). In addition, we should add that in many of the aforementioned studies, solid phase extraction (SPE) with silica-based cartridges was performed to purify the broccoli extracts (Campas-Baypoli et al. 2009, 2010; Guo et al. 2013a; Liang et al. 2006; Azizi et al. 2011; Abdulah et al. 2009; Matusheski et al. 2001; Sivakumar et al. 2007), while high-speed countercurrent chromatography (Liang et al. 2008), preparative LC (Matusheski et al. 2001), and low-pressure column chromatography (Liang et al. 2005) have also been used to purify the broccoli extracts.

Several analytical methods such as reversed-phase LC (Campas-Baypoli et al. 2009, 2010; Shen et al. 2010; Li et al. 2012; Guo et al. 2013a; Han and Row 2011; Xu et al. 2012; Liang et al. 2005, 2006, 2008; Totušek et al. 2011; Azizi et al. 2011; Bertelli et al. 1998; Jones et al. 2010; Martínez-Hernández et al. 2013; Liu et al. 2009; Abdulah et al. 2009; Matusheski et al. 2001; Nakagawa et al. 2006; Gu et al. 2012a; Sivakumar et al. 2007), and gas chromatography (GC; Shen et al. 2010; Guo et al. 2011, 2013b; Azizi et al. 2011; Bertelli et al. 1998; Ghawi et al. 2013; Matusheski et al. 2001; Moon et al. 2010) have been used to determine SF in broccoli. In some of the aforementioned publications, GC (Shen et al. 2010; Azizi et al. 2011; Bertelli et al. 1998; Matusheski et al. 2001), mass spectrometry (MS; Liang et al. 2005, 2008; Matusheski et al. 2001; Nakagawa et al. 2006) and nuclear magnetic resonance (Liang et al. 2008) have only been used to confirm the presence of SF. However, it has been postulated that, in some cases, SF was thermally degraded in the injection ports of the GC equipment (Campas-Baypoli et al. 2010; Jin et al. 1999), and usually, the GC analysis times were longer than those of LC. For these reasons, it was decided that LC should be employed in this study. It should also be said that almost all the aforementioned LC studies employed C_{18}-based analytical columns and UV or diode array (DAD) as detectors, although a C_8 stationary phase (Azizi et al. 2011) and MS (Jones et al. 2010) or evaporative light scattering (Nakagawa et al. 2006) detectors have occasionally been used. This scant use of MS detectors could be due to the high amount of SF detected in broccoli that makes it unnecessary to develop extremely sensitive methods.

Thus, we sought to develop a new LC-DAD method to determine SF in broccoli (florets, stems, and leaves) as well as to propose and, for the first time, optimize an exclusive, efficient, and as environment-friendly as possible hydrolysis and extraction procedures for each of the three broccoli matrices assayed. Accordingly, another goal of the present study

was to perform a complete validation of the proposed method, which has not been usually done in most of the aforementioned researches, and apply it to the analysis of SF in broccoli samples from two different cultivars (Parthenon and Marathon).

Materials and Methods

Reagents and Materials

SF standard (det. purity, 98 %; molecular weight, 177.3), ammonium formate, and hydrochloric acid were obtained from Sigma Aldrich Chemie GbmH (Steinheim, Germany). LC grade ethanol, methanol, DCM, acetonitrile, ethyl acetate, heptanes, and 1-butanol were supplied by Lab-Scan Ltd. (Dublin, Ireland), while LC methyl *t*-butyl ether (MTBE) and sodium sulfate anhydrous were acquired from Panreac Química S.L.U. (Barcelona, Spain). Syringe filters (17 mm, nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA), Albet 140 quantitative filter paper was supplied by Albet-Hahnemuehle S.L. (Barcelona, Spain), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA, USA). A Vibromatic mechanical shaker, an ultrasonic bath, and a drying oven were purchased from J. P. Selecta S. A. (Barcelona, Spain). A R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), Strata® (Phenomenex, Torrance, CA, USA) SI-1 Silica 55-μm SPE cartridges (3 mL with 500 mg of sorbent), and a 10-port Visiprep vacuum manifold (Supelco, St. Louis, MO, USA) were used for all extractions. A Vortex mechanical mixer from Heidolph (Schwabach, Germany) and a pH meter supplied by Schott (Mainz, Germany) were also employed. All the broccoli samples were ground in a Moulinette chopper device from Moulinex (Paris, France).

Standard Solutions

Standard stock solutions were prepared by dissolving approximately 5 mg of SF standard (yellow oil liquid) in 10 mL of acetonitrile at a final concentration of approximately 500 mg/L. These solutions were further diluted with ethanol to prepare the intermediate and working solutions. Each quality control (QC) sample was prepared using different amounts of samples depending on the broccoli matrix (0.25 g dry weight (DW) of florets and stems and 0.50 mg DW of leaves), spiked before and after the proposed extraction treatment with three different amounts of SF within the linear range. The concentrations of the different QC samples were as follows: low QC level, 6 mg/kg; medium QC level, 160 mg/kg; and high QC level, 600 mg/kg. All the broccoli samples, which were used to prepare the QC samples or in the validation studies, contain endogenous levels of SF, which must be taken into account when comparing the results. Thus, to calculate the signals for the different broccoli samples used in the recovery, matrix effect, and validation experiments, the SF areas corresponding to endogenous SF levels must also be determined. Those areas were subtracted from the total area obtained for the spiked broccoli samples, and the resulting areas were compared with the standard solutions for the recovery and matrix effect studies. Meanwhile, the final area values were employed to construct the calibration curves or to study other validation parameters. Stock solutions were stored in glass containers and kept in the dark at −20 °C, while intermediate, working, and matrix-matched solutions were stored in glass containers and kept in the dark at + 4 °C. All solutions were stable for over 1 month.

Chromatography Systems

An Agilent Technologies (Palo Alto, CA, USA) 1200 series LC-DAD and 1100 series LC-MS, controlled by ChemStation software (Rev.A.10.02 (1757); Agilent Technologies), were used in this study. Both systems consisted of a vacuum degasser, a quaternary solvent pump, and an autosampler with a column oven. One of the 1100 LC systems was equipped with a DAD, while the other LC was coupled to a single quadrupole MS analyzer with an electrospray (ESI) interface. It must be specified that the LC-MS system was used only for confirmatory purposes. A Synergi™ Hydro-RP C_{18} 4 μm (150×4.6 mm) analytical column (Phenomenex) was used for the LC analyses and was protected with a C_{18} security guard cartridge (4×3.0-mm i.d.; Phenomenex). As a result of the findings of the optimization study, the mobile phase selected was a mixture of 20 mM ammonium formate in water and acetonitrile (55:45, *v*/*v*), which was applied at a flow rate of 0.6 mL/min in isocratic mode. The injection volume was set at 5 μL (a draw speed of 50 μL/min), the temperature at 25 °C, and the detection wavelength at 196 nm. The operating conditions of the ESI interface in positive mode employed in the confirmation studies were drying gas (N_2) temperature, 350 °C; drying gas flow (N_2), 10 L/min; nebulizer gas (N_2) pressure, 60 psi; capillary voltage, 4,000 V; fragmentor voltage, 100 V. Full-scan LC-MS spectra were obtained by scanning from m/z 70 to 300 (Fig. 2). Chromatograms were analyzed by means of the Data Analysis program in ChemStation; the analytes were quantified using peak areas.

Sample Procurement and Treatment

Plant Material

Fresh broccoli samples (leaves, florets, and stems) from two different cultivars (Parthenon and Marathon) were supplied by farmers from the region of Castilla y León (Spain) and were harvested after 6 months of seed growth. All broccoli samples

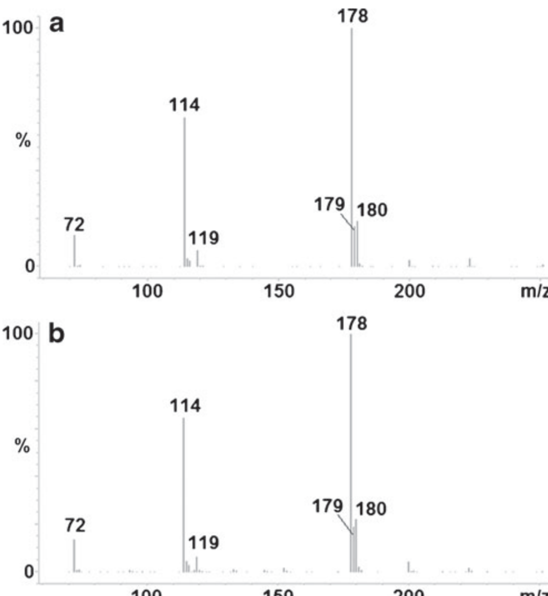

Fig. 2 MS spectra of the SF assigned peak in standard solution (**a**) and broccoli stem sample (**b**) (Marathon)

were dried at 40 °C in an oven and a significant weight loss was observed in all cases (>75 %). Subsequently, they were ground to a fine powder with a Moulinette chopper and stored at +4 °C until their analysis.

Conversion of GRA to SF

Briefly, 0.25 g (florets and stems) or 0.50 g (leaves) of dried sample was mixed with 5 mL (florets and stems) of acidic water (pH 6.0 using 0.1 M hydrochloric acid) or 7 mL (leaves) of acidic water (pH 5.0 using 0.1 M hydrochloric acid), which contained, in all cases, ascorbic acid at a concentration of 4 mg/g of dry weight of broccoli. The mixture was mechanically shaken in a Vortex device for 1 min and afterwards was incubated in an oven at 45 °C for 2 h (florets), 55 °C for 1 h (stems), or 35 °C for 4 h (leaves).

Sample Extraction and Cleanup

Subsequent to hydrolysis, 25 mL of MTBE was added to the mixture; this process was followed by sonication for 1 min. Next, anhydrous sodium sulfate was added (5.5 g for florets and stems, 7.0 g for leaves); the resulting mixture was filtrated through a filter paper (Albet 140) and the eluent was collected. In order to perform a sample cleanup, an SPE procedure was carried out with SI-1 silica cartridges. Prior to the sample being loaded, conditioning took place with 3 mL of MTBE, the silica cartridges were washed with 3 mL of ethyl acetate (which was then discarded), and the SF eluted with 2 mL of methanol. The extracts obtained were evaporated to dryness in a rotary evaporator, and the dry residue was reconstituted with 1 mL of ethanol, passed through a nylon 0.45-μm filter, and injected into the LC-DAD system.

Method Validation

Validation was carried out in accordance with different international guidelines (U.S. Food and Drug Administration 2001; Tompson et al. 2002) determining the selectivity, carryover effect, reinjection reproducibility, stability, sensitivity, as well as linearity, precision, and recovery.

To determine the selectivity of the proposed method, a set of extracts from non-spiked broccoli samples (n=6) were injected onto the LC-DAD and the results compared with those obtained from standard SF solutions (retention time (RT), UV, and MS spectra). Standard calibration curves based on the external standard method were used to quantify SF in broccoli samples and to calculate the limits of detection (LOD) and quantification (LOQ) since the matrix and sample treatment did not affect the analyte signal. The standard solutions used to construct the calibration curve were made by preparing different solutions containing variable amounts of SF over a concentration range of 0.6–200 mg/L (from ~2.5 to 800 mg/kg in matrix with the proposed sample treatment). In addition, calibration curves were fitted by the peak area against the analyte concentrations. Linearity was evaluated by visual analysis of the plots, a calculation being made of the determination coefficients (R^2), and by our back-calculation of the concentrations of the individual calibration standards.

The LOD and LOQ of the method were determined as, respectively, three and ten times the standard deviation of the intercept for the calibration curve (external standard method) divided by the slope. In order to confirm those limits, SF standard solutions at the corresponding LOD and LOQ values were injected onto the LC-DAD system. The carryover effect was assessed in each matrix by injecting broccoli samples with a high-concentration sample (high QC) or calibration standard and evaluating the responses at the RT of the analyte of blank samples or ethanol. Reinjection reproducibility was evaluated by reinjecting previously acceptable standards (low and high QCs) three times per day (5 days). The stability of the SF during sample preparation, after long-term (frozen at the intended storage temperature for stock solutions or stored at + 4 °C for matrix matched and standard solutions, 1 month in both cases) and short-term (room temperature (25 °C), 2 weeks) storage, as well as the autosampler stability, has also been evaluated. To perform these experiments, a set of standard and matrix-matched solution samples prepared in ethanol from a freshly made stock solution of SF were used. Precision experiments were performed concurrently by repeated sample analysis, but by using broccoli samples spiked at three

concentrations of SF (low, medium, high QC levels) on the same day (n=6), which were treated according to the procedure described in this study. In each run, a calibration curve was established and replicates (n=6) of each spiking level were analyzed.

Results and Discussion

Optimizing the LC-DAD Conditions

Determining SF in broccoli matrices has been usually carried out in reversed-phase mode by means of C_{18}-based stationary phase columns with water and acetonitrile as the mobile phase components (Campas-Baypoli et al. 2010; Guo et al. 2013a; Han and Row 2011; Liang et al. 2005, 2006, 2008; Campas-Baypoli et al. 2009; Totušek et al. 2011; Liu et al. 2009; Abdulah et al. 2009; Matusheski et al. 2001; Sivakumar et al. 2007). Initially, SF standard solutions and mobile phases composed of water and acetonitrile in isocratic elution mode were used to select the optimal analytical column. Several C_{18} stationary phases with different polarities and lengths were tested—Synergi^{TM} 4 μm Hydro-RP 80Å (150×4.6 mm), Synergi^{TM} 4 μm Fusion-RP 80Å (250×4.6 mm), and Gemini® 3 μm C_{18} 110Å (50×2.0 mm)—all of which were provided by Phenomenex. Moreover, a hydrophilic interaction liquid chromatography (HILIC) column (Luna® 3 μm HILIC 200Å, 50×2.0 mm; Phenomenex) was also tested due to the emphasis that it is actually being placed on this type of chromatography to separate polar compounds in food matrices. The HILIC column was discarded due to the low RTs obtained (<1 min), which means that the HILIC separation mechanism was not the suitable one for this compound. Meanwhile, the shortest C_{18} column (Gemini®) was not selected because it was also not possible to elute SF in a reasonable period of time (RT<2 min). Regarding the Synergi^{TM} columns, it should be said that it was possible to determine SF with both of them, although, as can be expected, a higher flow rate of the mobile phase was required when the longest column (Synergi^{TM} Fusion-RP) was used in order to obtain analysis times comparable with the Synergi^{TM} Hydro-RP (RT<6 min). Consequently, as one of the goals of this study was to develop the quickest possible LC-DAD method using as few reagents as possible, the Synergi^{TM} Hydro-RP column was chosen. It should be remarked that in only four of the previously mentioned studies (Guo et al. 2013a; Totušek et al. 2011; Jones et al. 2010; Nakagawa et al. 2006) were analytical columns of the same length (150 mm) employed; in the other studies, longer columns (250 or 300 mm) were used.

As has been previously mentioned, mobile phases composed of water and acetonitrile were used in the preliminary stage. However, ammonium formate was tested as the mobile phase component in order to improve the peak shape and to facilitate the application of the optimized chromatographic conditions to potential MS analyses. As has been previously mentioned, the use of MS was not required in most cases, but its purpose was confirmatory as SF is present at high concentrations in broccoli. Nevertheless, it is occasionally necessary to develop highly sensitive methodologies based on MS detection to determine lower amounts of SF and metabolites (biological matrices; Agrawal et al. 2006), and the use of ammonium salts could be beneficial as they improve chemical ionization and facilitate MS detection. The influence of salt concentration (5–30 mM) on the separation was studied, and it was observed that there was a decrease in peak width and a slightly shorter analysis time when the concentration was increased up to 20 mM. As no significant improvement at higher values was observed, 20 mM was selected as the optimal buffer concentration. It should also be pointed out that the use of ammonium formate as a mobile phase component has not been reported in any of the aforementioned publications. Once the mobile phase components had been selected, the percentage of organic modifier and the flow rate were optimized with a view to reducing analysis time as far as possible, but avoiding co-elution with matrix compounds. For these reasons, broccoli samples (florets, leaves, and stems) were injected with the treatment described in "Sample Procurement and Treatment." Following these experiments, a mobile phase was selected composed of 20 mM ammonium formate and acetonitrile (55:45, v/v) in isocratic elution mode at a flow rate of 0.6 mL/min as these were the optimal conditions which allowed baseline separation of SF from the matrix compounds in a shorter period of time. Moreover, it was not necessary to introduce a post-time to elute any other matrix components, which might have been retained, from the column as the sample treatment proposed had eliminated all of them. The influence of the column temperature was also investigated (from 25 to 45 °C at 10 °C intervals), and as expected, a slight decrease in RTs was observed as the temperature increased, although this improvement did not compensate for the loss of symmetry in the case of temperatures over 25 °C. Therefore, 25 °C was chosen as the optimal value. Consideration was given to the possibility of enhancing the LOD and LOQ by injecting larger sample volumes (2–10 μL). It was found that the S/N ratio increased when up to 5 μL was injected, above which the ratio did not improve significantly and a loss of symmetry and peak shape was evident, especially when high SF concentrations were injected. Finally, following UV scanning to obtain the best S/N values, 196 nm was selected as the optimal wavelength for SF detection.

Under the chromatography conditions described above, it was possible to analyze SF (RT~4 min) in broccoli by LC-DAD with an overall run time of 5 min (Fig. 3), which, to our knowledge, is the fastest proposal that has been published. Moreover, it should be mentioned that in only four of the previously mentioned studies was the RT of SF lower than

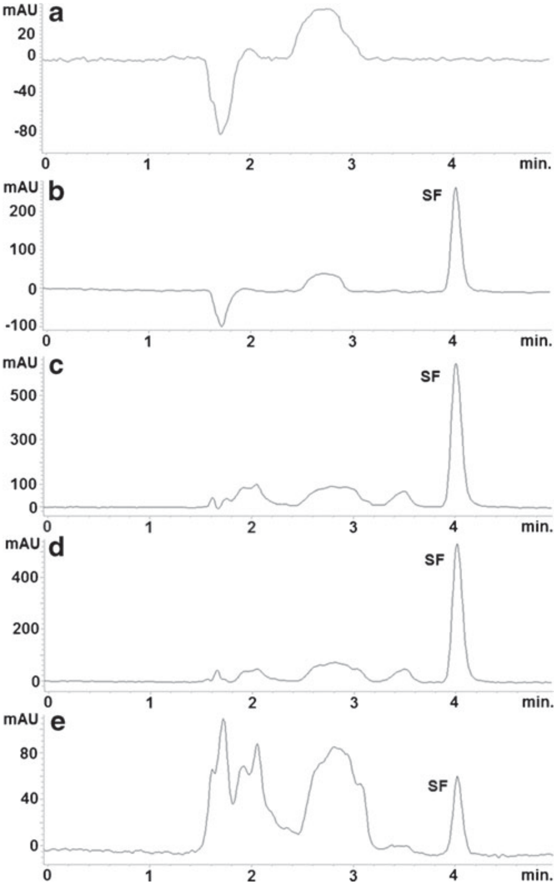

Fig. 3 Representative LC-DAD (λ=196 nm) chromatograms obtained from ethanol (**a**), a standard solution (50 mg/L) of SF (**b**), a broccoli floret sample (Parthenon) (**c**), a broccoli stem sample (Parthenon) (**d**), and a broccoli leaf sample (Parthenon) (**e**). The LC-DAD conditions are described in "Chromatography Systems"

8 min (6 min: Li et al. 2012; 5.7 min: Sivakumar et al. 2007; 5.5 min: Totušek et al. 2011; 4.6 min: Matusheski et al. 2001), but in all cases, the overall run times (>8 min) and the mobile phase flow rates (>0.8 mL/min) were higher than those proposed in this paper, while in other LC publications (Campas-Baypoli et al. 2009, 2010; Shen et al. 2010; Han and Row 2011; Liang et al. 2005, 2006, 2008; Totušek et al. 2011; Liu et al. 2009; Abdulah et al. 2009; Matusheski et al. 2001), these parameters, especially overall analysis time, were quite different.

Sample Treatment

Optimizing the Conversion of GRA to SF

To optimize the hydrolysis of GRA to SF, several trials were conducted with broccoli samples (florets, leaves, and stems) by varying most of the aforementioned parameters: (1) the pH of acidic water (3–7) using 0.1 M hydrochloric acid to reach those pH values; (2) the volume of acidic water (2, 5, 7, and 10 mL); (3) hydrolysis time (0, 1, 2, 4, and 7 h); (4) temperature (25, 35, 45, 55, and 65 °C); and (5) the amount of ascorbic acid (0, 1, 4, 8, and 12 mg of ascorbic acid per gram of dry weight of broccoli). The results of those tests are summarized in Fig. 4. In order to perform these experiments, the amount of sample to be used was initially selected. Some tests were conducted by varying the weight of the sample (0.05–0.75 g). It was found that the optimal values were 0.25 g for florets and stems and 0.50 g for leaves as when higher amounts were analyzed, the SPE procedure required more time and, in some cases, the DAD signal was too high, with the peaks becoming wider and starting to appear somewhat deformed.

With regard to selecting the pH (Fig. 4a), the highest SF content was detected in broccoli leaves at pH 5, while in florets and stems, the optimal pH was 6. It was also concluded that 5 mL of the corresponding acidic solutions provided the maximum amount of SF in broccoli florets and stems, although 7 mL should be used when analyzing broccoli leaves (see Fig. 4b). Subsequently, the influence of temperature was tested. In this case, different values were obtained for each of the broccoli matrices analyzed (see Fig. 4c). That of 35 °C was selected as the optimal value in broccoli leaves, while the best results for florets and stems were obtained with 45 and 55 °C, respectively. Moreover, several hydrolysis times were assayed (Fig. 4d); the results again showed differences between the three broccoli matrices analyzed. It was found that 1 h was enough to obtain the maximum conversion of GRA to SF in broccoli stems, but longer times were required for florets (2 h) and leaves (4 h). It was also concluded that this parameter has a strong influence on the formation of SF as without this step the SF content was quite low. Finally, the influence of the presence of ascorbic acid on the hydrolysis process was tested (see Fig. 4e). It was found that its presence has an influence on SF content in the three matrices; the best results were obtained when using 4 mg/g. On examination of these results, it can be concluded that the conversion of GRA to SF should be optimized for each broccoli part as differences were observed in the action of most of the parameters studied, especially temperature and hydrolysis time. This finding could be of interest for potential readers as it is the first time that a study has been made of the possible differences in the conversion of GRA to SF depending on the broccoli matrix. It should be specified that in none of the previous publications was there optimization and use of different hydrolysis parameters for each broccoli part. Finally, the optimized hydrolysis conditions have been demonstrated to be useful for obtaining broccoli extracts with the highest content of sulforaphane.

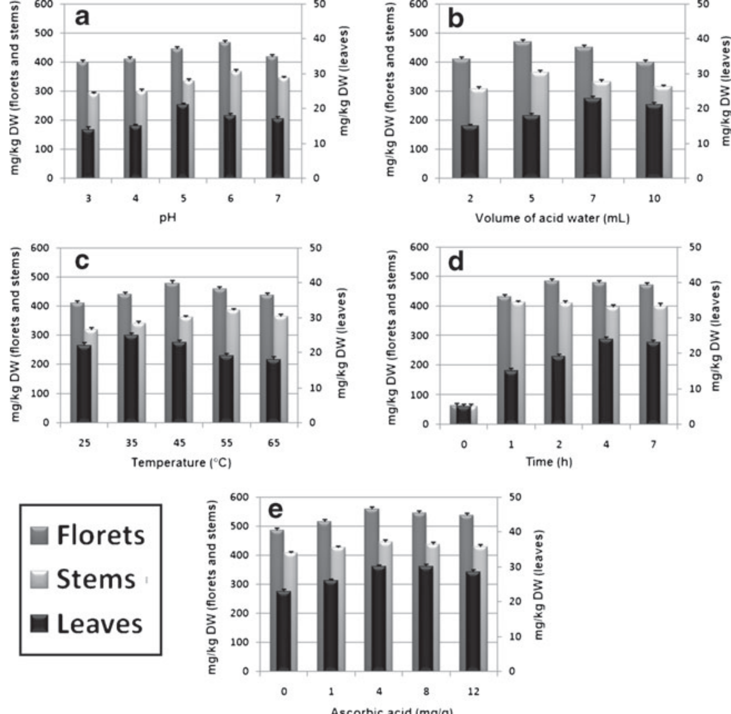

Fig. 4 Effects of various parameters on the conversion of GRA to SF: pH of acidic water (**a**); volume of acidic water (**b**); temperature (**c**); time (**d**); and presence of ascorbic acid (**e**). All the samples were treated after hydrolysis with the SPE described in "Sample Extraction and Cleanup." Data represent the mean of three replicates±the standard deviation of the mean (*narrow bars*). *DW* dry weight

Optimizing the Extraction and Cleanup Procedures

Once hydrolysis conditions were optimized, several tests were performed in order to select the most adequate solvent to extract SF from the broccoli matrices. As seen previously, DCM has been commonly used to extract SF (Campas-Baypoli et al. 2009, 2010; Han and Row 2011; Guo et al. 2011, 2013b; Liang et al. 2006; Totušek et al. 2011; Azizi et al. 2011; Bertelli et al. 1998; Jones et al. 2010; Martínez-Hernández et al. 2013; Ghawi et al. 2013; Liu et al. 2009; Abdulah et al. 2009; Haina et al. 2010; Matusheski et al. 2001; Nakagawa et al. 2006; Sivakumar et al. 2007). However, one of the goals of the present study was to develop an extraction method as environment-friendly as possible. For this reason, the use of chlorinated compounds (DCM or chloroform) should be avoided. Consequently, it was decided that the suitability of other more recommendable solvents such as heptane, 1-butanol, ethyl acetate, or MTBE should be tested and a comparison made of their performance against that of DCM. Promising results were obtained when MTBE was employed as the signal obtained with this solvent was quite similar to DCM (~96 %); however, the use of the other organic solvents was discarded due to the lower signals obtained (see Fig. 5). Tests were conducted at different spiking concentrations (6, 160, and 600 mg/kg of SF) with DCM and MTBE used as the extractant solvents; the previous results were confirmed: in all cases, the MTBE recoveries were very close to those obtained with DCM (data not shown). As a result, it was decided that the experiments would be continued with MTBE as it possessed lower toxicity than DCM and provided quite similar recovery results. Afterwards, a study was made of the amount of MTBE necessary for maximum extraction efficiency. Several volumes were tested (10–30 ml), and the results showed that 25 mL was enough to obtain recoveries higher than 92 % for the different spiking concentrations (6, 160, and 600 mg/kg of SF) and broccoli matrices assayed (see Fig. 6). It should be specified that for samples

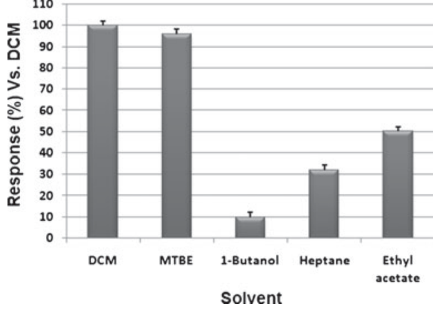

Fig. 5 Recovery (in percent) from several solvents compared to recovery from DCM after spiking broccoli samples at the medium QC level (160 mg/kg). Data represent the mean of three replicates±the standard deviation of the mean (*narrow bars*)

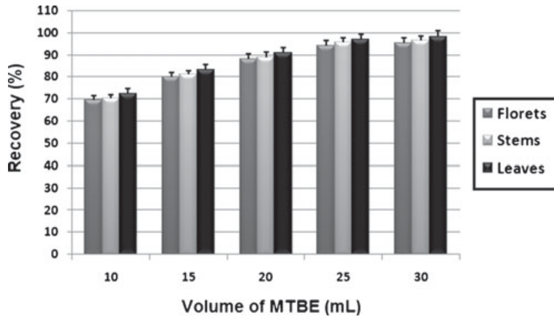

Fig. 6 Recovery (in percent) from different volumes of MTBE after spiking broccoli samples at the medium QC level (160 mg/kg) with the proposed sample treatment (described in "Sample Procurement and Treatment"). Data represent the mean of three replicates±the standard deviation of the mean (*narrow bars*)

with lower SF content, 20 mL would be more than enough to obtain recoveries higher than 88 %, but as it is impossible to have an accurate knowledge of SF content without analyzing the sample, it is recommended that 25 mL of MTBE should be used for efficient extraction. In two previous studies (Campas-Baypoli et al. 2010; Elbarbry and Elrody 2011), it was stated that subsequent to extraction, the sample should be stored at room temperature for 1 h. Therefore, a study was made of the influence of this parameter on the extraction of SF. Several waiting times were assayed (0, 1, 2, and 4 h), with the results (data not presented) showing no significant differences among the four experiments, and so it was concluded that it was unnecessary to carry out this step. Anhydrous sodium sulfate was added to the mixture (5.5 g floret and stems; 7.0 g leaves) as the drying agent for removing traces of water; it was then filtrated through a filter paper (Albet 140) and the eluent collected. In preliminary studies, this solution was evaporated to dryness and reconstituted with ethanol and injected into the LC-DAD system with no SPE cleanup. It was observed that no interferences affected the determination of SF and that the recovery percentages were good (>90 %), but some matrix components eluted at higher RTs (see Fig. 7). To elute all matrix compounds from the column without SPE cleanup, it was necessary to employ longer chromatographic run times and gradient elution programs in order. Moreover, there was also an increase in the pressure of the system after injecting the samples without a post-time step. Consequently, the decision was taken to perform an SPE procedure (cleanup) as this has provided satisfactory results in previous studies (Campas-Baypoli et al. 2009, 2010; Han and Row 2011; Totušek et al. 2011; Bertelli et al. 1998; Martínez-Hernández et al. 2013; Liang et al. 2008; Haina et al. 2010; Nakagawa et al. 2006; Sivakumar et al. 2007). Silica-based cartridges have been proven to be the best option to extract SF, which may be attributed to the weak polarity of SF, easily selectively absorbed by a weak polarity column such as silica (Han and Row 2011). Prior to use, the cartridges were conditioned with MTBE as it was found after performing some preliminary experiments that its use provided similar results to DCM, which was usually employed (Campas-Baypoli et al. 2009, 2010; Han and Row 2011; Bertelli et al. 1998; Sivakumar et al. 2007). Different volumes were tested (1–5 mL), and it was found that 3 mL was enough to perform this task (data not shown). Next, the extracts (25 mL) were loaded onto the cartridges, after which a washing stage was applied as this can reduce most of the interference during the separation of SF. Ethyl acetate was used to perform this task as this has been successfully employed previously (Campas-Baypoli et al. 2009, 2010; Han and Row 2011; Bertelli et al. 1998; Nakagawa et al. 2006; Sivakumar et al. 2007). Different volumes were tested (1–5 mL), and it was found that 3 mL was enough to wash out unnecessary compounds, but not SF (data not shown). Finally, the elution solvent and its optimal volume were selected. Three different solvents were assayed (methanol, ethanol, and acetonitrile), and it was found that methanol provided better results than ethanol, while acetonitrile was not able to extract SF from the cartridge. Several elution volumes were tested (1–3 mL), and it was observed that elution with 2 mL of methanol was enough to obtain adequate recoveries (data not shown). After the optimized SPE procedure, the resulting extract was placed in a 20-mL conical flask and evaporated to dryness at 25 °C. Afterwards, the solvent used to reconstitute the sample was also studied. Two environment-friendly solvents (water and ethanol) were tested, and the best results were obtained with ethanol (data not shown). It was also found that 1 mL of this solvent was enough to obtain satisfactory results. Consequently, the dry residue was reconstituted in 1 mL of ethanol and passed through a syringe filter, after which a 5-μL aliquot was injected into the LC-DAD system. Finally, it should be specified that the optimal SPE conditions could be applied to the three broccoli sample types as no significant differences in terms of extraction efficiency and cleanup of the sample were observed after analyzing the three different broccoli matrices.

Extraction Efficiency

The recovery and efficiency of the sample treatment were determined in six replicates at three concentrations (low, medium, and high QC levels); a comparison of the SF peak areas was made from (1) broccoli samples spiked with different amounts of SF (6, 160, and 600 mg/kg) and then treated according to the aforementioned procedure to obtain the recovery percentages (A samples) and (2) broccoli samples treated as described above and afterwards spiked with 6, 160, and 600 mg/kg of SF (B samples). The resulting recovery percentages (see Table 1) ranged from 92 to 102 %; meanwhile, the relative standard deviation (%RSD) was lower than or equal to 5 % in all cases, indicating that the sample treatment procedure selected was a suitable one. Moreover, as may be observed from the data

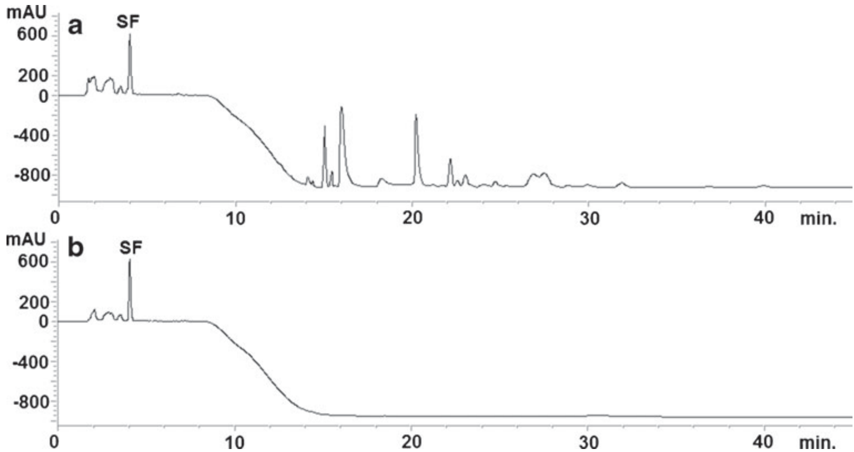

Fig. 7 Representative LC-DAD (λ=196 nm) chromatograms obtained from broccoli floret samples (Parthenon) extracted with MTBE without SPE treatment (**a**) and with SPE treatment (**b**) (as described in "Results and Discussion"). The gradient elution program was as follows: (1) 0 min, 20 mM ammonium formate in water and acetonitrile (55:45, *v*/*v*); (2) 5 min, 20 mM ammonium formate in water and acetonitrile (55:45, *v*/*v*); (3) 10 min, 20 mM ammonium formate in water and acetonitrile (0:100, *v*/*v*); (4) 45 min, 20 mM ammonium formate in water and acetonitrile (0:100, *v*/*v*). The other LC-DAD conditions are described in "Chromatography Systems"

summarized in Table 1, the recovery percentages were slightly lower when analyzing samples spiked at higher concentrations. These recovery values are comparable with previous data obtained using DCM or ethyl acetate as the extractant solvents, in which recoveries in excess of 90 % were obtained. However, the proposed sample extraction procedure has the advantages of using MTBE instead of DCM, the former being selected for the first time to extract SF, and the shorter chromatographic run due to the use of the SPE, permitting the elimination of certain matrix components that eluted at higher RTs, as occurred when using only ethyl acetate without SPE (see Fig. 7).

Method Validation

To assess the selectivity of the method, extracts from broccoli samples (florets, stems, and leaves) were injected into the LC-DAD system. As it is not possible to obtain broccoli samples free of SF, the UV and MS of SF, as well as the RT in the standard solutions, were compared with the matrix data. As can be seen in Fig. 3, the RTs matched perfectly in standard solutions and matrix samples. It was also observed that the UV spectra were almost identical in standard and matrix samples. Meanwhile, an analysis of the MS results showed that the most abundant ion (100 % of relative abundance) was 178 m/z ($[M+H]^+$) in standard and matrix samples, and the relative percentages of other abundant ions (72 m/z (12 %) $[M\text{-}C_4H_9SO]^+$, 114 m/z (63 %) $[M\text{-}CH_3OS]^+$, and 119 m/z (6 %) $[M\text{-}NCS]^+$), which were not isotopes of the most abundant ion (178 m/z), as stated in most validation guidelines, were quite similar in all cases (Fig. 2).

As has already been seen, a standard calibration curve in ethanol based on the external standard method was used to quantify SF in broccoli samples. It was possible to use non-matrix standard calibration curves since no matrix effect was noticed. This finding was confirmed by comparing the SF peak areas of blank samples B (broccoli samples treated as described above and afterwards spiked with the same amounts of SF) and standard solutions at the three QC levels (see Table 1); moreover, the slopes of the external standard calibration curve (35.1 (slope)±1.0 (confident interval)) and the

Table 1 Recovery data obtained for broccoli samples treated according to the procedure described in "Results and Discussion" ($n=6$)

Compound	Concentration (mg/kg)	Evaluation of the sample treatment			Evaluation of the matrix effect		
		Mean (%)±RSD (%)			Mean (%)±RSD (%)		
		Florets	Stems	Leaves	Florets	Stems	Leaves
Sulforaphane	6	97±5	99±4	102±4	97±5	100±5	97±4
	160	94±4	95±5	97±5	99±4	98±5	95±5
	600	92±5	92±4	94±5	98±5	102±4	98±5

RSD relative standard deviation

standard addition calibration curves for each type of broccoli sample (florets, 34.0±1.1; stems, 34.4±1.5; leaves, 34.3±0.8) fell within the same confidence interval. In addition, a comparison was made of the concentration values of broccoli samples determined by the external standard and the standard addition methods. It was found that the results were quite similar with both methods as the differences between the data obtained with both calibration methods were lower than 3 % (data not shown). It should be noted that the confidence interval of the intercept from the external standard method (43.8±89.2) included zero. Linearity was evaluated by calculating the regression equation and R^2, the latter determined as >0.99. The deviation of the back-calculated standard concentrations was equal to or less than 5 % in all cases from the nominal values. Absence of bias was confirmed by a *t* test and by studying the distribution of residuals (data not shown).

The LOD (0.2 mg/L equivalent to 0.8 mg/kg) and LOQ (0.6 mg/L equivalent to 2.5 mg/kg) values were calculated as described in "Materials and Methods." It should be said that in only two of the previously mentioned LC-based publications were the LODs of the method specified; in one case, this was lower (0.020 mg/L; Han and Row 2011), while in the other it was higher (0.56 mg/L; Campas-Baypoli et al. 2010). However, the sensitivity of the method described in this paper has been proven to be good enough to measure SF content in all the broccoli samples.

No peak area variations were observed at SF elution time when solvent blanks (ethanol) were injected after running samples spiked with high concentrations of SF (Fig. 3a); thus, it was possible to discard a carryover effect when using the proposed method. An evaluation of reinjection reproducibility revealed %RSD values <2.5 % in all cases (data not shown), which demonstrated that the samples could be safely reanalyzed within 5 days in the case of instrument failure. No variations were evident in any of the stability tests performed, namely, room temperature matrix stability, long-term stability, and autosampler stability, as we found no significant differences when comparing SF peak values obtained from the stability study samples with those of fresh samples (data not shown). In all cases, the absolute %RSD values were <6 %. The intra-day precision (%RSD) ranged from 1 to 4 % in all cases, while the %RSD values for the inter-day assays ranged from 2 to 5 %. These results indicate that the proposed method is accurate. Moreover, while an internal standard is recommended in most validation guidelines, it was not necessary in this case due to the high degree of precision achieved.

Application of the Method

The validated method was applied to determine SF content in 30 broccoli samples (florets, leaves, and stems) from two different cultivars (Parthenon and Marathon). The SF content of each broccoli sample is summarized in Table 2. As might be expected, SF was detected in all the samples, and its content was quite different depending on the part and variety of broccoli. The highest SF content was found in broccoli floret samples from Parthenon cultivars (>500 mg/kg); the lowest amount of SF was detected in broccoli leaf samples from the Marathon variety (<18 mg/kg). It can also be concluded that the Parthenon broccoli samples analyzed displayed more SF content than the Marathon samples and that the highest amounts of SF were obtained from florets in both varieties. Finally, it should be mentioned that these results agree to a large extent with the scientific data available in broccoli (Campas-Baypoli et al. 2010; Li et al. 2012; Liang et al. 2006; Matusheski et al. 2001; Nakagawa et al. 2006; Gu et al. 2012b) as differences in the SF content within broccoli parts and cultivars, quite similar to those described in this paper, have previously been found. Finally, it was demonstrated that broccoli by-products (leaves) possessed a significant SF content. These products, which are usually discarded, could be used as complements in animal diets or as nutraceutical reservoirs that reduce their environmental impact and at the same time give them a certain economic value.

Table 2 Concentration (in milligrams per kilogram, dry weight) of sulforaphane in broccoli samples (five samples of each broccoli part per cultivar)

Broccoli part	Cultivars	
	Parthenon Min.–max. values	Marathon Min.–max. values
Florets	525–556	90–107
Stems	415–446	25–40
Leaves	22–30	17–24

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Conflict of interest Ana M. Ares declares that she has no conflict of interest. José Bernal declares that he has no conflict of interest. María T. Martín declares that she has no conflict of interest. José L. Bernal declares that he has no conflict of interest. María J. Nozal declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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V.6. PAPER VI. Effect of temperature and light exposure on the detection of total intact glucosinolate content by LC-ESI-MS in broccoli leaves

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Effect of Temperature and Light Exposure on the Detection of Total Intact Glucosinolate Content by LC-ESI-MS in Broccoli Leaves

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Abstract Total intact glucosinolate content in broccoli leaf extracts (*Ramoso calabrese* cultivar) has been determined by liquid chromatography coupled to tandem mass spectrometry with the aim of detecting potential differences in this value due to the effects of the drying temperature or the working and storage conditions (temperature and light exposure). Those broccoli leaf extracts were obtained with two different sample treatments based on heating the sample (microwave or oven), and using boiling water as extraction solvent. Significant differences were observed in the total intact glucosinolate content depending on the drying temperature and light exposure under typical working conditions. Meanwhile, those differences were less remarkable when the extracts were stored at low temperature and protected from light exposure.

Keywords Intact glucosinolates · Broccoli leaves · LC-ESI-MS · Light exposure · Temperature

Abbreviations

ALY	Glucoalyssin
DW	Dry weight
ESI	Electrospray ionization
BC	Glucobrassicin
GER	Glucoerucin
GIB	Glucoiberin
GLSs	Glucosinolates
GRA	Glucoraphanin
GST	Gluconasturtiin
4ME	4-Methoxyglucobrassicin
MW	Microwave
NEO	Neoglucobrassicin
4OH	4-Hydroxyglucobrassicin
SIN	Sinigrin

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Introduction

Broccoli (*Brassica oleracea* L. var. *Italica*) is a good source of many health promoting compounds and potentially protective phytochemicals including lipids, proteins, amino acids, phenolics, carotenoids, selenium, vitamins or glucosinolates (GLSs, Ares et al. 2013). GLSs are β-thioglucoside *N*-hydroxysulfates, with a side chain derived from amino acids and a sulfur-linked β-D-glucopyranose moiety (see Fig. 1). GLSs and/or their breakdown products (isothiocyanates) have long been known for their fungicidal, bactericidal, nematocidal and allelopathic properties, and they have recently gained research interest because of their anticancer activity (Ares et al. 2013; Moreno et al. 2006). Those compounds are present in all broccoli parts, including leaves (Ares et al. 2013, 2014; Domínguez-Perles et al. 2010; Hennig et al. 2012; Sasaki et al. 2012), which economic value could be increased, as leaves could be used as a GLSs source, and at the same time, the environmental impact would be reduced because these by-products have been usually discarded.

GLSs are usually determined according to the presence (intact or non-intact desulfo-derivatives) of a sulfate group, and in most cases, the sample treatments include heating, which is required to deactivate the myrosinase enzyme and obtain a better extraction of intact GLSs (Domínguez-Perles et al. 2011). As can be expected, the influence of the temperature on GLS content in broccoli has been previously studied (Hanschen et al. 2012; Hennig et al. 2012; Oliviero et al. 2012; Song and Thornalley 2007; Van Eylen et al. 2008, 2009). In those publications, the effect of different thermal procedures

Fig. 1 Structures of the studied glucosinolates (*GLSs*)

Glucobrassicin (GBC)
Glucoerucin (GER)
Glucoiberin (GIB)
Glucoraphanin (GRA)
Gluconasturtiin (GST)
Sinigrin (SIN)
4-Metoxyglucobrassicin (4ME)
4-Hydroxyglucobrassicin (4OH)
Glucoalyssin (ALY)
Neoglucobrassicin (NEO)

on the GLSs content has been investigated, but once the broccoli samples have been previously freeze-dried, frozen, or were placed in heated solvent baths (water, methanol or water and methanol mixtures). In the present work, it has been studied for the first time the influence of the temperature of the drying process on the intact GLS content in fresh broccoli leaf samples, which have not been previously treated with any of the above mentioned procedures. In addition, we have also pioneered the research about the effects of the working and storage conditions (temperature and light exposure) on total intact GLSs content in broccoli leaf extracts. Those extracts were obtained using two previously optimized sample treatments based on the deactivation of myrosinase by heating the sample (microwave [MW] or oven), and employing boiling water as extraction solvent (Ares et al. 2014). Furthermore, as water has been previously used to extract GLSs in broccoli (Jones et al. 2010), the results of the present research could be of interest for many readers.

Our aim, therefore, was to determine the total intact GLS content in broccoli leaf extracts (*Ramoso calabrese* cultivar) in

order to determine potential differences in this value due to the effects of the drying temperature or the working and storage conditions (temperature and light exposure). To obtain the individual GLS concentration, required to calculate the total GLS content, it was employed a previously validated liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC-ESI-MS) method (Ares et al. 2014).

Materials and Methods

Reagents and Materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), glucobrassicin (GBC), gluconasturtiin (GST) and sinigrin (SIN), which was used as the external standard (ES), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). SIN was used as an external standard to quantify the GLSs from broccoli where individual standards were not available (glucoalyssin [ALY]; 4-hydroxyglucobrassicin [4OH]; neoglucobrassicin [NEO]; 4-methoxyglucobrassicin [4ME]), as has normally been the case (Domínguez-Perles et al. 2011; Pérez-Balibrea et al. 2011). It must be specified that those GLSs were selected, as in a previous study (Ares et al. 2014), there were detected in leaves from a *R. calabrese* broccoli cultivar. Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie (Steinheim, Germany), was employed to identify and confirm the presence of ALY, 4OH, and NEO due to the absence of individual standards for these GLSs. LC grade methanol and acetonitrile were both supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst formic acid was purchased from Sigma Aldrich Chemie. Syringe filters (17 mm, Nylon 0.45 μm) were purchased from Nalgene (Rochester, NY, USA), quantitative filter paper (Albet 140) was supplied by Albet-Hahnemuehle S.L. (Barcelona, Spain), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA,USA). An Eppendorf Centrifuge 5810R (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), a vacuum filter system (Supelco, St. Louis, MO, USA), and an R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), were used for all the extractions. An MW model MG-3924 W from LG electronics (Madrid, Spain), a drying oven from Selecta (Barcelona, Spain), as well as a Vortex mechanical mixer from Heidolph (Schwabach, Germany) were also employed. The samples were ground with a Moulinette chopper device from Moulinex (Paris, France).

Standard Solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each GLS in 10 ml of ultrapure water to a final concentration of approximately 300 mg/l. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Broccoli leaf samples (50 mg) were spiked with different amounts of the available GLS standards for calibration at different concentrations (between

Table 1 Molecular weight, quantification and confirmation ions/transitions (ESI-MS detection in negative EIC mode), limit of quantification (LOQ, μg/g) and concentration (μg/g dry weight, n=5) for each of the intact glucosinolates (GLSs) analyzed in broccoli leaves (*Ramoso Calabrese* cultivar)

Compound name (abbreviation)	Molecular weight	Quantification ions	Confirmation ions	Confirmation transitions	LOQ[a]	Concentration found (min–max values)[a]
Aliphatic GLSs						
Alkyl GLSs						
Glucoiberin (GIB)	423	422	423, 424	422>97	0.12	34–36
Glucoraphanin (GRA)	437	436	437, 438	436>97	0.23	150–163
Glucoalyssin (ALY)[b]	451	450	451, 452	450>97	0.16	0.5–0.9
Glucoerucin (GER)	421	420	421, 422	420>97	0.06	2–4
Alkenyl GLSs						
Sinigrin (SIN)	359	358	359, 360	358>97	0.16	ND
Aromatic GLSs						
Gluconasturtiin (GST)	423	422	423, 424	422>97	0.04	1–4
Indole GLSs						
4-Hydroxyglucobrassicin (4OH)[b]	464	463	464, 465	463>97	0.16	28–33
Glucobrassicin (GBC)	448	447	448, 449	447>97	0.11	25–34
4-Methoxyglucobrassicin (4ME)[b]	478	477	478, 479	477>97	0.16	55–67
Neoglucobrassicin (NEO)[b]	478	477	478, 479	477>97	0.16	125–130

ND not detected

[a] Data obtained from Ares et al. (2014)

[b] Quantified with SIN calibration curves

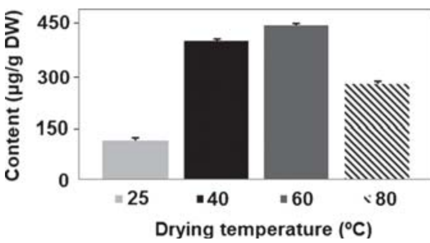

Fig. 2 Effect of drying temperature on total intact GLS content in broccoli leaves (*Ramoso calabrese* cultivar). Data represent the mean of three replicates ± the standard deviation of the mean (*narrow bars*). *DW* dry weight

LOQ [see Table 1] and 400 μg/g [ESI-MS]). These matrix-matched standards were extracted by means of the same procedure as described for samples. All standard solutions were stored in amber glass vials and kept in the dark at 4 °C.

Sample Procurement and Treatment

Plant Material

Fresh broccoli leaf samples (*R. calabrese*) were harvested after 6 months of growth from seed. These samples were dried in an oven at four different temperatures (25 °C, 40 °C, 60 °C and 80 °C) in order to check the effect of the drying temperature on the total intact GLS content. All broccoli samples were stored at +4 °C until analysis.

Sample Preparation

Broccoli leaf extraction conditions were determined in a previous study (Ares et al. 2014). Briefly, 50 mg of dried ground broccoli leaf powder was put into a beaker and water (23 ml for the MW heating treatment; 24 ml for the oven heating treatment) was added. The resulting mixture was heated in a MW for 3.5 min at 70 W, or during 30 min at 60 °C in an oven. The resulting mixture was cooled in an ice-water bath for 30 s and centrifuged at 11,000 rpm for 8 min at 10 °C. The supernatant was collected, filtered under vacuum (Albet 140), and evaporated to dryness at 55 °C in a rotary evaporator; the dry residue was reconstituted with 1 ml of ultrapure water, filtered through a nylon 0.45-μm filter, and injected into the LC ESI-MS system.

LC-ESI-MS System

The chromatographic and detection conditions were optimized in a previous publication (Ares et al. 2014). A s series LC-MS Trap XCT instrument was used in conjunction with ESI in negative ion mode. The analytical column was a Gemini® 3 μm C_{18} 110 Å (150×4.60 mm) protected by a Gemini® C_{18} security guard cartridge (4×3.0 mm), both from Phenomenex (Torrance, CA, USA). The mobile phase selected was a mixture of (A) formic acid in water (0.5 %, v/v) and (B) formic acid in acetonitrile (0.5 %, v/v) applied at a flow rate of 1 ml/min in a gradient mode as follows: (1) 0 min (A–B, 100:0, v/v); (2) 0–13 min (A–B, 100:0, v/v); (3) 13–18 min (A–B, 40:60, v/v); (4) 18–26 min (A–B, 40:60, v/v); (5) 26–30 min (A–B, 100:0, v/v); (6) 30–35 min (A–B, 100:0, v/v). The injection volume and column temperature were set at 20 μl and 30 °C, respectively. Compounds were identified by comparing the MS and MS/MS data (retention time, ions, and confirmation transition) with those obtained from pure standard solutions when commercially available, or with data obtained from analyzing the certified rapeseed material. Otherwise, one GLS (4ME) was tentatively assigned by comparing the information obtained (MS) with available

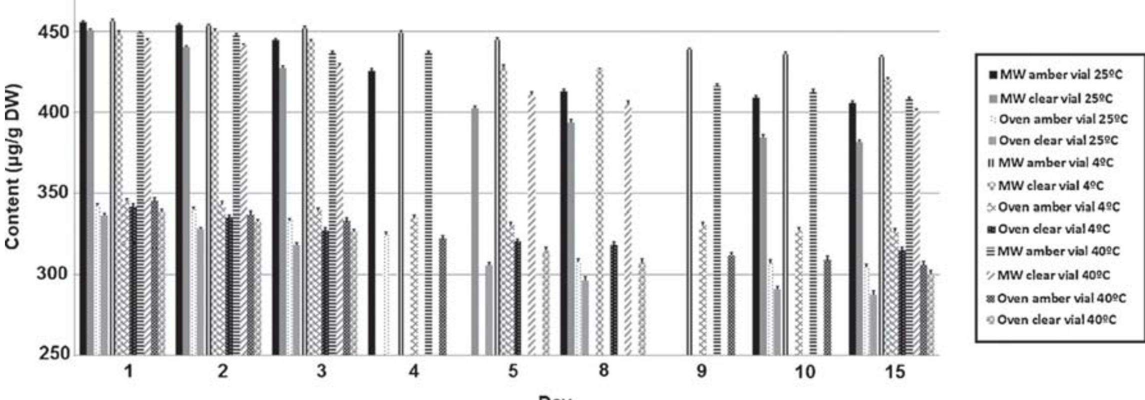

Fig. 3 Effect of the working and storage conditions (temperature and light exposure) on total intact GLS content in broccoli leaf water extracts obtained with two different sample treatments (MW and oven). Data represent the mean of three replicates ± the standard deviation of the mean (*narrow bars*). *DW* dry weight

bibliographic data (Fernández-León et al. 2012; Pérez-Balibrea et al. 2011). The MS ions and MS/MS transitions monitored for each compound are summarized in Table 1. All the transitions corresponded to fragmentation of the abundant precursors (ion *m/z* $[M–H]^-$ to ion *m/z* 97 ($[SO_3H]^-$) (Fernández-León et al. 2012). Optimal MS and MS/MS conditions were set as follows: capillary voltage, 3,500 V; drying gas (N_2) flow, 10 l/min; drying gas (N_2) temperature, 350 °C; Nebulizer pressure, 25 psi; trap drive, 35; skimmer, 40 V: octopole RF amplitude, 130 V; capillary exit, 105.0 V; fragmentation scanning, 30–200 %; isolation width (*m/z*), 4.0; fragmentation width (*m/z*), 10.0; max. accumulation time, 200 ms; ion charge control (ICC), 200,000; delay, 5 ms. To protect the ion source from matrix constituents, the MSD Trap system automatically controlled the run at different times: at $t_{0–5\ \mathrm{min}}$ to waste, $t_{5–28\ \mathrm{min}}$ to mass and $t_{28–30\ \mathrm{min}}$ to waste.

Results and Discussion

Effect of Drying Temperature on the Total Intact GLS Content

Several tests were performed to check the effect of the drying temperature on the GLS content from broccoli leaf samples. Four different temperatures (25 °C, 40 °C, 60 °C and 80 °C), which have been selected on basis of the existing scientific literature and previous experiments, were tested. To obtain the corresponding GLS concentration, in order to calculate the total GLS content, the peak areas of each analyte were plotted onto their respective calibration curves. Meanwhile, to quantify four GLSs (ALY, 4OH, NEO, 4ME), the calibration curve of sinigrin was used (external standard) as previously described. As shown in Fig. 2, the amount of GLSs quantified increased with the temperature up to 60 °C; meanwhile, at higher temperatures a decrease was observed in the total GLS content which is especially relevant for indole-based GLSs like GBC, 4ME or NEO (data not shown). This finding was in agreement with previous studies (Hanschen et al. 2012; Hennig et al. 2012). Taking into account those results, 60 °C was chosen as the optimal drying temperature. Once the drying temperature has been selected, we studied the water content of the broccoli leaves. The samples were weighed before and after the drying process at different times (2, 4, 6, 8, 12, 16, 20, 24 h), and the dried material percentage remained constant (~15 % of the fresh weight, data not shown) after 12 h of drying (overnight).

Effect of Working and Storage Conditions (Temperature and Light Exposure) on the Total Intact GLS Content

We also studied the influence of the working and storage temperature (4 °C — refrigerator, 25 °C — benchtop, 40 °C — oven) and light exposure (clear and amber glass vials), which were typical conditions, on the total intact GLS content on broccoli leaf extracts (water) during 15 days. In addition, these experiments were performed on extracts obtained with two different sample treatments (MW and oven), which were developed and optimized in a previous work (Ares et al. 2014), in order to check if the heating step provoked differences in the total GLS content. The quantification of the GLSs was done as described in the previous section. Moreover, it should be also specified that all the extracts were only injected 4 days (first, second, third and 15th), while there were alternative injected during the test time (15 days). As can be observed in Fig. 3, significant differences were observed in the total GLS content when comparing the extracts obtained with the different heating procedures (higher content for MW treatment) under the same working and storage conditions. This finding was in good agreement with previous results (Ares et al. 2014), although it was not directly related with the working and storage conditions. In addition, the slightest variation in the total intact GLS content was observed for both classes of samples treatments when the extracts were stored in amber vials at 4 °C, as the GLS contents at the end of the test time (day 15) were approximately 6 % and 9 % lower than the initial values for MW and oven treatments, respectively. Moreover, it was also observed that the total intact GLS content decreased as the days go by in a similar way for both types of extracts at the tested temperatures (4 °C, 25 °C and 40 °C) when using glass amber vials. Meanwhile, the highest decrease in the total intact GLS content (>20 % of the initial value) was detected for both treatments when the extracts was placed in glass vials at 25 °C. After examining those results, it can be concluded that broccoli leaf extracts (water) should be stored at 4 °C in glass amber vials. Furthermore, under typical working conditions of temperature (25 °C) and light exposure (glass vials) the total intact GLS content was significantly lower.

Conclusions

The highest total intact GLS content in broccoli leaves (*R. calabrese* cultivar) was obtained when using a drying temperature of 60 °C. In addition, this drying process should be performed during 12 h (overnight) in order to obtain a constant dried material percentage. There were not found significant differences related to the working and storage conditions on the total intact GLS content within samples obtained with different heating procedures. Furthermore, the variation of the

total intact GLS content during the test time (15 days) was lower (<9 % of the initial value) when the broccoli leaf extracts (water) were stored at 4°C in amber glass vials. Meanwhile, light exposure must be reduced as much as possible when working at typical laboratory temperatures, especially 25 °C, in order to avoid a remarkable decrease in the total intact GLS content (>20 % of the initial value). Finally, we have demonstrated that the drying temperature, working and storage conditions should be studied and controlled in order to obtain and maintain the highest total intact GLS content in broccoli leaf extracts.

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Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

Conflict of Interest Ana M. Ares declares that she has no conflict of interest. María J. Nozal declares that she has no conflict of interest. José L. Bernal declares that he has no conflict of interest. José Bernal declares that he has no conflict of interest.

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V.7. PAPER VII. Fast determination of intact glucosinolates in broccoli leaf by pressurized liquid extraction and ultra high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry

FAST DETERMINATION OF INTACT GLUCOSINOLATES IN BROCCOLI LEAF BY PRESSURIZED LIQUID EXTRACTION AND ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO QUADRUPOLE TIME-OF-FLIGHT MASS SPECTROMETRY

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Abstract

In this study, we investigate for the first time the suitability of an environmentally sustainable extraction technique (pressurized liquid extraction, PLE) in conjunction with a fast separation technique (ultraperformance liquid chromatography, UHPLC) coupled to a selective mass spectrometry (MS) detector (quadrupole time-of-flight, qTOF) to extract, separate and quantify fifteen intact-glucosinolates (GLSs) in broccoli leaves. Firstly, we have developed and optimized by means of an experimental design an efficient extraction procedure based on PLE, giving complete extraction within 15 min. UPLC separation utilizing a C_{18} analytical column and a mobile phase composed by formic acid in water (0.5%, v/v) and formic acid in acetonitrile (0.5%, v/v) in gradient elution mode at 0.3 mL/min, resulted in baseline-separated peaks and a run time of 13 min. The method was fully validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, precision, trueness; meanwhile a study of the matrix effect was also performed. A good selectivity, low LODs and LOQs, ranging from 2 to 26 µg/g, wide linear ranges from LOQ to 2500 µg/g, and satisfactory precision and trueness, were obtained for the studied GLSs. Finally, the proposed method was successfully applied to the analysis of intact-GLSs in fifteen broccoli leaf samples from three different cultivars (*Parthenon, Nubia,* and *Naxos*). Nine intact-GLSs were detected in all the varieties, although in different concentrations depending on the broccoli cultivar. This study demonstrates the potential use of PLE as an environmentally sustainable alternative to extract intact-GLS from broccoli leaves, and that UPLC-qTOF-MS allowed a rapid, selective and sensitive determination of intact-GLSs in this matrix.

Keywords: Broccoli leaves; Intact-glucosinolates; Pressurized liquid extraction; UPLC-qTOF-MS.

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is highly valued by large groups of the population due to its flavour, but also due to some health promoting effects, such as anticancer, antibacterial, antimicrobial, or antioxidant properties, which have been mainly attributed to glucosinolates (GLSs) and their degradation products, as well as phenolic compounds, carotenoids and dietary essential minerals (Ares, Nozal, & Bernal, 2013; Martínez-Hernández, Gómez, García-Talavera, Artés-Hernández, Monedero-Saiz, Sánchez-Álvarez, & Artés, 2013). GLSs are β-thioglucoside N-hydroxysulfates, with a side chain (R) derived from amino acids and a sulfur-linked β-D-glucopyranose moiety, which could be found in all broccoli parts, and some of these compounds appear to be protective against chemical carcinogens (Tolrà, Alonso, Poschenrieder, Barceló, & Barceló, 2000). Following tissue damage, these compounds undergo enzymatic hydrolysis by myrosinase to glucose and a variety of degradation products (isothiocyanates, nitriles, thiocyanates, epithiocyanates, epithionitriles and oxazolidines) (Francisco, Velasco, Moreno, García-Viguera, & Cartea, 2010), which are responsible for flavour and most biological activities of GLSs (Mohn, Cutting, Ernst, & Hamburger, 2007). Intensive broccoli cultivars generate a high amount of waste products, mainly leaves, which are usually discarded despite their similar composition to the edible parts of the plant. Although most of the investigation has been focused on broccoli edible parts (sprouts, florets, heads, stems, roots), some research has been conducted with the aim of determining nutritional ingredients from broccoli leaves in order to give them certain economic value, as nutraceutical reservoirs, and at the same time to reduce the environmental impact (Ares, Nozal, Bernal, & Bernal, 2014a). In the scarce published scientific literature related to this matrix, they could be found some works where GLSs were studied (Ares et al., 2014a; Ares, Nozal, Bernal, & Bernal, 2014b; Branca, Li, Goyal, & Quiros, 2002; Domínguez-Perles, Martínez-Ballesta, Carvajal, García-Viguera, & Moreno, 2010; Hennig, Verkerk, Bonnema, & Dekker, 2012; López-Berenguer, Martínez-Ballesta, García-Viguera, & Carvajal, 2008; Sasaki, Neyazaki, Shindo, Ogawa, & Momose, 2012). Different methods for the analysis of GLSs in broccoli leaves according to the presence (intact or non-intact desulfo-derivatives) of a sulfate group have been reported in literature. Although the desulfation step decreases the polarity of GLSs and improves their chromatographic resolution in reversed-phase liquid chromatography (RPLC), it is a time consuming procedure, and faster methods have been proposed for the direct analysis of intact-GLSs (Glauser, Schweizer, Turlings, & Reymond, 2012; Mohn et al., 2007). Typically, extraction of intact-GLSs is carried out in heated water (Ares et al., 2014a, 2014b) or heated methanol and water mixtures (Domínguez-Perles et al., 2010; López-Berenguer et al., 2008; Sasaki et al., 2012). As can be seen all these treatments include heating, which is necessary to inactivate myrosinase and avoid the degradation of the intact-GLSs. It must be commented that similar sample treatments (solid-liquid extraction protocols) were used in most cases to extract those compounds from other broccoli parts. Nowadays, traditional extraction techniques, which usually require long extraction times, large amounts of samples, sorbents and organic solvents, are being replaced by novel extraction techniques that are known to be more

environmentally friendly (Mustafa, & Turner 2011). Pressurized liquid extraction (PLE), especially using water as a solvent, is an emerging greener technology compared to conventional extraction techniques, and it has been already successfully used to obtain bioactive compounds in foods and plants. To our knowledge, PLE has never been applied to extract intact-GLSs from broccoli leaves. Furthermore, it has been only once developed and optimized an exclusive PLE protocol to extract GLSs (Mohn et al., 2007). In this study, the authors have validated a protocol to extract and analyze intact-GLSs in *Isatis tinctoria* leaf material, which was also applied to perform a qualitative analysis of other cruciferous plants, as broccoli heads. Finally, PLE has been also tested to extract phytochemicals, including GLSs in vegetables (broccoli heads) and fruits (Alarcón-Flores, Romero-González, Martínez Vidal, & Garrido Frenich, 2013). However, in this study, the authors used a previous established PLE conditions for the analysis of isoflavones in soy related matrices.

The analysis of intact-GLSs in broccoli leaves is usually performed by RP-HPLC with ultraviolet (UV) or diode array (DAD) (Ares et al., 2014a) and mass spectrometry (MS) (Ares et al., 2014a, 2014b; Domínguez-Perles et al., 2010; López-Berenguer et al., 2008; Sasaki et al., 2012) detectors; meanwhile C_{18} (Ares et al., 2014a, 2014b; Domínguez-Perles et al., 2010; López-Berenguer et al., 2008) and C_{30} (Sasaki et al., 2012) based analytical columns were used in all cases. Among them, MS and in particular tandem mass spectrometry (MS/MS) has emerged as one of the preferred detection techniques offering enough sensitivity and an unambiguous identification and quantification of GLSs (Alarcón-Flores et al.,2013). In addition, conventional RPLC analysis of GLSs requires 20 min or longer analysis time. To reduce this, ultra performance liquid chromatography (UPLC) could be used instead, as it could be possible to obtain better resolutions and sensitivities, and shorter running times (Alarcón-Flores et al., 2013). The coupling of UPLC with different detectors (DAD, MS) has been scarcely employed to determine GLSs (Alarcón-Flores et al., 2013; Glauser et al., 2012; Gratacós-Cubarsí, Ribas-Agustí, García-Regueiro, & Castellari, 2010; Lee, Kwak, Um, Lee, Jang, & Choi, 2012; Lin, Sun, Chen, Zhang, Fan, & Li, & 2014), and broccoli (accessions and heads) has been analyzed in few of these works (Alarcón-Flores et al., 2013; Lee et al., 2012), but to our knowledge, GLSs content in broccoli leaves has never been investigated with UPLC.

Our aim, therefore, was to develop a new, robust UPLC-qTOF-MS method, which was sensitive and as rapid as possible, to separate and determine fifteen intact GLSs from broccoli leaves; to our knowledge this is the largest number of intact-GLSs studied in broccoli leaves to date. Moreover, we wish to propose and optimize, by means of a statistical analysis (Central Composite Orthogonal (CCO) design), an efficient, rapid, and environmentally sustainable extraction procedure based on PLE, which has never been done before. Accordingly, the proposed method was validated and applied in the analysis of GLSs in broccoli leaf samples from three different cultivars (*Parthenon, Nubia* and *Naxos*) to determine differences in GLSs content and to check the usefulness of the proposed method.

2. Materials and methods

2.1. Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (NAS) and sinigrin (SIN) were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). Rapeseed certified ERM® BC367 reference material from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), which was composed among other compounds by glucoalyssin (ALY), 4-hydroxyglucobrassicin (4-OH), neoglucobrassicin (NEO), napoleiferin (GNL), glucobrassicanapin (GBN), progoitrin (PRO), GNA, GBN, NAS, GBC, and SIN, was employed to optimize separation, whilst potentially it could be used to identify and confirm the presence of ALY, 4-OH, GNL, PRO, GBN and NEO in the samples, due to the absence of individual standards for these GLSs. Moreover, GTL was used as an external standard (ES) to quantify the GLSs from broccoli where individual standards were not available, while SIN was employed as internal standard (IS). Both compounds were selected as they are naturally absent from broccoli and their structures are similar to that of the GLSs studied.

LC grade methanol and acetonitrile were both supplied by Honeywell Burdick and Jackson (Muskegon, MI, USA), meanwhile formic acid was acquired from Sigma Aldrich Chemie Gbmh. Syringe filters (17 mm, PTFE 0.2 μm) were purchased from VWR International (West Chester, PA, USA), and ultrapure water was obtained using Milli-Q systems (Millipore, Billerica, MA, USA). Acetonitrile, LC-MS grade, was provided by Scharlau (Barcelona, Spain) and ethanol (99.7%) was from Solveco (Rosenberg, Sweden).

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of powder of each GLS in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Broccoli leave samples (1 g) were spiked with different amounts of the eight GLSs, which were individually available, for calibration at different levels of concentration between limit of quantification (LOQ) and 10 mg/L (calibration levels of LOQ, 80 μg/L (GIB, GRA, GNA, GTL, GER), 120 μg/L (GBC, NAS), 250, 500, 1000, 2500, 10000 μg/L), which corresponded to concentration in matrix between LOQ and 2500 μg/g (LOQ, 20 μg/g (GIB, GRA, GNA, GTL, GER), 30 μg/g (GBC, NAS), 62, 250, 125, 250, 625, 2500 μg/g), according to the unit conversion and the proposed sample treatment; meanwhile SIN (IS) was added in all cases at 500 μg/L (125 μg/g for matrix matched standards). These matrix-matched standards were treated according to the extraction procedure described in this manuscript. Each quality control (**QC**) sample was prepared using 1 g of broccoli leaves samples spiked before (**BF** samples) or after (**AF** samples) the proposed sample treatment with the same amounts of GLSs used in the calibration studies.

All standard solutions were stored in glass containers and kept in the dark at +4ºC, where they were stable for over two weeks.

2.3. Plant material

Freeze-dried broccoli leaves samples (15) from three different commercial cultivars (*Parthenon, Nubia* and *Naxos*) were kindly donated by CEBAS-CSIC (Murcia, Spain). These samples were stored at +4ºC until their analysis to measure the GLSs content.

2.4. Pressurized liquid extraction (PLE) of plant samples

Pressurized liquid extraction was carried out with a Dionex ASE 200 instrument (Thermo Fisher; Germering, Germany) with attached solvent controller. Extraction of freeze-dried broccoli leaves (*Nubia* cultivar) was carried out in 11 mL extraction cells, which were filled with glass beads mixture at the top and the bottom of the sample (1 g of glass beads in each part) to favour uniform distribution of the sample and the extraction solvent in order to maximize the extraction yields. Broccoli leaf extraction conditions were set as follows after performing an optimization study by means of a CCO design using the program MODDE 10.1 (Umetrics, Umeå, Sweden): (**i**) sample was loaded into cell; (**ii**) cell was filled with solvent; (**iii**) warming up time (extraction temperature, 60ºC); (**iv**) static extraction in which all system valves were closed; (**v**) cell was rinsed with 60% cell; (**vi**) solvent was purged from cell with N_2; (**vii**) depressurization. Between extractions, a rinse of the complete system was made in order to overcome any carry-over. The resulting extracts were diluted, protected from light and stored at +4ºC. Finally 100 µL of the diluted extract was dried under N_2 stream and reconstituted in 1 mL of water. The extract was filtered (PTFE) and injected onto the UPLC-qTOF-MS system.

2.5. UPLC-qTOF-MS system

The chromatographic system consisted of an Acquity UPLC system interfaced to a Xevo G2 qTOF instrument with electrospray ionization (ESI), both from Waters (Manchester, UK). The UPLC instrument was equipped with a binary solvent, an autosampler, and a column heater compartment. The system was controlled by Waters® Empower™ Chromatography software; while MassLynx™ (V 4.1, SCN 779, Waters Corp., Manchester, UK) was used for MS data acquisition and treatment. An UPLC BEH Shield RP18 1.7µm 110Å (2.1 x 100 mm) from Waters was used as analytical column to perform all the experiments. After an optimization study, the mobile phase selected was a mixture of (**A**) formic acid in water (0.5%, v/v) and (**B**) formic acid in acetonitrile (0.5%, v/v) applied at a flow rate of 0.3 mL/min in a gradient mode. The injection volume and column temperature were set at 5 µL and 30ºC, respectively. The ESI interface was operated in negative mode, and full-scan UPLC-qTOF-MS spectra were obtained by scanning from m/z 70 to 700. The mass spectrometer was calibrated using a solution of sodium formate. Data were collected in continuum mode and mass was corrected during acquisition using an external reference (Lock-Spray™) comprising a 10 µL/min solution of leucine-enkephalin (2 ng/µL) via a lock-spray interface. Compounds were identified by retention time and MS fragmentation spectra with

those obtained from pure standard solutions when commercially available or with the data provided after analyzing the rapeseed certified material. Otherwise, one GLS (4-methoxyglucobrassicin, 4-ME) was tentatively assigned by comparing the obtained information for MS with available bibliographic data (Ares et al., 2014a; Pérez-Balibrea, Moreno, & García-Viguera, 2011). GLSs were quantified with MS spectra in total ion chromatogram (TIC) by generating extracted ion chromatograms (XIC) with the precursor ions; meanwhile the most relevant fragments for each precursor ion were also used to confirm the presence of each GLS.

3. Results and discussion

3.1. Optimization of the extraction protocol

The selected extraction technique (PLE) utilizes solvents at elevated temperatures subjected to high enough pressures to keep them in a liquid state. By using high temperature, the extraction process become more efficient due to faster diffusion rates, thereby facilitating faster extractions (Björklund, Nilsson, & Bøwadt, 2000), which together with the higher pressure, allows for improved penetration within the sample matrix and leads to improve extraction efficiency compared to using the same solvent at ambient temperature (Mustafa, & Turner 2011). Furthermore, the dielectric constant decreased with increasing temperature, which implies that the solvent polarity can be tuned by changing the temperature. The effect is particularly drastic in water, whose dielectric constant at 250°C is the same as that for methanol at room conditions. Thus, water at high temperatures is an interesting alternative extraction solvent and could potentially replace many of the organic solvents conventionally used in extraction. However, special caution should be given to thermolabile compounds, as these could be degraded unless a careful optimization of the extraction parameters is conducted, and PLE equipments are not cheap (Mustafa, & Turner 2011). Nevertheless, these drawbacks could be compensated by the advantages of accomplishing efficient processing. Consequently, we decided to check the suitability of PLE as an alternative to extract GLSs from broccoli leaves. Some previous experiments were carried out prior to design and perform the optimization with a CCO design (data not shown), and it was decided that ethanol and water would be used as solvents to perform the PLE experiments. Moreover, the pressure during PLE might slightly influence the extraction yield, and, for that reason the pressure was consistently kept constant (Kronholm, Hartonen, & Riekkola, 2007). Consequently, optimization was carried out testing three independent variables: **i)** extraction temperature; **ii**) extraction time; **iii**) extraction solvent composition (ethanol and water). Extractions were performed at five different extraction temperatures, five extraction times and five ethanol and water mixtures. In addition, it should be considered that the warming-up time changed depending on the extraction temperature. All selected factors are interrelated, so their influence on the extraction efficiency was investigated by applying a CCO design with three central points, which involved a total of 17 experiments, which were made in random runs. The central composite design is probably the most widely used experimental design for fitting a second-order response surface. This design has the additional capability of intrinsic confirmation of results and estimation of experimental

error, using orthogonal designs (Gonçalves, Carvalho, Azenha, & Alpendurada, 2006). The parameters of the model were estimated by multiple linear regression using MODDE 10.1, which also provided the optimum conditions that maximize the extraction of GLSs. The goodness of fit of the model was evaluated by the coefficient of determination (R^2), the RSD and the lack of fit test for the model from the ANOVA. The optimal values, which provided the best results in terms of the amount of total extracted GLSs, were selected. It should be also commented that due to the differences observed in the PLE extract volumes, the selected extraction solvent mixture was added to the resulting PLE extracts up to 25 mL. In addition, it was also required to perform a dilution of the extract with water (1:10, v/v) because of the high amount of some of the studied GLSs, which were found in the broccoli leaf samples.

To assess the efficiency of the proposed sample preparation based PLE, they were compared the results obtained for broccoli leaves (**BF** and **AF**) samples spiked at three different GLS concentrations (low, medium and high **QC** levels). The resulting recovery values (see Table 1) ranged from 85 to 97% in all cases, indicating that the sample preparation procedure selected was adequate and efficient. Finally, a quantitative extraction method based on PLE was developed providing results not only good in terms of extraction efficiency, but also significant faster sample treatment times (< 15 min) that previous ones where intact GLSs were determined in broccoli leaves (20 min, Ares et al., 2014a, 2014b; > 30 min, Domínguez-Perles et al., 2010; > 40 min, López-Berenguer et al., 2008; > 20 min, Sasaki et al., 2012).

3.2. UPLC optimization

Although intact-GLSs have been widely studied in Broccoli, only a small number of these researches were done in broccoli leaves (Ares et al., 2014a, 2014b; Domínguez-Perles et al., 2010; López-Berenguer et al., 2008; Sasaki et al., 2012), and in none of the above cited works the separation of intact-GLSs was performed by UPLC. We decided to start separation optimization by using the mobile phase that was proposed and optimized in our previous study (Ares et al., 2014a), which was a mixture of formic acid in water (0.5%, v/v) and formic acid in acetonitrile (0.5%, v/v), and a C_{18} analytical column reccomended for UPLC analysis (UPLC BEH Shield RP18). Moreover, the optimization of the separation was done by using a GLSs mixture composed of the 15 compounds obtained after mixing the rapeseed certified material, individual GLSs standards and broccoli leaf samples, which were treated with the proposed sample treatment. Several experiments were conducted by means of different mobile phases composition and flow rates in order to separate all the analytes in the shortest time as possible. As can be seen in Figure 1, the fifteen intact-GLSs were separated in less than 8 min, and the overall run time was 13 min, which is much shorter than those methods described in the literature where intact-GLSs were analyzed in broccoli leaves (35 min, Ares et al., 2014a, 2014b; 40 min, Domínguez-Perles et al., 2010; 40 min, López-Berenguer et al., 2008; 25 min, Sasaki et al., 2012). In addition, it should be mentioned than in these researches the number of studied GLSs it was not given (López-Berenguer et al., 2008) or it was smaller (≤ 12) (Ares et al., 2014a, 2014b; Domínguez-Perles et al., 2010; Sasaki et al., 2012). Finally, it is also interesting to mention that the proposed UPLC method could be used with DAD or UV detectors as

the fifteen compounds were baseline separated, which it is not so usual when using UPLC as in most cases MS or MS/MS were selected as detectors, and it is not required a complete separation of the compounds. However, considering possible ion suppression, even in ESI-MS it is advantageous with separated peaks.

3.3. Mass spectrometry optimization.

A qTOF was selected for the detection and identification of intact-GLSs in broccoli leaves because its rapid acquisition rate makes it an ideal detector in combination with UPLC, which usually provides very sharp peaks (Glauser et al., 2012), the dynamic range of the qTOF is extended, which is an essential feature for GLSs analysis in broccoli leaves due to the high variation on the concentration depending on the GLSs, it is possible to perform a tentative identification and quantification of compounds when the individual standard is not available, and because qTOF has been previously used for quantitative analysis (Martínez Bueno, Ulaszewska, Gomez, Hernando, & Fernández-Alba, 2012). Intact-GLSs have been analyzed in negative ESI mode as the best results in terms of peak area and peak height were obtained in previous researches with these conditions (Ares et al., 2014a, 2014b; Domínguez-Perles et al., 2010; López-Berenguer et al., 2008; Sasaki et al., 2012). To establish the optimal MS and MS/MS conditions, individual standards, rapeseed certified material and matrix matched solutions were injected and the peak parameters were evaluated for each GLSs (the optimal conditions are given in subsection 2.5). Intact-GLSs showed an intense $[M-H]^-$ (precursor ions) on their full-scan spectra, which were used by generating XIC for each GLS to obtain the maximum sensitivity for quantitative analysis. Moreover, significant fragments obtained of the precursor ions for each intact-GLSs were selected to confirm their presence in the broccoli leaf samples. To check how the matrix influenced the ESI ionization, the peak areas of the intact-GLSs in standard solutions were compared with those obtained in **AF** samples. Broccoli leaves contain endogenous levels of GLSs which must be taken into account when comparing the results. Thus, to calculate the signals for the broccoli leaf samples, the GLSs areas corresponding to endogenous levels must be also determined. Those areas were subtracted from the total area obtained for the spiked broccoli leaves samples and the resulting areas were compared with the standard solutions. The response of all compounds at the three concentrations assayed was comprised between 95% and 104% in all the cases, as it could be observed in Table 1. Hence, it was concluded that the matrix (broccoli leaves) does not affected the ESI ionization of the analytes.

3.4. Validation of the UPLC-qTOF-MS method.

Validation was carried out following different International guidelines (International Conference on Harmonization, 2005; Thomson, Ellison, & Wood, 2002). The validation studies were performed using broccoli leaves from *Nubia* variety as we did to have enough quantity of broccoli leaves from other varieties to carry out the required experiments. Moreover, it should be mentioned that the validation was

not done for ALY, 4-OH, PRO, GBN, GNL and NEO due to the lack of individual standards, and SIN has not also been included in the validation study as it was the IS. To determine the selectivity of the proposed method, a set of non-spiked broccoli leaves (n=6) were injected onto the UPLC-qTOF-MS system, and the results were compared with those obtained from standard GLSs solutions, rapeseed certified material and the existing scientific literature. No interference of the matrix compounds was detected at the elution time of each GLS for MS. In addition, both MS spectra (matrix and standard solutions), were quite similar for the studied compounds (data not shown), and they were only observed some minor differences in relation to the ion intensity. The limit of detection (LOD) and LOQ values were determined experimentally as, respectively, three and ten times the standard deviation of the intercept for the calibration curve (standard addition) divided by the slope. In all cases, the LOD and LOQ values were below 8 µg/g and 26 µg/g, respectively. As it has been previously commented, they have been published few works where intact GLSs were determined in broccoli leaves, and only in one of those researches they have been calculated those limits (Ares et al., 2014a), which were slightly lower but with a different MS system (ion trap). However, the LODs and LOQs values obtained with the proposed method are good enough to determine intact-GLSs in broccoli leaves. Standard calibration curves could be used to quantify the GLSs in broccoli leaves, since the matrix and sample preparation did not significantly affect the analytes signal (see subsection 3.3). This finding was corroborated by the fact that the slopes of the standard and matrix-matched calibration curves overlapped at the confidence intervals (data not shown). Calibration curves (n=6) were constructed by plotting the signal ratio on the *y*-axis (analyte peak areas/IS area) against the analyte concentration on the *x*-axis. The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied, and the coefficient of the determination values (R^2) were above 0.99 in all cases. Moreover, the lack of bias was confirmed by a *t*-test, and a study of the distribution of residuals. Intra-day precision and trueness experiments were performed concurrently by repeated sample analysis using broccoli leaf samples spiked with three concentrations of GLSs (**QC** levels) on the same day of (n=6). Inter-day precision and trueness were assessed by an analysis of broccoli leaf samples spiked with three concentrations of the compounds studied (**QC** levels) over three consecutive days (n=6). In each run a calibration curve was established and replicates (n=6) of each spiked concentration level were analyzed. Precision was expressed as the percentage of relative standard deviation (% RSD) at the three concentrations (**QC** levels) for each analyte, and the resulting %RSD values were at all times lower than or equal to 9%. Trueness was calculated by means of relative error (% RE), and those values ranged from 3% to 7% for the intra-day readings, and from 4% to 8% for the inter-day values. These results indicate that the present method is both precise and has only minor systematic errors.

3.5. Application of the method

The validated method was applied to determine the intact-GLSs content in 15 broccoli leaves samples from three commercial cultivars *(Parthenon, Nubia* and *Naxos)*. The GLS content of each broccoli variety

is summarized in Table 2. As can be seen, nine intact-GLSs (ALY, GRA, GIB, GER, GBC, NAS, 4-ME, 4-OH and NEO) were detected and in all the varieties, although 4-OH was only quantified in *Naxos* cultivar; PRO and GBN were only detected in *Naxos* and *Parthenon* cultivars respectively; meanwhile GTL, SIN, GNL, GNA, and were not detected in any of the broccoli varieties investigated. Moreover, the absence of GTL and SIN is in good agreement with most of the existing bibliography (Ares et al, 2014a; Domínguez-Perles et al., 2010; López-Berenguer et al., 2008), and with the choice of both compounds as external and internal standards, respectively. In addition, the highest total content of GLSs was found in broccoli leaves samples from *Parthenon* cultivar, being the *Naxos* cultivar the poorest in GLS content. It should be remarked that GRA was the GLS found in higher amounts in all the broccoli varieties assayed. This finding is especially relevant, as this compound is the precursor of sulforaphane, which is a bioactive isothiocyanate with several health promoting effects (Agrawal, Winnik, Buckley, Mi, Chung, & Cook, 2006). Finally, it should be mentioned that the differences observed in GLS content depending on the cultivar are in agreement with the scientific data available for broccoli leaves (Ares et al., 2014a; Domínguez-Perles et al., 2010).

4. Conclusions

It has been developed a new and fast UPLC-qTOF-MS method to separate and quantify fifteen intact-GLSs in broccoli leaves. The extraction of these compounds from broccoli leaves was optimized by means of an experimental CCO design. The proposed extraction method based on PLE has proven to be efficient, fast, and environmental friendly. To our knowledge, this is the most complete study describing intact-GLSs content in broccoli leaves and a first report on their PLE extraction and UPLC-qTOF-MS determination in this matrix. Moreover, the proposed method was fully validated and the data demonstrated that the proposed method is consistent and reliable and that it could be employed standard calibration curves to quantify intact-GLSs in broccoli leaves as matrix effect was not significant. Finally, broccoli leaves from three different cultivars were analyzed, and nine of the studied GLSs were identified in those samples. The study provides clear evidence on usefulness of PLE as an alternative to extract intact-GLS from broccoli leaves, and that UPLC-qTOF-MS allowed a rapid, selective and sensitive determination of intact-GLSs in this matrix.

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Figure 1.- Representative UPLC-qTOF-MS (base peak intensity-BPI, negative mode) chromatogram obtained from a mixture of a broccoli leaf extract with rapeseed certified material and individual GLSs standards.

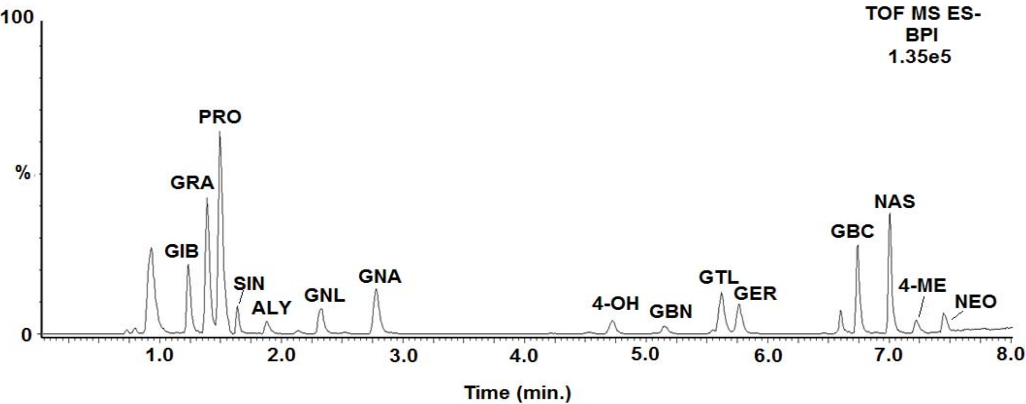

Table 1.- Data obtained for broccoli leaf samples treated accordingly to the procedures described in subsections 2.2, 3.1 and 3.3 (n=6).

	Evaluation of the sample preparation			Evaluation of the matrix effect		
Quality control (QC) sample	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
GIB	94 ± 2	92 ± 3	92 ± 5	95 ± 3	99 ± 4	97 ± 4
GRA	96 ± 5	93 ± 4	92 ± 6	96 ± 3	97 ± 3	99 ± 5
GNA	88 ± 4	89 ± 3	87 ± 6	101 ± 5	99 ± 4	104 ± 6
GTL	91 ± 3	92 ± 4	89 ± 3	99 ± 4	99 ± 6	98 ± 5
GER	89 ± 5	87 ± 6	90 ± 3	98 ± 6	102 ± 5	103 ± 6
GBC	86 ± 5	87 ± 5	85 ± 4	97 ± 7	99 ± 6	98 ± 5
NAS	88 ± 4	89 ± 4	91 ± 4	96 ± 4	97 ± 3	99 ± 5

low **QC**-20 µg/g (GIB, GRA, GNA, GTL, GER), 30 µg/g (GBC, NAS); medium **QC**-250 µg/g; high **QC**-2500 µg/g.

Table 2.- Concentration (µg/g, dry weight) of glucosinolates in broccoli leaves (five samples per cultivar).

Cultivars	***Parthenon***	***Nubia***	***Naxos***
Compound*	**Min.-Max. values**	**Min.-Max. values**	**Min.-Max. values**
GIB	130-173	110-130	n.d.-11
GRA	995-1136	885-1012	980-1090
PRO	ND	ND	ND-<LOQ
ALY	ND-18	ND-16	ND-23
GNL	ND	ND	ND
GNA	ND	ND	ND
4-OH	ND-<LOQ	ND-<LOQ	ND-19
GBN	ND-<LOQ	ND	ND
GER	ND-15	ND-17	ND-<LOQ
GBC	85-102	77-106	26-38
NAS	88-119	51-68	ND-30
4-ME	15-35	14-33	ND-18
NEO	19-29	20-31	ND-17

ND (not detected); *All other analytes were ND for all samples;

V.8. PAPER VIII. Development and validation of a liquid chromatography-tandem mass spectrometry method to determine intact glucosinolates in bee pollen

DEVELOPMENT AND VALIDATION OF A LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY METHOD TO DETERMINE INTACT GLUCOSINOLATES IN BEE POLLEN

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Abstract

A new method was developed to determine twelve intact-glucosinolates (GLSs) (glucoiberin, GIB; glucoraphanin, GRA; glucoerucin GER; gluconapin, GNA; glucotropaeolin, GTL; glucobrassicin, GBC; gluconasturtiin, NAS; glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4OH; 4-methoxyglucobrassicin, 4ME; neoglucobrassicin, NEO; sinigrin, SIN) in bee pollen, by means of liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). An efficient extraction procedure was proposed (average analyte recoveries were between 85% and 96%); this involved a solid-liquid extraction (SLE) with heated water, followed by a solid phase extraction (SPE) with a weak anion sorbent. Chromatography was performed on a Gemini® C_{18} analytical column with a mobile phase of formic acid in water (0.5%, v/v) and formic acid in acetonitrile (0.5%, v/v), at a flow rate of 1 mL/min. The method was fully validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, carry-over effect, reinjection reproducibility, precision and accuracy. The LOD and LOQ values were below 5 µg/kg and 16 µg/kg, respectively. The proposed method was applied to analyze intact-GLSs in bee pollen. Nine of the GLSs studied were identified in some the samples analyzed, and significant differences in GLS content were observed among the samples.

Keywords: Bee pollen; Glucosinolates; LC-MS/MS; Solid-liquid extraction; Solid phase extraction.

1. Introduction

Glucosinolates (GLSs) are β-thioglucoside N-hydroxysulfates, with a side chain (R) derived from amino acids and a sulfur-linked β-D-glucopyranose, and certain compounds appear to be protective against chemical carcinogens [1]. Following tissue damage, GLSs undergo enzymatic hydrolysis by myrosinase to glucose and a variety of degradation products (isothiocyanates, nitriles, thiocyanates, epithiocyanates, epithionitriles and oxazolidines) [2,3], which are responsible for flavour and most of the biological activities of GLSs [4]. Although most studies have focused their attention on determining GLSs in Brassica vegetables, due to the fact that these plants are rich in these bioactive compounds [5], it must be said that GLSs have been investigated in honey as a result of their potential use as botanical biomarkers [6]. In this study, the authors reported the presence of certain GLSs in unripe and mature honey with a methodology that involved a dilution of the sample with acidified water and a further solid-phase extraction (SPE) with C_{18} of the analytes prior to their analysis by liquid chromatography (LC) coupled to several detectors (diode array, DAD; tandem mass spectrometry, MS/MS). Although this method was not specifically developed for GLS analysis and was applied only for identification purposes. Therefore, if we take these findings into consideration, it is reasonable to suppose that such compounds could be found in bee pollen, and at the same time it could be also thought that GLSs might enter the beehive through the pollen collected by the bees from plants which are rich in them. Moreover, an analysis of GLSs in bee pollen may be of interest for several reasons; firstly, in order to check their potential presence, since, to the best of our knowledge, no research has been published in which GLSs have been analyzed in bee pollen; secondly, because if they are present in a product that would be consumed by humans, they would be positively affected by their beneficial health properties; also, because GLSs could be used as a parameter to that helps to identify the origin of pollen. These compounds have only once been studied in pollen [7], but the samples were obtained from plants of rapeseed and Indian mustard, not from a beehive. In this study, GLSs were determined by liquid chromatography and UV detection following overnight solid-liquid extraction (SLE), a partition of the resulting extracts, a precipitation of the protein material and an enzymatic desulfation; meanwhile, gas chromatography with mass spectrometry (MS) detection was used to identify and confirm the presence of certain GLSs. It should be highlighted that different methods for analyzing GLSs depending on the presence (intact or non-intact desulfo-derivatives) of a sulfate group have been reported in the literature. Although the desulfation stage decreases the polarity of GLSs and improves their chromatographic resolution in reversed-phase liquid chromatography (RPLC), it is a time-consuming procedure, and faster methods have been proposed for the direct analysis of intact-GLSs [4,8]. The extraction methods employed with intact-GLSs mainly involved the use of heated water or mixtures of methanol and water [8-14], although in some research studies an SPE with C_{18} and anion exchange cartridges [15], anion exchange SPE [16], or a pressurized liquid extraction [4,17], were also conducted to isolate the compounds. All these treatments include heating, which is necessary to deactivate the myrosinase enzyme and obtain a better extraction of intact-GLSs. In addition, an analysis of intact-

GLSs was usually performed by RPLC with ultraviolet (UV) or diode array (DAD) [6,7,9,11,15] and mass spectrometry (MS) [8-14,16-19] detectors, while C_{18} [6,8-12,15,17-19] and C_{30} [16] analytical columns were used in all cases. Among these, MS and in particular tandem mass spectrometry (MS/MS) has emerged as one of the preferred detection techniques, offering enough sensitivity and an unambiguous identification and quantification of GLSs [17]. The aim of this study was to develop a new, robust method, of maximum possible sensitivity, selectivity and rapidness, to determine intact-GLSs in bee pollen. To the best of our knowledge, this is the first study in which extraction, separation and detection procedures for intact-GLSs have been developed and optimized in this matrix. The proposed method was validated and applied in an analysis of intact-GLSs in bee pollen to determine potential differences in their content and to corroborate the argument that these compounds may be found in such a matrix.

2. Materials and methods

2.1. Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (NAS) and sinigrin (SIN), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). GER was to be used as an external standard to quantify some GLSs from bee pollen where individual standards were not available (glucoalyssin, ALY; 4-hydroxyglucobrassicin, 4OH; neoglucobrassicin, NEO; 4-methoxyglucobrassicin, 4ME), as this has not been detected in any bee pollen sample. Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), which was composed, among other compounds, of GNA, ALY, 4OH, NEO, NAS, GBC and SIN, was employed to optimize separation, whilst potentially it could be used to identify and confirm the presence of ALY, 4OH and NEO in the samples, due to the absence of individual standards for these GLSs. One GLS (4ME) is not present in the rapeseed certified material, and its individual standard was not available; however, it could be tentatively assigned by using the LC-MS/MS data summarized in our previous research [9] and the available bibliographic data [20,21]. In addition, broccoli leaf extracts [9] were used to identify, confirm the presence of 4ME, and optimize separation. LC grade methanol and acetonitrile were both supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst ammonium hydroxide, formic and acetic acids were purchased from Sigma Aldrich Chemie Gbmh. Syringe filters (17 mm, Nylon 0.45 μm) were obtained from Nalgene (Rochester, NY, USA), and ultrapure water was obtained by means of Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). An Eppendorf Centrifuge 5810R (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), an R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), Strata® C_{18}-E (3 mL with 500 mg of sorbent) SPE cartridges from Phenomenex (Torrance, CA, USA), Bond Elut NH_2 (3 mL with 500 mg of sorbent) SPE cartridges from Agilent Technologies (Palo Alto, CA, USA), a 10-

port Visiprep vacuum manifold (Supelco, St. Louis, MO, USA), and a Vibromatic mechanical shaker (purchased from J.P. Selecta S.A., Barcelona, Spain) were used for the extractions.

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each glucosinolate in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Bee pollen samples (1 g) were spiked before (**BF** samples) or after (**AF** samples) sample treatment, as described in subsection 2.3, with different amounts of the available glucosinolate standards in order to prepare the matrix-matched standards for validation (quality control (**QC**) samples and calibration curves), matrix effect, and sample treatment studies. All standard solutions were stored in glass containers and kept in the dark at +4ºC, where stability was determined for more than two weeks (data not shown).

2.3. Sample procurement and treatment

2.3.1.Samples

Commercial bee pollen samples (n=6, **C1-C6**) were purchased in local markets (Valladolid, Spain) and these were from different Spanish regions. Corbicular bee pollen samples (n=8, **O1-O8**) from organic apiaries were supplied by the Centro Apícola Regional (CAR) of Marchamalo (Castilla la Mancha, Spain). The samples from the CAR underwent preliminary analysis by LC-MS/MS in order to verify the presence of intact-GLSs. Once it was confirmed that some of them contained no residues from these compounds, subsamples of them were used as blank samples to prepare matrix-matched samples for validation (**QC** and calibration curves), matrix effect, and sample treatment studies. Finally, the pollen samples collected were mixed and dried at 45ºC in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in the dark at -20ºC until analysis.

2.3.2. Sample treatment

Briefly, a pollen sample and heated water (70ºC) were transferred to a centrifuge tube. The mixture was shaken for 10 min at 960 oscillations per minute in a Vibromatic and then centrifuged for 10 min at +4°C and 10414 g. The supernatant was collected and loaded onto a weak anion exchange SPE cartridge which was previously conditioned at about 1 mL/min by means of a suction system. The SPE cartridge was then washed; the rinse was discarded and, after 5 min of drying time, the analytes were eluted. The resulting solution was evaporated to dryness at 40ºC in a rotary evaporator; the dry residue was reconstituted, filtered through a nylon 0.45 μm filter, and injected into the LC-MS/MS system.

2.4. LC-MS/MS system

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD Trap XCT instrument, which was used in conjunction with electrospray ionization (ESI) in negative

ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data were analyzed using Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. The analytical column was a Gemini® 3μm C_{18} 110Å (150 x 4.60 mm) protected by a Gemini® C_{18} security guard cartridge (4 mm x 3.0 mm), both from Phenomenex (Torrance, CA, USA). Subsequent to the optimization study, the mobile phase selected was a mixture of (A) formic acid in water (0.5%, v/v) and (B) formic acid in acetonitrile (0.5%, v/v) applied at a flow rate of 1 mL/min in a gradient mode. The injection volume and column temperature were set at 20 μL and 30ºC, respectively. The ESI interface was operated in negative mode after studies had been performed with standard solutions of the more relevant MS parameters. Compounds were identified by comparing the MS and MS/MS data (retention time, spectra, ions and transitions) with those obtained from pure standard solutions when commercially available, or with data obtained from analyzing the certified rapeseed material and broccoli leaf extracts. MS/MS was used to quantify and confirm the presence of intact-GLSs. The ESI-MS/MS analyses were performed by means of multiple reaction monitoring (MRM) in ultra scan mass range mode, enabling smart fragmentation and scanning from m/z 70 to 500. To protect the ion source from matrix constituents, the MSD Trap system automatically controlled the run at different times: at $t_{0\text{-}5min}$ to waste, $t_{5\text{-}27min}$ to mass and $t_{27\text{-}30min}$ to waste. During MS integration a Gauss function was used as a smoothing algorithm.

2.5. Method validation

Validation was carried out in accordance with different international guidelines [22-24] determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, carry-over effect, reinjection reproducibility, precision and accuracy. As was previously stated in subsection 2.3.1, blank (GLSs-free) bee pollen samples were employed for the validation studies. Validation was not conducted for ALY, 4OH, NEO and 4ME due to the lack of individual standards. To determine the selectivity of the proposed method, a set of unspiked bee pollen samples (n=6) were injected onto the chromatographic system and the results were compared with those obtained for spiked bee pollen samples. The LODs and LOQs were experimentally determined by injecting a number of blank bee pollen samples (n=6), in which it had been previously confirmed that there were no intact-GLSs residues, and measuring the magnitude of background analytical response at the elution time of each compound. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively. Standard calibration curves were used to quantify the intact-GLSs in bee pollen, since the matrix and sample treatment did not significantly affect the analyte signal. The standard solutions used to construct the calibration curve were a preparation of various aqueous solutions containing variable amounts of eight commercial GLSs over a concentration range of LOQ to 1000 μg/kg (calibration levels of LOQ, 20, 50, 100, 200, 500, 1000 μg/kg). Calibration curves (n=6) were constructed by plotting the signal on the *y*-axis (analyte peak areas)

against the analyte concentration on the *x*-axis. The carry-over effect was assessed by the injection of spiked samples or standards with a high concentration of intact-GLSs (high **QC** or 1000 µg/L), and an evaluation of the responses at the retention time of each GLS. Reinjection reproducibility was evaluated by re-injection of previously acceptable standards (20 µg/L and 1000 µg/L) and **QCs** (low and high) three times per day; these had been stored for 1 week at +4ºC. Intra-day precision and accuracy experiments were performed concurrently by repeated sample analysis using bee pollen samples spiked with three concentrations of GLSs (low, medium and high **QC** levels) on the same day (n=6). Inter-day precision and accuracy were assessed by an analysis of bee pollen spiked with three concentrations of the compounds studied (low, medium and high **QC** levels) over three consecutive days (n=6). In each run a calibration curve was established and replicates (n=6) of each spiked concentration level were analyzed. Precision was expressed as the percentage of relative standard deviation (% RSD) at the three concentrations for each analyte. Accuracy was calculated by means of relative error (% RE).

3. Results and discussion

3.1. Optimization of the extraction from bee pollen

As seen previously [7], GLSs were only once determined in a similar matrix (rapeseed and Indian mustard pollen) as desulfo derivatives, following a long extraction procedure which involved an overnight SLE with a methanol and chloroform mixture, a partition of the extracts with a hexane and water mixture and a further precipitation of the proteins. It should also be said that the use of chlorinated compounds (chloroform) are to be avoided in order to conduct extraction methods which are as environmental friendly as possible; moreover, the recovery percentages were not provided in the above-mentioned study. However, it was decided that an SLE should be tested as a sample treatment due to its simplicity, and that heated water would be used to extract the intact-GLSs; this was because of the good results it had provided in previous research, where intact-GLSs were determined in broccoli leaves [9]. In this study, several solvents were tested to extract intact-GLS (water, methanol, and a mixture (70:30, v/v) of methanol and water), the highest amount of GLSs being obtained when heated water was employed. It should be also mentioned that the temperature of the heated water was selected (70ºC), as it was commonly used to deactivate the myrosinase enzyme [14], and it did not affect the thermal stability of the compounds [25]. Different agitation sources were checked (Vibromatic, magnetic agitation or sonication), and the best results in terms of recoveries (data not shown) were obtained with the Vibromatic at maximum speed (960 oscillations per minute). In addition, the volume of heated water and the extraction time were also studied in order to ascertain their influence on extraction recoveries. In addition, a centrifugation stage (5 min at 10°C and 10414 g) was included in order to facilitate separation between the phases. Following this, the supernatant was collected, transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 40ºC; the dry residue was reconstituted, and filtered through a nylon 0.45 µm filter [9]. It should also be pointed out that no matrix interferences co-eluted

with the Intact-GLSs when bee pollen samples treated with the proposed extraction procedure were injected, and the recovery percentages were acceptable for all the compounds (>80 %). However, several matrix components eluted at both lower and higher retention times, which implied the need for longer run times to elute these compounds and avoid problems with the column. Therefore, the decision was taken to introduce an SPE after performing the SLE so as to remove as many matrix compounds as possible yet without affecting the analytes. As has been previously reported, C_{18} [6], weak anion exchange [16] or a combination of C_{18} and weak anion exchange [15] SPE cartridges were used to analyze GLSs in other matrices. It was decided, then, that the suitability of certain sorbents for SPE should be verified. As a result of several experiments, it was observed that weak anion exchange SPE sorbents provided the highest recovery rates and sufficiently clean chromatograms; meanwhile, lower rates of recovery and slightly cleaner chromatograms, or similar recoveries and less clean chromatograms, were obtained with the combination of the two different sorbents and C_{18}, respectively. We therefore chose weak anion exchange SPE sorbents to optimize extraction of intact-GLSs from the bee pollen. As bee pollen contains several substances such as proteins, sugars and lipids, direct elution of the cartridges caused matrix interference and unclean chromatograms were the result. To overcome this problem, a washing phase was introduced. Optimal drying times for the cartridges were also determined, and as no differences were observed between times of 5-20 min, a 5 min drying period was chosen to avoid delays in the extraction procedure. In addition, a solution of concentrated ammonium hydroxide in methanol had been previously used to elute GLSs from these SPE sorbents [15], we decided to test different amounts of this solution in order to obtain maximal elution. Next, the solution obtained was transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 40ºC. Reconstitution was deemed appropriate for enhanced effectiveness when extracting. In order to assess the efficiency of the proposed sample treatment, a comparison was made of the results obtained for blank bee pollen spiked at three different GLS concentrations (low, medium and high **QC** levels), either prior to (**BF** samples) or following (**AF** samples) sample treatment. The recovery values (Table 1) ranged from 85%-96% in all cases, which indicated that the sample treatment procedure selected was appropriate and efficient. The results were sound not only in terms of extraction efficiency, but also regarding the clean nature of the chromatograms. It was not possible to make a comparison with other sample treatments as, in the only study in which GLSs had been determined in rapeseed and Indian mustard pollen [7], the authors did not provide any data on recovery/extraction efficiency. However, it should be highlighted that our proposal is much faster than the overnight extraction procedure employed in the latter study.

3.2. LC optimization

As mentioned previously, GLS determination has generally been performed in RPLC by means of C_{18} based stationary phase columns; such was the case of the only study in which GLS was determined in rapeseed and Indian mustard pollen [7]. We recently published a paper concerning the analysis of intact-GLSs in broccoli [9], where we optimized the chromatographic conditions by selecting a Gemini® 3µm C_{18} 110Å (150 x 4.60 mm) together with formic acid in water (0.5%, v/v) and formic acid in acetonitrile

(0.5%, v/v) as, respectively, the analytical column and mobile phase components. The chromatographic separation in this study was optimized for simultaneous DAD and MS/MS detection, since the high concentration of intact-GLSs detected in most cases meant they could be quantified by DAD; meanwhile, MS/MS was employed to confirm the presence of the compounds and to quantify GLSs found in low concentrations. However, as in the present study it would be only employed MS/MS due to the low amounts of intact-GLSs expected, we decided to optimize the chromatographic conditions proposed in our previous study in order to reduce analysis time as much as possible whilst maintaining the baseline separation of the compounds. Individual standards, spiked bee pollen samples, rapeseed certified material, and broccoli leaf extracts were used to optimize separation of the twelve intact-GLSs. Tests were also carried out to study the influence of column temperature (between 20ºC and 45ºC at 5ºC intervals), which produced different retention times and peak symmetries. As expected, the former decreased slightly and the S/N ratio was also enhanced as the temperature increased; however, a significant loss of symmetry, which did not compensate for the decrease in analysis time or improvement in the S/N ratio, was observed at temperatures over 30ºC. As a result, the latter was the working temperature chosen. The possibility of enhancing the method's sensitivity by injecting greater sample amounts was also considered; this meant testing the injection of blank bee pollen samples spiked with intact-GLSs in amounts ranging from 5 to 30 µL. The results showed an increase in S/N when up to 20 µL was injected, above which this ratio did not significantly improve and the chromatographic peaks began to appear somewhat deformed. It was, therefore, decided that 20 µL should be the corresponding injection volume. With the chromatography conditions described above, the intact-GLSs were separated in under 26.5 min, and the overall run time was 30 min (see Figure 1). These compounds have been widely studied in vegetables, but very little research has been carried out on bee products. It should be specified that the analysis time was much higher (>50 min) in the only method devoted to analyzing twelve GLSs in pollen from plants of rapeseed and Indian mustard [7]; meanwhile, six GLS were detected in honey [6] in less than 25 min, but in this study the method was not exclusively conducted for GLS analysis and certain matrix compounds coeluted with the analytes. Finally, the overall runtime of our previous proposal [9] was reduced by 5 min as a result of a slight modification of the mobile phase conditions at the end of the chromatographic run. It was not possible to further reduce analysis time in order to maintain the separation between two pairs of compounds (GER and GBC; 4ME and NAS). Consequently, the advantages of the proposed chromatographic conditions have been demonstrated, as it was possible to separate a large number of intact-GLSs in a shorter analysis time than that required in previous research into related matrices.

3.3. MS optimization

Intact-GLSs were previously analyzed in negative ESI mode [8,9,12-14,18,19], the best results being obtained in terms of peak area and peak height. To establish optimal ESI-MS/MS conditions, several experiments (flow injection analysis) were conducted in order to choose these optimum ESI-MS/MS

(negative mode) parameters and achieve maximum sensitivity by the infusion mode (5 µL/min) of standard and matrix-matched solutions (1000 µg/L) of intact-GLSs, the individual standards of which were available. In ESI-MS, the compounds exhibited intense [M-H] in full-scan spectra. These ions were selected as precursors to obtain product ions for MS/MS analyses in MRM mode. The MRM transition that provided the highest signal was used for quantification; meanwhile, a second MRM transition was used for confirmation. Such ions/transitions have been commonly used in the existing literature devoted to analyzing GLSs by MS/MS [8,9,12,13,16,18,19]. To ascertain how the matrix influenced the detection of GLSs, the peak areas of the GLSs in standard solutions were compared with those obtained in **AF** samples (bee pollen samples spiked after sample treatment). The response of all compounds in the three concentrations assayed was between 87% and 104% in all cases, as shown in Table 1. Moreover, if a comparison is made between GLS peaks in standard and matrix-matched solutions at the same concentration levels, a slight variation in analyte signals can be observed. Thus, it was concluded that the matrix did not significantly affect ESI ionization of the analytes.

3.4. Validation of the method

To assess the selectivity of the method, extracts from blank bee pollen samples together with samples spiked with GLSs were injected (see Figure 1). As can be seen, no chromatographic interference was observed at GLS retention time in any of the blank samples analyzed. In order to identify GLS peaks in bee pollen samples (spiked and with endogenous GLS content), a comparison was made of the mass spectra of GLS peaks in standard solutions and bee pollen samples with endogenous or spiked GLS content; this was conducted at comparable concentrations and was measured under the same conditions. Both mass spectra were quite similar (data not shown), although some minor differences in ion intensity were observed and certain low intensity ions appeared only in bee pollen spectra. Percentages relating to the intensity of quantification and confirmation transitions for GLS peaks in standard solutions and bee pollen samples were also compared. In all cases, the tolerance rates for relative transition intensities were below 10% (data not shown), which is lower than the maximum rates permitted when mass spectrometric techniques are used [23]. Therefore, it can be concluded that the method was selective for determining GLSs in bee pollen. LODs and LOQs were determined experimentally as indicated in subsection 2.5. It can be observed that the LOD and LOQ values were quite similar in all cases, ranging from 1 to 5 µg/kg (LOD) and 3 to 16 µg/kg (LOQ). To reiterate, no LOD or LOQ values were reported in the only publication analyzing GLSs in a similar matrix (rapeseed and Indian mustard pollen [7]), so it was not possible to make a proper comparison. As mentioned in subsection 2.5, standard calibration curves could be employed as no significant matrix effect was observed (see Table 1); the slopes of the standard and matrix-matched calibration curves overlapped at the confidence intervals (data not shown). The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied, while the coefficient of the determination values (R^2) was above 0.99 in all cases. The lack of bias was confirmed by a *t* test and a study of the distribution of residuals. It should also be pointed out that no peaks were observed at the retention

times for the analytes when solvent or GLS-free bee pollen samples were injected subsequent to running samples with high concentrations of the compounds in question. This observation indicates a negligible carry-over effect when the proposed method is used to analyze intact-GLSs in bee pollen. An evaluation of reinjection reproducibility revealed %RSD values < 3% in all cases (data not shown), demonstrating that the samples could be safely reanalyzed within one week. Finally, intra- and inter-day precision (% RSD values) were at all times lower than or equal to 8%. Accuracy (% RE values) ranged from 3% to 8% for the intra-day readings, and from 4% to 9% for the inter-day values. These results indicate that the present method is both precise and accurate, as a result of which, although it is recommended in most validation guidelines, an internal standard was not necessary in this study.

3.5. Application of the method

The validated method was applied to determine intact-GLS content in 13 bee pollen samples obtained from organic apiaries (**O1-O8**) and markets (**C1-C6**). All the samples were analyzed in triplicate. The GLS content of each sample is summarized in Table 2. As can be seen, nine intact-GLSs (GRA, GIB, GNA, SIN, GBC, NAS, GTL, NEO and 4ME) were found in certain samples analyzed over a wide concentration range (LOQ-2226 µg/kg); meanwhile, ALY, 4OH, and GER were not detected in any of the samples. Moreover, no residues of the intact-GLSs under study were detected in two of the samples (**O1** and **O6**), which were used as blanks. SIN and GNA were found at high concentrations (> 600 µg/kg) in three and one of the analyzed bee pollen samples, respectively; meanwhile GRA was the intact-GLS which was detected most in the samples (10 of 13). This finding is especially relevant if it considered that this compound is the precursor of sulforaphane, which is a bioactive isothiocyanate with a potential role not only in the prevention of cancer, but also in that of chronic and degenerative diseases such as diabetes, atherosclerosis and cardiovascular disorders [26,27]. In addition, certain GLSs were previously detected in rapeseed and Indian mustard pollen samples (11 and 6, respectively) [7], but in this case the authors provided the normalized GLS composition of these samples rather than the concentration of each GLS content. Finally, it should be added that these are quite interesting results, as intact-GLSs have never been reported in bee pollen samples. Moreover, qualitative and quantitative comparisons indicated that the samples contained several intact-GLSs that could provide a basis for bee pollen selection or for determining origin.

4. Conclusions

A new analytical method has been developed and optimized to simultaneously identify and quantify twelve intact-GLSs in bee pollen. The proposed extraction method based on an SLE with heated water and an SPE procedure with weak anion sorbents has proven to be efficient and suitable, as the results were good not only in terms of extraction efficiency, but also regarding the clean nature of the chromatograms. This LC-ESI-MS/MS method was fully validated and the data demonstrated that it is consistent and reliable, with a wide linear range of applicability, and that standard calibration curves could be employed to quantify intact-GLSs in bee pollen as a matrix effect was not significant. An

analysis of different bee pollen samples proved the utility of this method. Nine of the GLSs examined were identified in certain samples, and significant differences in GLS content were observed among the samples analyzed. GRA was the most commonly detected compound and SIN was found at the highest concentrations. Our observation of intact-GLSs in bee pollen samples is an interesting finding hitherto unreported.

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Figure 1.- Representative LC-MS (extracted ion chromatogram-EIC, negative mode) obtained from: (**A**) a bee pollen sample (**O7**), which contained endogenous 4ME among other GLSs, spiked with the eight

individual GLSs standards and with rapeseed certified material; (**B**) a blank (GLSs-free) bee pollen sample.

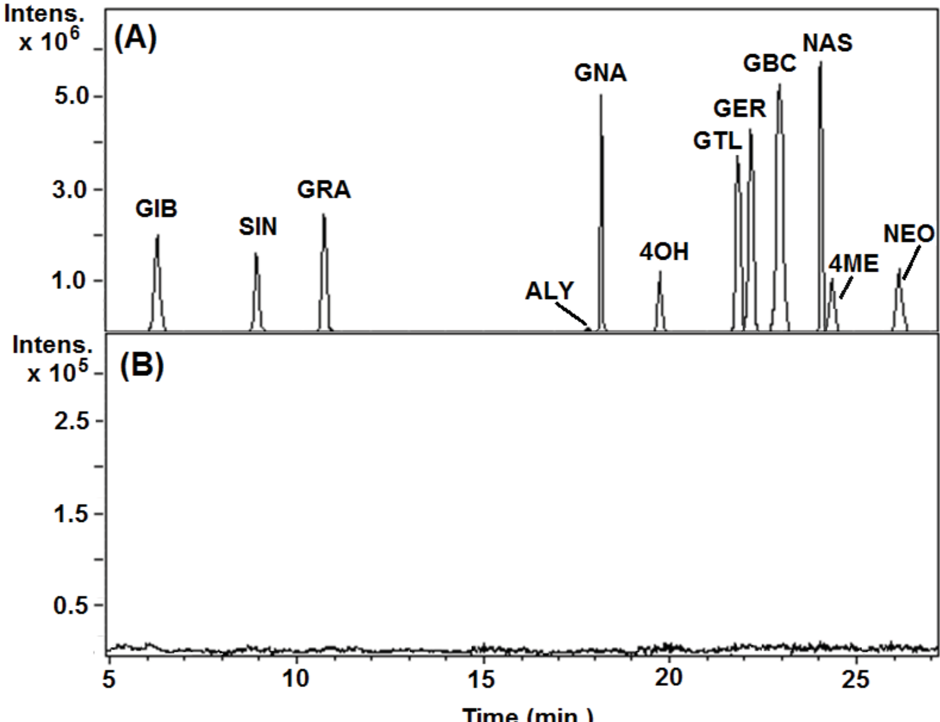

Table 1.- Recovery data obtained for blank bee pollen samples treated accordingly to the procedures described in subsections 2.3, 3.1 and 3.3 (n=6).

	Evaluation of the sample treatment			Evaluation of the matrix effect		
Quality control (QC) sample	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
GIB	86 ± 3	87 ± 4	89 ± 4	96 ± 6	102 ± 6	99 ± 5
SIN	94 ± 4	90 ± 4	92 ± 3	98 ± 5	100 ± 6	97 ± 6
GRA	96 ± 3	93 ± 3	91 ± 4	103 ± 6	101 ± 5	100 ± 6
GNA	85 ± 4	88 ± 5	86 ± 5	93 ± 6	90 ± 6	88 ± 7
GTL	90 ± 5	94 ± 3	91 ± 4	98 ± 5	102 ± 7	104 ± 6
GER	87 ± 5	90 ± 4	86 ± 4	95 ± 6	101 ± 6	93 ± 5
GBC	88 ± 4	85 ± 5	86 ± 5	87 ± 6	89 ± 7	93 ± 6
NAS	89 ± 5	91 ± 4	93 ± 4	88 ± 5	93 ± 6	90 ± 6

low **QC**-10 µg/kg (GIB, GRA), 20 µg/kg (SIN, GNA, GTL, GER, GBC, NAS); medium **QC**-200 µg/kg; high **QC**-1000 µg/kg.

Table 2.- Results of the analysis of intact-GLSs in bee pollen samples (means of triplicate analyses; μg/kg, dry weight)[A].

Sample	GIB	SIN	GRA	GNA	GTL	GBC	NAS	4ME[B]	NEO[B]
#C1	<LOD	<LOD	13	31	141	38	48	<LOD	<LOD
#C2	20	43	12	<LOD	<LOD	<LOD	<LOD	21	<LOD
#C3	4	<LOD	117	<LOD	<LOD	96	<LOQ	<LOD	25
#C4	5	<LOD	9	196	70	102	43	<LOD	<LOD
#C5	8	<LOD	25	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD
#C6	305	<LOD	75	1772	344	183	145	<LOD	<LOD
#O1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
#O2	6	980	<LOD	<LOD	17	<LOQ	<LOD	33	<LOD
#O3	<LOQ	2226	7	<LOD	15	14	<LOD	<LOD	<LOD
#O4	5	688	7	<LOQ	<LOQ	<LOD	<LOD	<LOD	<LOQ
#O5	<LOD	113	8	243	14	<LOD	<LOD	<LOD	<LOD
#O6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
#O7	<LOD	<LOD	6	54	<LOQ	<LOD	<LOQ	40	<LOD
#O8	5	19	8	22	<LOD	<LOQ	<LOD	<LOD	28

[A]All other analytes were <LOD for all samples; [B]Quantified with GER calibration curves; **O**: bee pollen samples from organic apiaries, **C**: commercial bee pollen samples.

V.9. PAPER IX. A specific and validated method to quantify intact glucosinolates in honey by liquid chromatography coupled to tandem mass spectrometry

A SPECIFIC AND VALIDATED METHOD TO QUANTIFY INTACT GLUCOSINOLATES IN HONEY BY LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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ABSTRACT

A specific method was developed to quantify twelve intact-glucosinolates (GLSs) in honey, by using liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization (ESI). An efficient extraction procedure was proposed (average analyte recoveries were between 88% and 104%); this involved a dilution of the honey with heated water, followed by a solid phase extraction (SPE) with a weak anion exchange sorbent. Chromatography was performed on a C_{18} analytical column with a mobile phase of formic acid in water (0.5%, v/v) and formic acid in acetonitrile (0.5%, v/v). The method was fully validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, carry-over effect, reinjection reproducibility, precision and accuracy. The LOD and LOQ values were below 3 μg/kg and 8 μg/kg, respectively. The proposed method was applied to analyze intact-GLSs in honey from different botanical origins. Four of the GLSs studied were identified in several of the samples analyzed.

Keywords: Anion exchange sorbent; Glucosinolates; Honey; LC-MS/MS; Solid phase extraction.

INTRODUCTION

Glucosinolates (GLSs) are secondary plant metabolites that are mainly found in vegetables of the genus Brassica, such as cabbage, rape, or broccoli. They are β-thioglucoside N-hydroxysulfates with a side chain (R) which determines the GLS class (aliphatic, aromatic, or indolic),[1,2] derived from amino acids and a sulfur-linked β-D-glucopyranose moiety. Interest in GLSs has been mainly focused on several observations such as:[3-5] **i**) these compounds have antinutritional effects in cattle; **ii**) they are involved in plant defense mechanisms against depredation; **iii**) some of these compounds appear to be protective against chemical carcinogens; **iv**) they coexist with myrosinase in plants, especially in Brassica vegetables, and in the presence of water are rapidly hydrolyzed by an enzyme (myrosinase) into their breakdown products (isothiocyanates, thiocyanates, nitriles or indoles); additionally, some of them are well known for their anticancer activity or their fungicidal, bactericidal, nematocidal and allelopathic properties. As can be expected, most studies have focused their attention on determining GLSs in Brassica vegetables, but it must be said that GLSs have been investigated in honey as a result of their potential use as botanical biomarkers.[6] In this study, research was also conducted into the potential of flavonoid glycosides and glucosinolates (GLSs) as botanical biomarkers in Argentinean *Diplotaxis* honeys. The authors reported the presence of certain GLSs in honey (fresh (unripe) and mature), and it was observed that GLS composition varied between fresh (unripe) and mature honey, which could be explained by the degradation of GLSs to their breakdown products during the ripening of the honey in the beehive.[6-8] Consequently, the authors suggested that Brassicaceae honeys could share some chemical markers of the GLS family, and that the presence or absence of these compounds might be used as a bio-indicator of honey freshness. A detailed analysis of GLSs in honey may, then, be of interest for several reasons; for instance, if they are present in a bee product that would be consumed by humans, they would be positively affected by the beneficial health properties of GLSs and related products; also, because GLSs could be used as a botanical biomarker or as a parameter to establish honey freshness. It should be said that GLSs have been scarcely investigated in honey.[6,9] In the first of these studies,[6] the methodology applied involved a dilution of the sample with acidified water and a further solid-phase extraction (SPE), with a C_{18} sorbent of the analytes prior to their analysis by liquid chromatography (LC), coupled to several detectors (diode array, DAD; tandem mass spectrometry, MS/MS); however, the method proposed was not specifically developed for GLS analysis and was employed only for identification purposes. Meanwhile, in the other analysis,[9] GLS and its degradation product were extracted with a mixture of organic solvents; the quantitative examination was by the palladium chloride method, and the analytes were isolated and identified by gas chromatography coupled to mass spectrometry (GC-MS). It should also be mentioned that different methods for analyzing GLSs depending on the presence (intact or non-intact desulfo-derivatives) of a sulfate group have been reported in the literature. Although the desulfation stage decreases the polarity of GLSs and improves their chromatographic resolution in reversed-phase liquid chromatography (RPLC), it is a time-consuming procedure, and faster methods have been proposed

for the direct analysis of intact-GLSs.[10,11] In addition, the extraction methods employed with intact-GLSs in vegetable matrices have mainly involved the use of heated water or mixtures of methanol and water,[4,10,12-15] although some research studies have employed an SPE with C_{18} and anion exchange cartridges,[16,17] or a pressurized liquid extraction.[11] All these treatments include heating, which is necessary to deactivate the myrosinase enzyme and obtain a better extraction of intact-GLSs. Moreover, analysis of intact-GLSs was usually performed by RPLC with ultraviolet or diode array[4,6,13,16] and mass spectrometry (MS)[4,10,12-15,18,19] detectors, while C_{18}[4,6,10,12-14,16,19] and C_{30}[17] based analytical columns were used in all cases. Among these, MS and in particular tandem mass spectrometry (MS/MS) has emerged as one of the preferred detection techniques, offering sufficient sensitivity and an unambiguous identification and quantification of GLSs.[18]

The aim of this study was to develop a specific method, of maximum possible sensitivity and selectivity to quantify intact-GLSs in honey. To the best of our knowledge, this is the first study in which extraction, separation and quantification procedures for intact GLSs have been developed and optimized in this matrix by using LC-MS/MS. Accordingly, the proposed method was validated and applied in an analysis of intact-GLSs in honey samples from four very different botanical origins (heather, multifloral, rosemary and orange blossom) to determine potential differences in the content of intact-GLSs, to corroborate the argument that some of these compounds may be found in such a matrix, and to check the usefulness of the proposed method to perform a specific GLS analysis in this matrix.

MATERIALS AND METHODS

Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (NAS) and sinigrin (SIN), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), which was composed, among other compounds, of glucoalyssin (ALY), 4-hydroxyglucobrassicin (4OH), neoglucobrassicin (NEO), NAS, GBC, GNA, and SIN, was employed to optimize separation, whilst it was potentially capable of identifying and confirming the presence of ALY, 4OH and NEO in the samples, due to the absence of individual standards for these GLSs. One GLS (4-mehtoxyglucobrassicin, 4ME) is not present in the rapeseed certified material, and its individual standard was not available; however, it could be tentatively assigned by using the available bibliographic data.[20,21]Moreover, broccoli leaf extracts were used to identify and confirm the presence of 4ME, and optimize separation. SIN was used as an external standard (ES) to quantify the GLSs from broccoli where individual standards were not available, while GTL was employed as an internal standard (IS), as neither of the compounds was detected in any honey sample. LC grade methanol and acetonitrile were both supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst ammonium

hydroxide, formic and acetic acids were purchased from Sigma Aldrich Chemie Gbmh. Syringe filters (17 mm, Nylon 0.45 μm) were obtained from Nalgene (Rochester, NY, USA), and ultrapure water was obtained by means of Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). The extractions involved use of the following: an Eppendorf Centrifuge 5810R (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), an R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), Strata® C_{18}-E (3 mL with 500 mg of sorbent) SPE cartridges from Phenomenex (Torrance, CA, USA), Bond Elut NH_2 (3 mL with 500 mg of sorbent) SPE cartridges from Agilent Technologies (Palo Alto, CA, USA), a 10-port Visiprep vacuum manifold (Supelco, St. Louis, MO, USA), and a Vibromatic mechanical shaker (purchased from J.P. Selecta S.A., Barcelona, Spain).

Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each glucosinolate in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Honey samples (4 g) were spiked before (**BF** samples) or after (**AF** samples) sample treatment, as described in subsection *Sample treatment*, with different amounts of the available glucosinolate standards in order to prepare the matrix-matched standards for validation (quality control (**QC**) samples and calibration curves), matrix effect, and sample treatment studies. All standard solutions were stored in glass containers and kept in the dark at +4ºC, where stability was determined for more than two weeks (data not shown).

Sample procurement and treatment

Samples

Several honey types were selected according to their different color, composition and botanical origin. Samples (mature honey) from different regions of Spain were kindly donated by the Regional Centre for Research into Beekeeping and Honey Production, at Marchamalo (Guadalajara, Spain). All honey samples underwent a preliminary analysis by LC-ESI-MS/MS in order to check for the presence of intact-GLSs Once the latter was confirmed absent in several samples, subsamples of the corresponding honeys were used as blank specimens to prepare matrix-matched ones for validation (**QC** and calibration curves), matrix effect, and sample treatment studies.

Sample treatment

Briefly, a homogenized honey sample was diluted in heated water, and the resulting solution was loaded onto a weak anion exchange SPE cartridge at about 1 mL/min by means of a suction system. The SPE cartridge was then washed; the rinse was discarded and, after a 5-minute drying time, the analytes were eluted. The resulting solution was evaporated to dryness at 40ºC in a rotary evaporator; the dry residue was reconstituted, filtered through a nylon 0.45 μm filter, and injected into the LC-MS/MS system.

LC-MS/MS system

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD Trap XCT instrument, which was used in conjunction with electrospray ionization (ESI) in negative ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and an MSD Trap Control version 4.2. Data were analyzed by Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. The analytical column was a Gemini® 3µm C_{18} 110Å (150 x 4.60 mm) protected by a Gemini® C_{18} security guard cartridge (4 mm x 3.0 mm), both of which were from Phenomenex (Torrance, CA, USA). Subsequent to the optimization study, the mobile phase selected was a mixture of (A) formic acid in water (0.5%, v/v) and (B) formic acid in acetonitrile (0.5%, v/v) applied at a flow rate of 1 mL/min in a gradient mode. The injection volume and column temperature were set at 20 µL and 30ºC, respectively. The ESI interface was operated in negative mode after studies had been performed with standard solutions of the more relevant MS parameters. To protect the ion source from matrix constituents, the MSD Trap system automatically controlled the run at different times: at $t_{0\text{-}5min}$ to waste, $t_{5\text{-}27min}$ to mass and $t_{27\text{-}30min}$ to waste. During MS integration a Gauss function was used as a smoothing algorithm.

Method validation

Validation was carried out in accordance with different international guidelines[22,23] determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, carry-over effect, reinjection reproducibility, precision and accuracy. It should be noted that basic but efficient chemometric statistical tools from Excel (Microsoft Office 2010, Microsoft Corporation, Redmond, WA, USA), ChemStation (Rev.A.10.02) and Data Analysis for LC/MSD Trap 2.2 software, both from Agilent Technologies, were employed to analyze the data obtained in order to validate the method. As was previously stated in the subsection *Samples*, blank honey samples were employed for the validation studies. Validation was not conducted for ALY, 4OH, NEO and 4ME due to the lack of individual standards; neither has GTL been included in the validation study as it was the IS. To determine the selectivity of the proposed method, a set of blank (GLS-free) honey samples (n=6) were injected onto the chromatographic system and the results were compared with those obtained for spiked honey samples. The LODs and LOQs were experimentally determined by injecting a number of blank honey samples (n=6), in which it had been previously confirmed that there were no intact-GLS residues, and measuring the magnitude of background analytical response at the elution time of each compound. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively. Standard calibration curves from LOQ to 1000 µg/L (calibration levels of LOQ, 25 (GIB and GRA), 50, 100, 200, 500, 1000 µg/L) were used to quantify intact-GLSs in honey samples as no significant matrix effect was observed that could affect the ionization of the analytes (see subsection *MS optimization*). Blank honey was treated accordingly with the proposed procedure and spiked with variable amounts of seven

commercial GLSs over a concentration range of LOQ to 250 µg/kg (calibration levels of LOQ, 6 (GIB and GRA), 12, 25, 50, 125, 250 µg/kg), and GTL (IS) was added in all cases at 25 µg/kg (100 µg/L for standard calibration curves). All calibration curves (n=6) were constructed by plotting the signal on the *y* axis (analyte peak areas) against analyte concentration on the *x* axis. The carry-over effect was assessed by the injection of spiked samples or standards with a high concentration of intact-GLSs (high **QC** or 1000 µg/L), and an evaluation of the responses at the retention time of each GLS. Reinjection reproducibility was evaluated by reinjection of previously acceptable standards (25 µg/L and 1000 µg/L) and **QCs** (low and high) three times per day; these had been stored for 1 week at +4ºC. Intra-day precision and accuracy experiments were performed concurrently by repeated sample analysis using honey samples spiked with three concentrations of GLSs (low, medium and high **QC** levels) on the same day (n=6). Inter-day precision and accuracy were assessed by an analysis of honey samples spiked with three concentrations of the compounds studied (low, medium and high **QC** levels) over three consecutive days (n=6). In each run a calibration curve was established and replicates (n=6) of each spiked concentration level were analyzed. Precision was expressed as the percentage of relative standard deviation (%RSD) at the three concentrations for each analyte. Accuracy was calculated by means of relative error (%RE).

RESULTS AND DISCUSSION

Optimization of the extraction procedure

As seen previously, GLSs have been the object of scant analysis in honey, and in only one case,[6] the compounds were analyzed by LC. In this study, C_{18} SPE cartridges were used to analyze GLSs and flavonoid compounds in unripe and mature honey, but the extraction procedure was not optimized/developed specifically for GLS analysis. Nevertheless, we decided to use SPE due to our experience in analyzing honey,[24] as it is quite useful in terms of removing as many honey compounds as possible without affecting the analytes. It should also be specified that anion exchange [17] or a combination of C_{18} and anion exchange[16] SPE cartridges have been previously used to analyze GLSs in other matrices (vegetables). As a result, the decision was taken to test the suitability of certain SPE sorbents to extract intact-GLSs from the honey. As a result of several preliminary experiments, it was observed that anion exchange SPE sorbents provided the highest recovery rates (data not shown) and sufficiently clean chromatograms; meanwhile, lower rates of recovery and slightly cleaner chromatograms, or similar recoveries and less clean chromatograms, were obtained with the combination of the two different sorbents and C_{18}, respectively. We therefore chose weak anion SPE sorbents to optimize extraction of intact-GLSs. Firstly, the amount of honey and the volume of heated water to dissolve it were selected. Heated water was used as the solvent, as it has provided good results in previous research,[4,24] and as heating is useful not only to facilitate the dissolution of the honey, but also to avoid a potential enzymatic hydrolysis of GLSs. As honey contains sugars and substances such as pigments and phenolic compounds, direct elution of the cartridges resulted in matrix interference, and unclean chromatograms were the result. To overcome this problem, a washing phase was introduced.

Optimal drying times for the cartridges were also determined, and as no differences were observed between times of 5-20 min, a 5-minute drying period was chosen to avoid delays in the extraction procedure. In addition, as a solution of concentrated ammonium hydroxide in methanol had been previously used to elute GLSs from these SPE sorbents,[16] we decided to test different amounts of this solution in order to obtain maximal elution. The solution obtained was transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 40ºC. Reconstitution was deemed appropriate for enhanced effectiveness when extracting. In order to assess the efficiency of the proposed sample treatment, a comparison was made of the results obtained for blank (GLS-free) honey spiked at three different GLS concentrations (low, medium and high **QC** levels), either prior to (**BF** samples) or following (**AF** samples) sample treatment. The recovery values (Table 1) ranged from 88%-104% in all cases, indicating that the sample treatment procedure selected was appropriate and efficient. The results were sound not only in terms of extraction efficiency, but also regarding the clean nature of the chromatograms. It was not possible to make a comparison with other sample treatments as, in the two studies in which GLSs had been determined in honey,[6,9] the authors did not provide any data on recovery/extraction efficiency.

Study of chromatographic conditions

We decided to optimize the chromatographic conditions proposed in our previous study[4] in order to reduce analysis time as much as possible whilst maintaining the baseline separation of the compounds with this different matrix. Individual standards, spiked honey samples, rapeseed certified material, and broccoli leaf extracts were used to optimize separation of the twelve intact-GLSs. Several experiments were conducted by means of different mobile phase compositions, and the best results were obtained with the chromatographic conditions described in the subsection *LC-MS/MS system*. With the chromatography conditions described above, the twelve intact-GLSs were separated in less than 26.5 min, and the overall run time was 30 min (see Figure 1). It was not possible to further reduce analysis time in order to maintain the separation between the two pairs of compounds (GER and GBC; 4ME and NAS). It should also be specified that six GLS were detected in honey by RPLC in under 25 min,[6] but in this study the method was not exclusively conducted for GLS analysis, and subsequently the overall run time was much higher (65 min), the GLSs studied were different, and certain matrix compounds seemed to coelute with some of the analytes. In addition, the analysis time was much shorter than that employed in the GC-MS analysis (70 min),[9] although it should be taken into account that this is not a true comparison, as the separation tecnhique was not the same.

MS optimization

Intact-GLSs were previously analyzed in negative ESI mode, [10,14,15,18,25,26] the best results being obtained in terms of peak area and peak height. To establish the best ESI-MS/MS conditions, several experiments (flow injection analysis) were conducted for optimal ESI-MS/MS (negative mode) parameters and in order to achieve maximum sensitivity by the infusion mode (5 µL/min) of standard and matrix-matched solutions

(1000 μg/L) of intact-GLSs, the individual standards of which were available. In ESI-MS, the compounds exhibited intense $[M-H]^-$ in full-scan spectra. These ions were selected as precursors to obtain product ions for MS/MS analyses in MRM mode. The MRM transition that provided the highest signal was used for quantification; meanwhile, a second MRM transition was used for confirmation. Such ions/transitions have been commonly used in the existing literature devoted to analyzing GLSs by MS/MS.[10,14,15,17] To ascertain how the matrix influenced GLS detection, the peak areas of the GLSs in standard solutions were compared with those obtained in **AF** samples (honey samples spiked after sample treatment). The response of all the compounds in the three concentrations assayed and for the different botanical origins was between 92% and 108% in all cases, as shown in Table 2. Moreover, if a comparison is made between GLS peaks in standard and matrix-matched solutions at the same concentration levels, a slight variation in analyte signals can be observed. Thus, it was concluded that the matrix did not significantly affect ESI ionization of the analytes.

Method Validation

To assess the selectivity of the method, extracts from blank honey samples together with samples spiked with GLSs were injected (see Figure 1). As can be seen, no chromatographic interference was observed at GLS retention time in any of the blank samples analyzed. In order to identify GLS peaks in honey samples (spiked and with endogenous GLS content), a comparison was made of the mass spectra of GLS peaks in standard solutions and honey samples with endogenous or spiked GLS content; this was conducted at comparable concentrations and was measured under the same conditions. Both mass spectra were quite similar (data not shown), although some minor differences in ion intensity were observed and certain low intensity ions appeared only in honey spectra. Therefore, it can be concluded that the method was selective for determining GLSs in honey. LODs and LOQs were determined experimentally as previously indicated. It can be observed that the LOD and LOQ values were quite similar in all cases, ranging from 1 to 3 μg/kg (LOD) and 3 to 8 μg/kg (LOQ). To reiterate, no LOD or LOQ values were previously reported in the publication analyzing GLSs in honey by LC-MS, so it was not possible to make a proper comparison. Standard calibration curves could be employed as no significant matrix effect was observed (see Table 2); the slopes of the standard and matrix-matched calibration curves overlapped at the confidence intervals (data not shown). The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied; the coefficient of the determination values (R^2) was above 0.99 in all cases (data not shown). The lack of bias was confirmed by a *t* test and a study of the distribution of residuals. It should also be pointed out that no peaks were observed at the retention times for the analytes when solvent or GLS-free honey samples were injected subsequent to running samples with high concentrations of the compounds. This observation indicates a negligible carry-over effect when the proposed method is used to analyze intact-GLSs in honey. An evaluation of reinjection reproducibility revealed %RSD values < 3% in all cases (data not shown), demonstrating that the samples could be safely reanalyzed within one week. Finally, intra- and inter-day precision (%RSD values) were at all times lower than or equal to 7% (data not shown). Accuracy (%RE values) ranged from 3% to 7% for the intra-day readings, and from 4% to 8% for the inter-day values

(data not shown). Finally, the validation study demonstrated that the proposed method is consistent, precise, accurate and reliable, with a wide linear range of applicability, and that standard calibration curves could be employed to quantify intact-GLSs in honey since a matrix effect was not significant.

Application of the method

With the proposed method honey samples from different botanical origins (multifloral, rosemary, heather and orange blossom) were analyzed to search for residues of intact-GLSs. Sulforaphane, which is the breakdown product of GRA, has been previously detected in some honey samples.[8] In our study all the samples were examined in triplicate. As can be seen, four aliphatic intact-GLSs (GRA, GIB, GNA, and ALY) were found in certain samples at low concentration levels (5-22 μg/kg, see Figure 2), although in some cases it was possible to detect intact-GLSs but not quantify their presence; meanwhile, SIN, GTL, GBC, 4OH, 4ME, NEO, NAS, and GER were not detected in any of the samples. Moreover, no residues of the intact-GLSs under study were detected in some of the samples, which were used as blanks. These are quite interesting results, as intact-GLSs have never been reported and quantified in honey samples from different botanical origins. In addition, the lowest number of intact-GLS were detected in honeys from heather botanical origin (GRA and GIB); GRA was detected in only four samples at trace levels (<10 μg/kg), which could be related to the appearance of SFN in some samples. As the GLSs concentrations found were low (<22 μg/kg), it is not possible to establish significant differences in relation to GLS content. Furthermore, the presence of intact-GLSs in several honey samples confirmed the results previously published relating to the presence of these compounds. Only aliphatic GLSs were detected, a finding which also tallied with the suggestion made by Truchado et al.,[6] who stated that this GLS class could be more chemically stable than other classes, such as indolic GLSs. Finally, it has been demonstrated that there is a need to develop a specific analytical method to determine intact-GLSs in honey; such a method should, as we have proposed, be very sensitive, as the content observed was lower than 30 μg/kg. The proposed method might also be of interest to other researchers as a new analytical and quantitative tool to be used in further investigative work on these compounds, for instance, as chemical markers for determining the botanical origin of honey, or as a bio-indicator of its freshness, as was previously suggested.[6]

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FIGURE CAPTIONS

Figure 1.- Representative LC-ESI-MS (extracted ion chromatogram-EIC, negative mode) obtained from: (**A**) a multifloral blank (GLSs-free) honey sample, spiked with the eight individual GLSs standards, and a broccoli leaf extract which contained (GIB, GRA, ALY, 4OH, GER, GBC, NAS, 4ME, and NEO)[4]; (**B**) a blank (GLSs-free) multifloral honey sample.

Figure 2.- Representative LC-ESI-MS/MS chromatograms (MRM, negative mode) using the quantification transitions obtained for multifloral honey samples with endogenous GLS content: (**A**) GIB (18 µg/kg); (**B**) GRA (5 µg/kg); (**C**) ALY (< LOQ); (**D**) GNA (12 µg/kg); (**E**) a multifloral honey sample spiked with GTL (internal standard-IS, 25 µg/kg).

Table 1.- Recovery data obtained for spiked honey samples treated accordingly to the described procedure in subsection *Optimization of the extraction procedure* (n=6).

Compound	Rosemary			Orange Blossom			Multifloral			Heather		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
GIB	90 ± 4	94 ± 5	96 ± 3	96 ± 6	99 ± 5	102 ± 4	93 ± 5	90 ± 3	88 ± 5	93 ± 3	101 ± 5	95 ± 4
SIN	92 ± 3	98 ± 3	96 ± 5	92 ± 4	90 ± 4	98 ± 5	94 ± 3	98 ± 5	95 ± 4	101 ± 4	99 ± 4	103 ± 5
GRA	96 ± 4	104 ± 3	103 ± 5	96 ± 5	100 ± 4	103 ± 3	99 ± 4	95 ± 4	96 ± 5	93 ± 4	100 ± 6	96 ± 5
GNA	99 ± 6	100 ± 4	96 ± 5	95 ± 4	97 ± 5	101 ± 4	91 ± 5	96 ± 6	89 ± 4	92 ± 3	98 ± 5	93 ± 3
GER	91 ± 5	88 ± 4	90 ± 4	95 ± 5	101 ± 4	100 ± 6	97 ± 4	98 ± 5	92 ± 4	100 ± 4	104 ± 5	98 ± 4
GBC	100 ± 4	98 ± 3	96 ± 3	90 ± 5	88 ± 4	92 ± 4	95 ± 6	97 ± 5	98 ± 5	95 ± 5	102 ± 4	97 ± 4
NAS	89 ± 4	93 ± 5	95 ± 5	93 ± 4	99 ± 5	98 ± 5	96 ± 5	94 ± 5	98 ± 4	103 ± 4	100 ± 4	96 ± 3

Table 2.-Matrix effect data obtained for spiked honey samples treated accordingly to the procedures described in subsections *MS optimization* (n=6).

Compound	Rosemary			Orange Blossom			Multifloral			Heather		
	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC	Low QC	Medium QC	High QC
GIB	104 ± 4	106 ± 5	102 ± 4	103 ± 3	101 ± 5	97 ± 4	92 ± 5	94 ± 4	95 ± 5	107 ± 4	98 ± 5	103 ± 4
SIN	105 ± 5	101 ± 4	104 ± 4	101 ± 4	105 ± 6	98 ± 5	102 ± 4	106 ± 5	98 ± 6	99 ± 5	100 ± 4	96 ± 5
GRA	104 ± 5	96 ± 6	99 ± 5	98 ± 5	94 ± 4	92 ± 3	107 ± 4	104 ± 4	106 ± 5	102 ± 4	99 ± 6	106 ± 5
GNA	103 ± 4	100 ± 5	104± 6	104 ± 4	100 ± 5	98 ± 4	94 ± 6	97 ± 5	92 ± 5	107 ± 5	99 ± 4	104 ± 4
GER	105 ± 3	104 ± 4	98 ± 3	105 ± 6	98 ± 4	99 ± 5	93 ± 4	96 ± 3	98 ± 4	103 ± 4	100 ± 6	106 ± 5
GBC	100 ± 5	107 ± 5	104 ± 4	100 ± 5	105 ± 4	103 ± 4	105 ± 4	99 ± 6	107 ± 5	105 ± 5	98 ± 4	104 ± 5
NAS	106 ± 4	104 ± 4	100 ± 5	105 ± 4	101 ± 5	102 ± 4	104 ± 3	108 ± 5	102 ± 4	98 ± 3	100 ± 4	105 ± 4

Figure 1

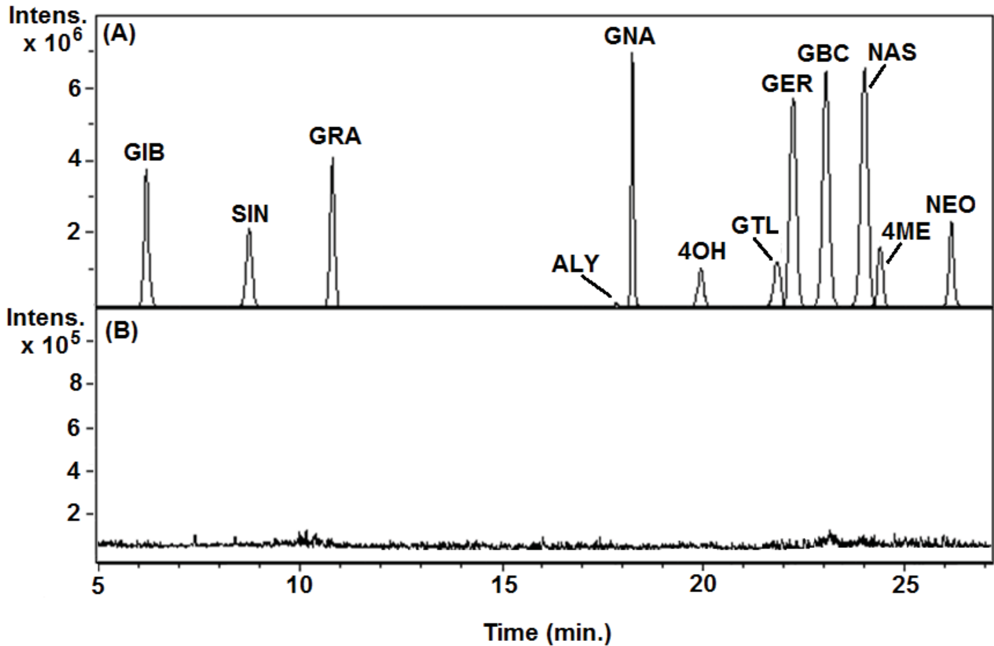
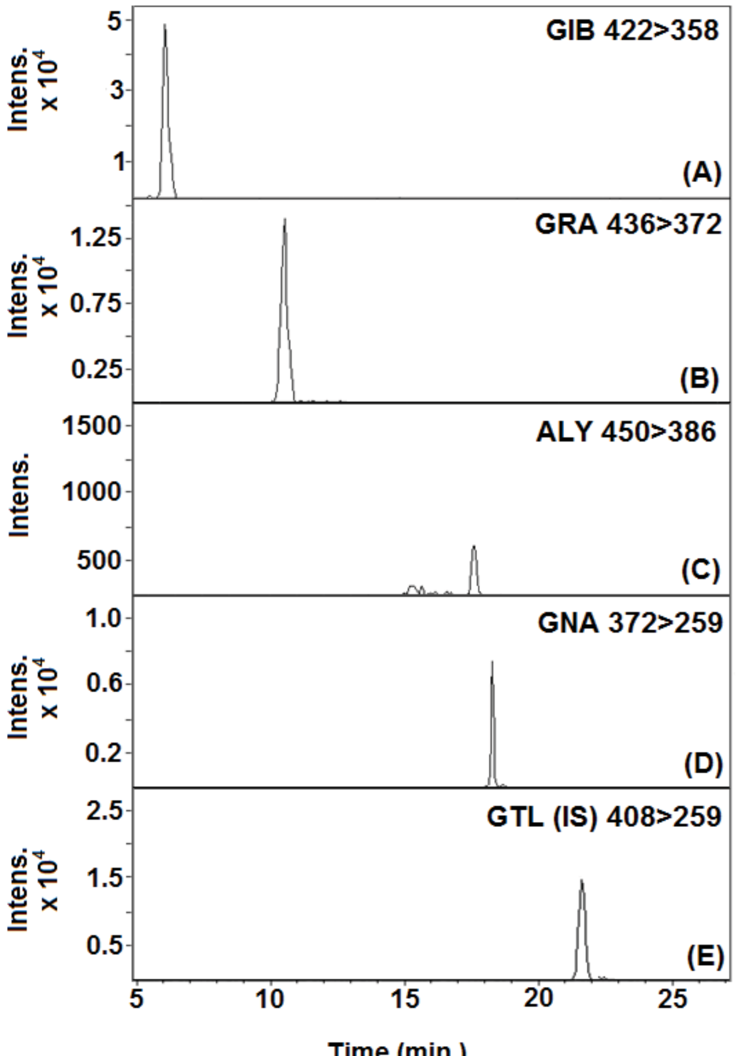


Figure 2



V.10. PAPER X. Analysis of intact glucosinolates in beeswax by liquid chromatography-tandem mass spectrometry

ANALYSIS OF INTACT GLUCOSINOLATES IN BEESWAX BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A new method was developed to determine twelve intact-glucosinolates (glucoiberin; glucoraphanin; glucoerucin; gluconapin; glucotropaeolin; glucobrassicin; gluconasturtiin; glucoalyssin; 4-hydroxyglucobrassicin; 4-methoxyglucobrassicin; neoglucobrassicin; sinigrin) in beeswax by means of liquid chromatography tandem mass spectrometry with electrospray ionization. An efficient extraction procedure was proposed (average analyte recoveries were between 88% and 101%); this involved the dissolution of melted beeswax, a solvent extraction, and a concentration step in a rotary evaporator. Chromatography was performed on a C_{18} analytical column with a mobile phase of formic acid in water (0.5%, v/v) and formic acid in acetonitrile (0.5%, v/v), at a flow rate of 1 mL/min. The method was fully validated in terms of selectivity, limits of detection and quantification, linearity, precision and accuracy. The limits of detection and quantification values were below 7 µg/kg and 23 µg/kg, respectively. The proposed method was applied to analyze beeswax samples collected from organic apiaries.

1. Introduction

Glucosinolates (GLSs) are secondary plant metabolites that occur in vegetables of the genus Brassica, such as cabbage, rape, or broccoli. They are β-D-thioglucoside-(Z)-*N*-hydroxyiminosulfate compounds with a side chain (R) derived from amino [1]. When plant tissues are damaged, GLSs undergo enzymatic hydrolysis by myrosinase to glucose and a variety of degradation products (isothiocyanates, nitriles, thiocyanates, epithiocyanates, epithionitriles and oxazolidines) [2,3], which are responsible for flavour and most of the biological activities related to GLSs [4], although certain GLSs appear to be protective against chemical carcinogens [2]. Although most studies have focused their attention on determining GLSs in Brassica vegetables, due to the fact that these plants are rich in these bioactive compounds [5], it must be said that GLSs have been investigated and detected in honey as a result of their potential use as botanical biomarkers [6]. Consequently, it may be supposed that residues of these compounds can be found in other bee products, for instance, beeswax. Moreover, an analysis of GLSs in beeswax may be of interest for several reasons; firstly, in order to check their potential presence, since, to the best of our knowledge, no research has been published in which GLSs have been analyzed in this matrix; secondly, because if they are present in a product that could be considered a beehive reservoir, they could be transmitted to other bee products that would be consumed by humans, who would be positively affected by their beneficial health properties; and also because GLSs could be used as a parameter to help identify the origin of beeswax. GLSs have been studied in only one bee matrix, namely, honey [6,7]. The methodology employed in the first study [6] involved a dilution of the sample with acidified water and a further solid-phase extraction prior to GLS analysis by liquid chromatography (LC); meanwhile, in the other analysis [7], GLS and its degradation products were extracted with a mixture of organic solvents, the analytes being isolated and identified by gas chromatography coupled to mass spectrometry (GC-MS). It should be highlighted that different methods for analyzing GLSs, in accordance with the presence (intact or non-intact desulfo-derivatives) of a sulfate group, have been reported in the literature. Regarding the analysis of intact-GLSs, solid-phase extraction has been successfully used to extract intact-GLSs with other matrices, especially vegetables [8,9]; similar results have been seen with pressurized liquid extraction [4,10]. Nevertheless, the preferable and simplest choice is a solvent extraction with heated water or mixtures of methanol and water [11-17]. Consequently, taking into account our experience and the favorable results obtained in recent research [18-20], we decided to use solvent extraction as the sample treatment. Analysis of intact-GLSs was usually performed by LC with ultraviolet (UV) or diode array (DAD) [6,8,12,14,21] and mass spectrometry (MS) [9-17,20,21] detectors, while C_{18} [6,8,10,11-15,20,21] and C_{30} [9] analytical columns were used in most cases. Among these, MS, and in particular tandem mass spectrometry (MS/MS), has emerged as one of the preferred detection techniques, providing as it does sufficient sensitivity and unambiguous identification and quantification of GLSs [10]. The main goal of this study was to develop and validate a new, robust method of maximum possible sensitivity and selectivity to determine intact-GLSs in beeswax. To the best of our knowledge, this is the

first study in which extraction, separation and detection procedures for intact-GLSs have been developed and optimized in this matrix. A further goal of this study was to apply the proposed method as a pioneer analysis of beeswax samples to verify the possible presence of residues of intact-GLS.

2. Materials and methods

2.1. Reagents and materials

Glucoiberin (GIB), glucoraphanin (GRA), glucoerucin (GER), gluconapin (GNA), glucotropaeolin (GTL), glucobrassicin (GBC), gluconasturtiin (NAS) and sinigrin (SIN), were purchased from Phytoplan Diehm & und Neuberger GmbH (Heidelberg, Germany). Rapeseed certified ERM® BC367 reference material, from Sigma Aldrich Chemie Gbmh (Steinheim, Germany), which was composed, among other compounds, of glucoalyssin (ALY), 4-hydroxyglucobrassicin (4OH), neoglucobrassicin (NEO), GNA, NAS, GBC and SIN, was employed to optimize separation, whilst potentially it could be used to identify and confirm the presence of ALY, 4OH and NEO in the samples, due to the absence of individual standards for these GLSs. One GLS (4-methoxyglucobrassicin, 4ME) is not present in the rapeseed certified material, and its individual standard was not available; however, it could be tentatively assigned by using the available bibliographic data [22,23]. In addition, broccoli leaf extracts were used to identify and confirm the presence of 4ME, and optimize separation. Moreover, SIN was used as an external standard (ES) to quantify the GLSs where individual standards were not available, while GTL was employed as internal standard (IS). Hexane (Pestiscan grade), acetonitrile and isopropanol (both of LC grade) were supplied by Lab-Scan Ltd. (Dublin, Ireland), whilst formic acid and ethanol (LC grade) were purchased from Sigma Aldrich Chemie Gbmh and Panreac (Barcelona, Spain), respectively. Syringe filters (17 mm, Nylon 0.45 μm) were obtained from Nalgene (Rochester, NY, USA), and ultrapure water was obtained by means of Millipore Milli-RO plus and Milli-Q systems (Bedford, MA, USA). An R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), and a IKA® C-MAG HS 7 magnetic stirrer with heating and ceramic heating plate (IKA®-Werke GmbH & Co. KG, Staufen, Germany) were also used.

2.2. Standard solutions

Standard stock solutions were prepared by dissolving approximately 3 mg of each glucosinolate in 10 mL of ultrapure water to a final concentration of approximately 300 mg/L. These solutions were further diluted with ultrapure water to prepare the intermediate and working solutions. Beeswax samples (0.7 g) were spiked before (**BF** samples) or after (**AF** samples) sample treatment, with different amounts of the standards in order to prepare the matrix-matched standards for validation (quality control (**QC**) samples and calibration curves), matrix effect, and sample treatment studies. To obtain appropriate spiking of the

BF samples, beeswax was melted at 70ºC prior to the addition of the corresponding concentration of GLS. Next, the beeswax was cooled at room temperature and stored in the dark at -20ºC until analysis. Meanwhile, the GLS standards were included in the reconstitution solution when preparing the **AF** samples.. All standard solutions were stored in amber glass containers and kept in the dark at -20ºC; they were determined stable for more than two weeks.

2.3. Sample procurement and treatment

2.3.1. Samples

Ten beeswax samples collected from organic apiaries were supplied by the Centro Apícola Regional of Marchamalo (Guadalajara, Spain). Those samples were the object of a preliminary analysis by LC-MS/MS in order to verify the presence of intact-GLSs. Once the absence of residues from these compounds was confirmed, subsamples of the corresponding beeswax pools were used as blank (GLSs-free) samples to prepare matrix-matched samples for validation, matrix effect, and sample treatment studies. Finally, the beeswax samples were mixed and frozen at -20ºC. Following this, these frozen samples (spiked and non-spiked) were cut into thin layers, ground and pooled for optimum sample homogeneity; subsequently they were stored in the dark at -20ºC until analysis.

2.3.2. Sample treatment

Briefly, ground and homogenized beeswax was transferred to a 100 mL glass beaker and placed on a magnetic stirrer with the heating set at 70ºC. Once the beeswax had melted, 10 mL of hexane was added, and the mixture was shaken (medium speed) and heated for 2 min in order to dissolve the melted beeswax in hexane. Following this, a slightly heated ethanol and water mixture was added; the resulting mixture was shaken and heated for 5 min. Finally, the solution was straightaway transferred to an extraction funnel, the aqueous phase was collected, loaded onto a conical flask and evaporated to dryness in a rotary evaporator at 45ºC; the dry residue was reconstituted, filtered through a nylon filter and injected into the LC-ESI-MS system.

2.4. LC-MS/MS system

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD Trap XCT instrument, which was used in conjunction with electrospray ionization (ESI) in negative ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data were analyzed using Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. The analytical column was a Gemini® 3µm C_{18} 110Å (150 x 4.60 mm) protected by a Gemini® C_{18} security guard cartridge (4 mm x 3.0 mm), both from Phenomenex (Torrance, CA, USA).

Subsequent to the optimization study, the mobile phase selected was a mixture of (A) formic acid in water (0.5%, v/v) and (B) formic acid in acetonitrile (0.5%, v/v) applied at a flow rate of 1 mL/min in a gradient mode. The injection volume and column temperature were set at 20 µL and 30ºC, respectively. The ESI interface was operated in negative mode after studies had been performed with standard solutions of the more relevant MS parameters. Compounds were identified by comparing the MS and MS/MS data (retention time, spectra, ions and transitions) with those obtained from pure standard solutions when commercially available, or with data obtained from analyzing the certified rapeseed material and broccoli leaf extracts. MS/MS was used to quantify and confirm the presence of intact-GLSs. The ESI-MS/MS analyses were performed by means of multiple reaction monitoring (MRM) in ultra scan mass range mode, enabling smart fragmentation and scanning from m/z 70 to 500. To protect the ion source from matrix constituents, the MSD Trap system automatically controlled the run at different times: at $t_{0\text{-}5min}$ to waste, $t_{5\text{-}27min}$ to mass and $t_{27\text{-}30min}$ to waste. During MS integration a Gauss function was used as a smoothing algorithm.

3. Results and discussion

3.1. Optimization of the extraction from beeswax

In view of the absence of specific procedures in which intact-GLSs were determined in beeswax, it was decided that a solvent extraction should be developed and optimized as the sample treatment; this decision was the result of the simplicity of this extraction procedure and our experience with it [18,19]. Firstly, consideration was given to the amount of beeswax to be analyzed. Moreover, it was necessary to heat the beeswax (70ºC) during the sample treatment for several reasons: **i**) to obtain homogenous samples; **ii**) to facilitate its dissolution and extraction, as has proven to be effective in previously published studies [20]; **iii**) to deactivate the myrosinase enzyme, as this temperature is commonly used to perform this task [17], and it does not affect to the thermal stability of the GLSs [24]. Subsequently, the extraction procedure was carried out with a magnetic stirrer while heating. Different solvents (hexane and a hexane/isopropanol) and volumes were tested to dissolve the beeswax, as this provided good results in the above-mentioned research. Beeswax was more or less easily dissolved when using an hexane/isopropanol mixture, but recoveries were higher when using hexane (data not shown). Consequently, hexane was chosen to dissolve the beeswax. Afterwards, different solvents, which were immiscible with hexane, were assayed as extractants (ethanol, methanol, water and mixtures of both solvents with water), and the best results in terms of recoveries (data not shown) and separation between phases was obtained with an ethanol and water mixture. Different volume ratios of the hexane and ethanol/water mixture were tested in order to obtain optimal extraction conditions. The shaking time was also evaluated (2, 5 and 10 min), and it was observed that 5 min was sufficient for obtaining satisfactory recoveries (data not shown). Subsequently, the heated mixture was quickly transferred to an extraction funnel to avoid beeswax solidification, which affected the phase

separation; the aqueous phase was collected, transferred to a conical flask and gently evaporated to dryness in a rotary evaporator at 45ºC. Reconstitution was deemed appropriate for enhanced effectiveness when extracting. Therefore, the dry residue was reconstituted, and filtered through a nylon 0.45 μm filter. It should be highlighted that no matrix interferences co-eluted with the intact-GLSs upon injection of beeswax samples with the proposed extraction procedure (see Figure 1). In order to assess the efficiency of the proposed sample treatment, a comparison was made of the results obtained for blank beeswax spiked at three different GLSs concentrations (low, medium and high **QC** levels), either prior to (**BF** samples) or following (**AF** samples) sample treatment. The recovery values (Table 1) ranged from 88%-101% in all cases, which indicated that the procedure selected was appropriate and efficient. The results were sound not only in terms of extraction efficiency but also regarding its simplicity and solvent consumption, as the overall sample treatment consisted of three stages (dissolution, extraction and concentration), with 31 mL of solvent being used per sample.

3.2. LC optimization

Analysis of GLSs has generally been performed in RPLC by means of C_{18} based stationary phase columns; such was the case of our recent study devoted to analyzing twelve intact-GLSs in broccoli [12]. Consequently, we decided to optimize the chromatographic conditions proposed in our previous study for analysis of beeswax. Individual standards, spiked beeswax samples, rapeseed certified material, and broccoli leaf extracts were used to optimize separation of the twelve intact-GLSs. Several experiments were conducted by means of different mobile phase compositions. Tests were carried out to study the influence of the column temperature (between 20 and 50ºC) and the injection volume (between 5 and 30 μL) on the S/N ratio. The results showed an increase in the S/N when up to 20 μL was injected, and a significant loss of symmetry was observed at temperatures over 30ºC. Therefore, 30ºC and 20 μL were selected as optimal values. With the optimized chromatography conditions, the intact-GLSs were separated in under 26.5 min, and the overall run time was 30 min (see Figure 1), which is 5 min shorter than our previous proposal [12]. Moreover, it was not possible to further reduce analysis time in order to maintain the separation between two pairs of compounds (GER and GBC; 4ME and NAS). Finally, these compounds have been widely studied in vegetables, but no research has been carried out on beeswax. Thus, it is not possible to compare the analytical performance of our method with previous proposals.

3.3. MS optimization

Intact-GLSs were previously analyzed in negative ESI mode [9,11,12,15-17,21], the best results being obtained in terms of peak area and peak height. To establish optimal ESI-MS/MS conditions, several experiments (flow injection analysis) were conducted in order to choose these optimum ESI-MS/MS (negative mode) parameters and achieve maximum sensitivity by the infusion mode (5 μL/min) of standard and matrix-matched solutions (1000 μg/L) of intact-GLSs, the individual standards of which were available. The best results were found with the values described in subsection 2.4. In ESI-MS, the

compounds exhibited intense $[M-H]^-$ in full-scan spectra. These ions were selected as precursors to obtain product ions for MS/MS analyses in MRM mode. The MRM transition that provided the highest signal was used for quantification; meanwhile, a second MRM transition was used for confirmation. Such ions/transitions have been commonly used in the existing literature devoted to analyzing GLSs by MS/MS [9,11,12,15-17,21]. To ascertain how the matrix influenced the detection of GLSs, the peak areas of the GLSs in standard solutions were compared with those obtained in **AF** samples (beeswax samples spiked after sample treatment). The response of all compounds in the three concentrations assayed was between 87% and 105% in all cases, as shown in Table 1. Moreover, if a comparison is made between GLS peaks in standard and matrix-matched solutions at the same concentration levels, a slight variation in analyte signals can be observed. Thus, it was concluded that the matrix (beeswax) did not significantly affect ESI ionization of the analytes.

3.4. Validation of the method

Validation was carried out in accordance with different international guidelines [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf. (accessed 01.05.15), 25] determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, precision and accuracy. As was previously stated in subsection 2.3.1, blank (GLSs-free) beeswax samples were employed for the validation studies. Moreover, it should be mentioned that the validation was not done for ALY, 4-OH, PRO, GBN, GNL and NEO due to the lack of individual standards, and GTL has not also been included in the validation study as it was the IS. To determine the selectivity of the proposed method, a set of blank beeswax samples (n=6) were injected onto the chromatographic system and the results were compared with those obtained for spiked beeswax samples. As can be seen in Figure 1, no chromatographic interference was observed at GLSs retention times in any of the blank samples analyzed. In order to identify GLSs peaks in beeswax samples (spiked), a comparison was made of the mass spectra of GLSs peaks in standard solutions and beeswax samples with spiked GLSs content; this was conducted at comparable concentrations and was measured under the same conditions. Both mass spectra were quite similar (data not shown), although some minor differences in ion intensity were observed and certain low intensity ions appeared only in beeswax spectra. Moreover, percentages relating to the intensity of quantification and confirmation transitions for GLSs peaks in standard solutions and beeswax samples were also compared. In all cases, the tolerance rates for relative transition intensities were below 10% (data not shown). Therefore, it can be concluded that the method was selective for determining GLSs in beeswax. The LODs and LOQs were experimentally determined by injecting a number of blank beeswax samples (n=6), and measuring the magnitude of background analytical response at the elution time of each compound. The LODs and LOQs were estimated to be three and ten times the S/N ratio, respectively. It can be observed that the LOD and LOQ values were quite similar in all cases, ranging from 3 to 7 µg/kg (LOD) and 10 to 23 µg/kg (LOQ). To reiterate, no LOD or LOQ values were reported in beeswax, so it was not possible to make a proper comparison.

Standard calibration curves could be used to quantify intact-GLSs in beeswax, since the matrix and sample treatment did not significantly affect the analyte signal (see Table 1); the gradients of the standard and matrix-matched calibration curves overlapped at the confidence intervals. The standard solutions used to construct the calibration curve were a preparation of various aqueous solutions containing variable amounts of eight commercial GLSs over a concentration range of LOQ to 1000 µg/L (LOQ, 20, 50, 100, 200, 500, 1000 µg/L), which, according to unit conversion and proposed sample treatment, corresponded to concentration in matrix between LOQ and 1430 µg/g (LOQ, 28, 71, 142, 285, 714, 1428 µg/kg); GTL (IS) was added in all cases at 100 µg/L (142 µg/kg for matrix matched standards). Calibration curves (n=6) were constructed by plotting the signal on the *y*-axis (analyte peak areas) against the analyte concentration on the *x*-axis. The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied; meanwhile, the coefficient of the determination values (R^2) was above 0.99 in all cases. The lack of bias was confirmed by a *t* test and a study of the distribution of residuals. Intra-day precision and accuracy experiments were performed concurrently by repeated sample analysis using beeswax samples spiked with three concentrations of GLSs (low, medium and high **QC** levels) on the same day (n=6). Inter-day precision and accuracy were assessed by an analysis of beeswax spiked with three concentrations of the compounds studied (low, medium and high **QC** levels) over three consecutive days (n=6). In each run a calibration curve was established and replicates (n=6) of each spiked concentration level were analyzed. Precision was expressed as the percentage of relative standard deviation (%RSD) at the three concentrations for each analyte. Accuracy was calculated by means of relative error (%RE). Intra- and inter-day precision (%RSD values) were at all times lower than or equal to 7%. Accuracy (%RE values) ranged from 3% to 7% for the intra-day readings, and from 3% to 8% for the inter-day values. These results indicate that the present method is both precise and accurate.

3.5. Application of the method

Beeswax samples from organic apiaries were analyzed with the proposed method to search for residues of intact-GLSs. No residues of the twelve compounds under study were detected in any of the samples. This does not, however, mean that it was a wasted effort to develop a method for screening compounds that did not exist in these samples, since residues of GLSs in Brassicaceae honeys [6,7] have already been reported. As a result, it is reasonable to suppose that GLSs residues could appear in other bee matrices such as beeswax, and, therefore, sensitive and exclusive methods would be required to detect GLSs in this matrix because of the low concentrations expected.

4. Concluding remarks

A pioneer analytical method has been developed and optimized to simultaneously identify and quantify twelve intact-GLSs in beeswax. The proposed extraction method based on three steps (dissolution, liquid-

liquid extraction, and concentration) has proven to be efficient, simple and relatively cheap (a low consumption of solvents). This LC-ESI-MS/MS method was fully validated and the data demonstrated that it is consistent and reliable, with a wide linear range of applicability; standard calibration curves could be employed to quantify intact-GLSs in the beeswax due to the fact that a matrix effect was not significant. No residues, however, of the intact-GLSs studied were detected in any of the samples. In summary, the proposed method represents an innovative potential protocol for analyzing intact-GLSs residues at trace level in beeswax, being as it is the first analytical method devoted to investigating the presence of these compounds in this matrix. It might also be of interest as a starting-point for further investigation into the use of GLSs to determine the origin of the beeswax, since the presence or, as in the case of this study, absence of such compounds in beeswax samples could potentially be a determinant of the origin of the samples. Nevertheless, this would imply the analysis of a large number of samples from apiaries located close to different cultivars, including Brassicaceae crops.

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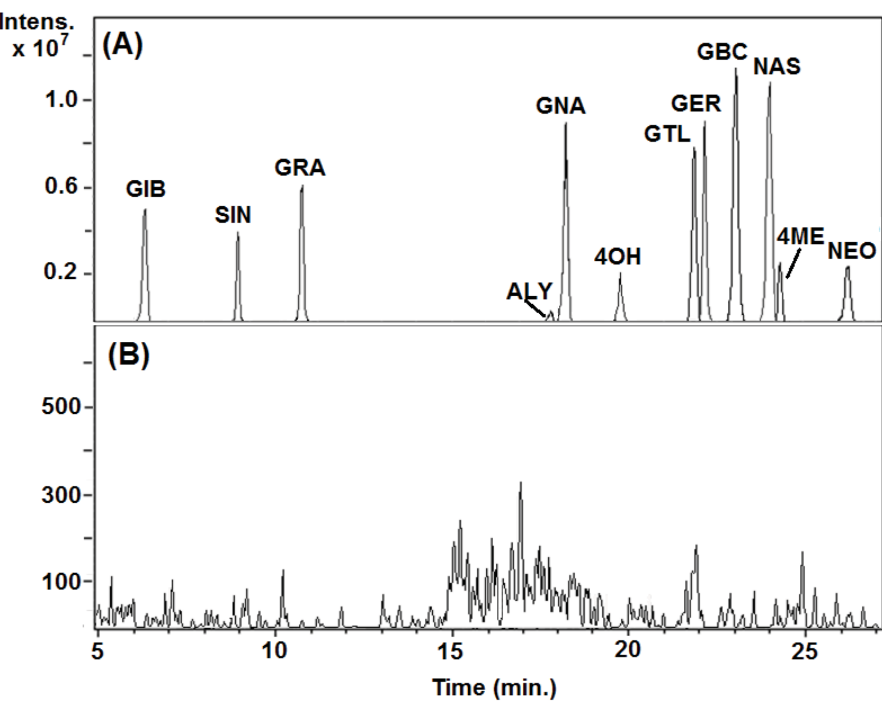

Figure 1.- Representative LC-MS (extracted ion chromatogram-EIC, negative mode) obtained from: (**A**) a blank (GLSs-free) beeswax sample, spiked with the eight individual GLSs standards, and a broccoli leaf extract which contained 4ME, NEO, 4OH and ALY, among other of the studied GLSs; (**B**) a blank (GLSs-free) beeswax.

Table 1.- Recovery data obtained for spiked beeswax samples treated accordingly to the procedures described in subsections 2.3, 3.1 and 3.3 (n=6).

	Evaluation of the sample treatment			**Evaluation of the matrix effect**		
Quality control (QC) sample	**Low**	**Medium**	**High**	**Low**	**Medium**	**High**
GIB	88 ± 4	91 ± 5	89 ± 4	100 ± 6	101 ± 5	98 ± 6
SIN	89 ± 4	93 ± 5	90 ± 4	103 ± 6	100 ± 5	105 ± 6
GRA	95 ± 5	92 ± 3	97 ± 4	104 ± 5	99 ± 6	102 ± 5
GNA	99 ± 4	96 ± 4	101 ± 3	88 ± 7	92 ± 7	89 ± 6
GER	88 ± 4	92 ± 5	90 ± 5	101 ± 5	96 ± 6	98 ± 6
GBC	99 ± 5	97 ± 4	96 ± 5	92 ± 7	88 ± 7	90 ± 6
NAS	91 ± 5	96 ± 5	93 ± 4	87 ± 6	89 ± 7	92 ± 6

low **QC**-28 µg/kg; medium **QC**-286 µg/kg; high **QC**-1428 µg/kg.

V.11. PAPER XI. Development and validation of a LC–MS/MS method to determine sulforaphane in honey

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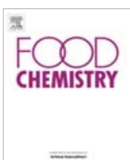

Analytical Methods

Development and validation of a LC–MS/MS method to determine sulforaphane in honey

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ABSTRACT

A new method was developed to determine sulforaphane (SFN) in honey using liquid chromatography tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI). An efficient extraction procedure was proposed (average analyte recoveries were between 92% and 99%); this involved a solid phase extraction (SPE) with a polymeric sorbent. Chromatography was performed on a Synergi™ Hydro analytical column with a mobile phase of 0.02 M ammonium formate in water and acetonitrile, at a flow rate of 0.5 mL/min. The method was fully validated in terms of selectivity, limits of detection (LOD) and quantification (LOQ), linearity, carry-over effect, reinjection reproducibility, precision and accuracy. The LOD and LOQ values were below 0.8 μg/kg and 2.6 μg/kg, respectively. The proposed method was applied to analyze SFN in honey from different botanical origins (rosemary, multifloral, orange blossom and heather), and SFN was detected at trace levels in some of the honey samples examined.

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1. Introduction

A natural, unprocessed and easily digested food, honey can be considered an important part of the human diet. In the last few years there has been observed an increase in honey consumption, which could be attributed to consumer interest in the positive health benefits provided by several honey constituents (Escudero, González-Martín, Rodríguez-Flores, & Seijo, 2015), such as sugars, proteins, vitamins, minerals, enzymes, free amino acids and several volatile compounds (Karabagias, Badeka, Kontakos, Karabournioti, & Kontominas, 2014). Several of the above-mentioned compounds have been suggested as floral markers to certify the botanical origin of a given honey, which is important for correct and legal labeling (Truchado et al., 2010). In this study, research was also conducted into the potential of flavonoid glycosides and glucosinolates (GLSs) as botanical biomarkers in Argentinean *Diplotaxis* honeys. The authors reported the presence of certain GLSs in honey (glucoiberin, glucoraphanin (GRA), 4-hydroxyclucobrassicin, glucobrassicanapin, glucobrassicin and neoglucobrassicin), and it was also observed that the GLS composition varied between fresh (unripe) and mature honey. The authors suggested that some of them could be degraded to isothiocyanates, indoles and other compounds during the ripening of the honey in the beehive. This conclusion tallied with that of a previous publication, in which it was postulated that nitriles appearing in honeys (*Taraxacum*) were possibly formed from GLSs supplied by *Brassicaceae* flowers (Soria, Martínez-Castro, de Lorenzo, & Sanz, 2008). GLSs are thioglucosides containing a cyano group and a sulfate group. These compounds coexist with myrosinase in plants, especially in Brassica vegetables, and in the presence of water are rapidly hydrolyzed into their breakdown products such as isothiocyanates, thiocyanates, nitriles or indoles (Ares, Nozal, & Bernal, 2013). GLSs and/or their corresponding breakdown products are well known for their fungicidal, bactericidal, nematocidal and allelopathic properties, and they have recently gained research interest because of their anticancer activity (Dinkova-Kostova & Kostov, 2012). Nowadays, a large body of research on functional foods, especially in anticarcinogens, has focused on a single bioactive isothiocyanate, sulforaphane (SFN) (Ares, Bernal, Martín, Bernal, & Nozal, 2014; Gu, Guo, & Gu, 2012), which is formed by hydrolysis of GRA; this is of interest on account of its potential role not only in the prevention of cancer, but also in that of chronic and degenerative diseases such as diabetes, atherosclerosis and cardiovascular disorders (Kuang et al., 2013). Thus, if we take into consideration the findings of Truchado et al. (2010), who detected GRA in unripe honey but not in mature honey, it is reasonable to suppose that SFN could be found in honey as a consequence of the degradation of GRA during the ripening process. An analysis of SFN in commercial honey could be of interest not only in order to check its potential presence, since, to the best of our knowledge,

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no research has been published in which SFN has been analyzed in commercial honey, but also because of the health benefits related to SFN and the positive nutritional and health effects associated with the consumption of honey. This compound has only once been studied in honey obtained from an experimental beehive (Świderski, Sterkowicz, Kaszychki, & Kołoczek, 2003), in which the authors used an SFN sugar extract to feed the bees. SFN was determined in herbhoney, which was previously diluted in a mixture of ethanol and water, and was extracted with chloroform by liquid chromatography (LC) and UV detection.

SFN has been widely determined in other matrices such as Brassica vegetables (Ares et al., 2014; Azizi, Amiri-Besheli, & Sharifi-Meher, 2011; Campas-Baypoli, Sánchez-Machado, Bueno-Solano, Ramírez-Wong, & López-Cervantes, 2010; Ghawi, Methven, & Niranjan, 2013; Guo, Guo, Wang, Zhuang, & Gu, 2013; Han & Row, 2011; Liang, Yuan, & Liu, 2013; Matusheski et al., 2001; Moon, Kim, Ahn, & Shibamoto, 2010; Nakagawa et al., 2006; Shen, Su, Wang, Du, & Wang, 2010; Sivakumar, Alboni, & Bacchetta, 2007) and biological ones (Agrawal et al., 2006; Dominguez-Perles et al., 2014; Hauder et al., 2011; Wang, Lin, Shen Khor, Nomeir, & Kong, 2011). Extraction of SFN from plant matrices was usually done by solvent extraction with an organic solvent such as dichloromethane (DCM) (Azizi et al., 2011; Campas-Baypoli et al., 2010; Ghawi et al., 2013; Guo et al., 2013; Han & Row, 2011; Matusheski et al., 2001; Nakagawa et al., 2006; Sivakumar et al., 2007), chloroform (Moon et al., 2010) or methyl t-butyl ether (MTBE) (Ares et al., 2014), and in many of the above-mentioned studies (Ares et al., 2014; Azizi et al., 2011; Campas-Baypoli et al., 2010; Guo et al., 2013; Matusheski et al., 2001; Sivakumar et al., 2007) solid phase extraction (SPE) with silica based cartridges was also performed to purify the plant extracts. In addition, SFN has been analyzed in biological matrices by a SPE with polymeric (Dominguez-Perles et al., 2014), C_{18} (Hauder et al., 2011), and C_2 (Agrawal et al., 2006) sorbents, or by extracting it with methanol containing formic acid (Wang et al., 2011).

Determining SFN has usually been achieved by LC (Agrawal et al., 2006; Ares et al., 2014; Azizi et al., 2011; Campas-Baypoli et al., 2010; Dominguez-Perles et al., 2014; Guo et al., 2013; Han & Row, 2011; Hauder et al., 2011; Liang et al., 2013; Matusheski et al., 2001; Nakagawa et al., 2006; Shen et al., 2010; Sivakumar et al., 2007; Wang et al., 2011), and gas chromatography (GC) (Ghawi et al., 2013; Guo et al., 2013; Matusheski et al., 2001; Moon et al., 2010; Shen et al., 2010). However, it has been postulated that in some cases SFN was thermally degraded in the injection ports of GC equipment (Campas-Baypoli et al., 2010), and usually the GC analysis times were longer than those of LC. Therefore, it was decided that LC should be employed in this study. It should also be mentioned that almost all the above-mentioned LC studies employed C_{18} based analytical columns and UV, diode array or MS detectors, although C_8 (Agrawal et al., 2006; Azizi et al., 2011) or C_{30} (Wang et al., 2011) stationary phases, and evaporative light-scattering detectors (Liang et al., 2013; Nakagawa et al., 2006) were occasionally used. Finally, MS/MS detection was employed here to increase the selectivity and sensitivity of the method.

The aim of this study was the development of a new and robust LC–ESI-MS/MS method, of maximum possible sensitivity, selectivity and rapidness, to determine SFN in commercial honey from different botanical origins. Although SPE and LC–ESI-MS/MS have been previously employed to determine SFN in other matrices, we optimize specific and efficient extraction and determination procedures for SFN from honey under new conditions; to the best of our knowledge, this is the first study in which extraction, separation and detection procedures for SFN have been developed and optimized in honeys from different botanical origins. Accordingly, the proposed method was validated and applied in an analysis of SFN in honey samples from four very different botanical origins (heather, multifloral, rosemary and orange blossom) to determine potential differences in SFN content and to corroborate the argument that this compound may be found in such a matrix.

2. Materials and methods

2.1. Reagents and materials

SFN standard (molecular weight: 177.3), ammonium formate, sodium hydroxide, formic and hydrochloric acids were obtained from Sigma Aldrich Chemie Gbmh (Steinheim, Germany). LC grade ethanol, methanol, DCM, and acetonitrile were supplied by Lab-Scan Ltd. (Dublin, Ireland), meanwhile LC methyl t-butyl ether (MTBE) was acquired from Panreac Química S.L.U. (Barcelona, Spain). Syringe filters (17 mm, Nylon 0.45 m) were purchased from Nalgene (Rochester, NY, USA), ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA,USA), and a Vibromatic mechanical shaker was purchased from (J.P. Selecta S.A., Barcelona, Spain). A R-210/215 rotary evaporator 109 (Buchi, Flawil, Switzerland), a filtration system (Millipore), Strata® C18-E (3 mL with 500 mg of sorbent) and Strata® X (6 mL with 200 mg of polymeric sorbent) from Phenomenex, Torrance, CA, USA), a Vortex mechanical mixer from Heidolph (Schwabach, Germany) and a 10-port Visiprep vacuum manifold (Supelco, St. Louis, MO, USA) were used for the extractions.

2.2. Standard solutions

Standard stock solution was prepared by dissolving approximately 5 mg of SFN in 10 mL of acetonitrile at a final concentration of approximately 500 mg/L. These solutions were further diluted with methanol to prepare the intermediate and working solutions. Honey samples (2.5 g) were spiked before (BF samples) or after (AF samples) sample treatment, as described in Section 2.3, with different amounts of SFN in order to prepare the matrix-matched standards for validation (quality control (QC) samples and calibration curves), matrix effect, and sample treatment studies. Each QC sample was prepared with 2.5 g of honey samples spiked with four different concentrations of SFN within the linear range. The concentrations of the different QC samples were as follows: QC level 1–4 μg/kg, QC level 2–40 μg/kg, QC level 3–400 μg/kg, and QC level 4–1200 μg/kg. Stock solution was stored in glass containers and kept in the dark at −20 °C. Meanwhile intermediate, working and matrix-matched solutions were stored in glass containers and kept in the dark at +4 °C. All solutions were stable for over 2 weeks.

2.3. Sample procurement and treatment

2.3.1. Samples

Several honey types were selected according to their different color, composition and botanical origin. Samples from different regions of Spain were kindly donated by the "Centro Apícola Regional" at Marchamalo (Guadalajara, Spain). Their botanical origin, which was confirmed by melissopalynological analysis, were: rosemary, *Rosmarinus officinalis* ($n = 10$); orange blossom, *Citrus* spp. ($n = 8$); multifloral ($n = 10$); and heather, *Erica* spp. ($n = 7$). All honey samples underwent a preliminary analysis by LC–ESI-MS/MS and the treatment previously proposed (Świderski et al., 2003) in order to check for the presence of SFN. Once absence of the latter was confirmed in several samples, subsamples of the corresponding honeys were used as blank samples to prepare

matrix-matched samples for validation (QC and calibration curves), matrix effect, and sample treatment studies.

2.3.2. Sample treatment

Briefly, 2.5 g of homogenized honey sample was diluted in 5 mL of water, and the resulting solution was loaded onto a Strata® X SPE cartridge (previously conditioned with 5 mL of methanol and 5 mL of water) at about 1 mL/min by means of a suction system. The SPE cartridge was then washed with 5 mL of a mixture of water and methanol (80:20, v/v); the rinse was discarded, and after 5 min of drying time the analytes were eluted with 2 mL of methanol, the resulting solution being passed through a syringe filter. Ten microliter aliquot was injected into the LC–ESI-MS/MS system.

2.4. LC–ESI-MS/MS system

The chromatographic system consisted of an Agilent Technologies (Palo Alto, CA, USA) 1100 series LC-MSD Trap XCT instrument, which was used in conjunction with electrospray ionization (ESI) in positive ion mode. The LC instrument was equipped with a vacuum degasser, a quaternary solvent pump, an autosampler and a thermostated column compartment. The system was controlled by an Agilent ChemStation for LC Rev A.10.02 and MSD Trap Control version 4.2. Data were analyzed by Quant Analysis for LC/MSD Trap 1.6 and Data Analysis for LC/MSD Trap 2.2, both from Agilent Technologies. The analytical column was a Synergi™ Hydro-RP C_{18} 4 μm (150 × 4.6 mm; Phenomenex, Torrance, CA, USA) protected by a C_{18} security guard cartridge (4 × 3.0 mm i.d.; Phenomenex). The mobile phase was a mixture of (A) 0.02 M ammonium formate in water and (B) acetonitrile applied at a flow rate of 0.5 mL/min in a gradient mode as follows: (i) 0–6.5 min (A–B, 52:48, v/v); (ii) 6.5–7.5 min (A–B, 5:95, v/v); (iii) 7.5–9.0 min (A–B, 5:95, v/v); (iv) 9.0–10.0 min (A–B, 52:48, v/v); (v) 10.0–14.0 min (A–B, 52:48, v/v). The injection volume and column temperature were set at 10 μL and 25 °C, respectively. The ESI interface was in positive mode after studies had been performed with standard solutions of the more relevant MS parameters. ESI-MS/MS analyses were performed by means of multiple reaction monitoring (MRM) in ultra scan mass range mode, enabling smart fragmentation and scanning from m/z 50 to 210. Optimal MS/MS conditions were set as follows: capillary voltage, 4500 V; drying gas (N_2) flow, 12 L/min; drying gas (N_2) temperature, 200 °C; Nebulizer pressure, 35 psi; trap drive, 35; skimmer, 15 V: octopole RF amplitude, 125 V; capillary exit, 58 V; max. accumulation time, 200 ms; ion charge control (ICC), 150,000. In addition, the MS parameter fragmentor was optimized to generate the highest signal for the most abundant product for quantification in MRM mode. Optimal fragmentation parameters were set as follows: scanning, 30–200%; isolation width (m/z), 4.0; fragmentation cut off, 44; fragmentation width (m/z), 10.0; fragmentation amplitude, 1.0. To protect the ion source from matrix constituents, the MSD Trap system was set as follow: t_{0-4min} to waste, t_{4-8min} to mass and $t_{8-14min}$ to waste. During MS integration a Gauss function was used as a smoothing algorithm.

2.5. Method validation

Validation was carried out following various international guidelines (Commission Decision 2002/657/EC, 2002; International Conference on Harmonization., 2005; Thompson, Ellison, & Wood, 2002) determining selectivity, limits of quantification (LOQ) and detection (LOD), as well as linearity, carry-over effect, reinjection reproducibility, precision and accuracy. As previously stated in Section 2.3.1, blank honey samples were employed for the validation studies. To determine the selectivity of the proposed method, a set of unspiked honey samples (n = 6) was injected onto the chromatographic system and the results were compared with those obtained for spiked honey samples. The LODs and LOQs were experimentally determined by injection of a number of blank honey samples (n = 6), in which the absence of SFN residues was previously confirmed, and measurement of the magnitude of background analytical response at the elution time of SFN in each honey sample for the different botanical origins investigated. The LODs and LOQs were estimated to be three and ten times the signal-to-noise (S/N) ratio, respectively. A matrix-matched standard calibration curve was used to quantify SFN in heather honey, as a matrix effect that affected analyte ionization was observed (see Section 3.3); meanwhile, SFN was quantified with standard calibration curves in honey samples from multifloral, rosemary and orange blossom botanical origins. Blank honey was treated accordingly with the proposed procedure and spiked with variable amounts of SFN over an analytical range between LOQ and 1200 μg/kg (calibration levels of LOQ, 8, 40, 80, 400, 1200 μg/kg) for matrix matched calibration curves, and LOQ and 1500 μg/L (LOQ, 10, 50, 100, 500 and 1500 μg/L) for standard calibration curves. All calibration curves (n = 6) were constructed by plotting the signal on the y axis (analyte peak areas for ESI-MS/MS) against analyte concentration on the x axis. The carry-over effect was assessed by injection of spiked samples or standards with a high concentration of SFN (QC4 or 1500 μg/L), and evaluation of the responses at the retention time of SFN. Reinjection reproducibility was evaluated by re-injection of previously acceptable standards (5 μg/L and 1500 μg/L) and QCs (QC1 and QC4) three times per day, which had been stored for 1 week at +4 °C. Intra-day precision and accuracy experiments were performed concurrently by repeated sample analysis with blank honey samples spiked at four different concentrations of SFN (QC levels) on the same day of (n = 6). Inter-day precision and accuracy were assessed by an analysis of blank honey samples spiked at four concentrations of SFN (QC levels) over three consecutive days (n = 6). Precision was expressed as the percentage of relative standard deviation (% RSD) at four concentration levels. Accuracy was calculated by means of relative error (% RE).

3. Results and discussion

3.1. Optimization of the extraction from honey

As seen previously (Świderski et al., 2003), SFN was determined in herbhoney by diluting the sample in an ethanol and water mixture of non-indicated specific composition and extracting the SFN with chloroform. Several preliminary tests were carried out with water and ethanol mixtures (100:0, 80:20, 50:50, v/v) to dilute the honey, and chloroform, DCM or MTBE as extraction solvents. Highest extraction efficiency was achieved when the honey was diluted in water and DCM (~60%) was employed as the extraction solvent, while much lower recovery rates were obtained with chloroform (~40%) and MTBE (~30%). In addition, several matrix compounds that co-eluted with SFN were extracted. Thus, it was not possible to perform efficient and adequate SFN extraction with this sample treatment, as the chromatograms were not sufficiently clean and the recoveries were not so high.

Consequently, the decision was taken to use an SPE procedure, as this provided satisfactory results in previous studies in broccoli and biological matrices, and, from our experience, it was successful for honey analysis. Silica-based cartridges have generally been used to extract SFN from broccoli, but these were not the best option in this study as honey is usually diluted in polar solvents (water or mixtures of alcohol and water), and these solvents should be avoided when using such cartridges. As has been previously reported, polymeric (Dominguez-Perles et al., 2014), C_{18} (Hauder et al., 2011) and C_2 (Agrawal et al., 2006) SPE sorbents

were used to analyze SFN in biological matrices. As a result of the number of scientific publications and our experience in analyzing honey, the decision was taken to check the suitability of polymeric (Strata® X) and C_{18} (Strata® C18-E) SPE sorbents to perform SPE. Following several experiments, it was observed that polymeric SPE sorbents (Strata® X) provided the highest recoveries (see Supplementary data, Table 1S) and cleanest chromatograms in all cases. We therefore chose polymeric sorbents to optimize the extraction of SFN from the honey.

Firstly, the amount of honey (1–4 g) and the most suitable solvent (water and ethanol mixtures: 100:0, 80:0, 50:50, v/v) and volume (3, 5 and 10 mL) to dissolve the honey were selected. After several tests, 2.5 g of honey and 5 mL of water were deemed the optimal values, as in this way the highest S/N ratio for securing maximum sensitivity was obtained. Prior to the loading of the diluted sample onto the SPE cartridges, some parameters were evaluated to optimize the extraction procedure. Firstly, different volumes of methanol and water were tested in order to precondition the cartridge; 5 mL of both applied sequentially was the most suitable. As honey contains sugars and substances such as pigments and phenolic compounds, direct elution of the cartridges resulted in certain matrix interference and unclean chromatograms were the result. To overcome this problem, a washing phase was introduced; several solvents were tested for this purpose: 0.1 M formic acid in water and water (5:95, v/v), 0.1 M hydrochloric acid in water and water (5:95, v/v), 0.1 M sodium hydroxide in water and water (5:95, v/v), and several water and methanol mixtures (95:5, 90:10, 80:20, 70:30, 50:50, v/v). Of these, the 80:20 (v/v) water and methanol mixture most effectively eliminated interference without compromising analyte detection; 5 mL of this solution was sufficient to obtain high recoveries and clean chromatograms for all the samples studied. Optimal drying times for the cartridges were also determined, and as no differences were observed between drying times of 5–20 min, a 5 min drying period was selected to avoid delays in the extraction procedure. Different solutions to elute the analytes were tested: methanol, the 80:20 (v/v) methanol and water mixture and acetonitrile. Methanol provided maximal elution. Following testing of the elution volumes (ranging from 1 to 3 mL), it was also found that 2 mL of methanol was sufficient to obtain satisfactory results. Consequently, the resulting solution was passed through a syringe filter, after which a 10 µL aliquot was injected into the LC–ESI-MS/MS system.

To assess the efficiency of the proposed sample treatment, a comparison was made of the results obtained for blank honey samples spiked at four different SFN concentrations (QC levels 1–4), either prior to (BF samples) or following (AF samples) sample treatment. The resulting recovery values (Table 1) ranged from 92% to 99% in all cases, which indicated that the sample treatment procedure selected was appropriate and efficient for all the concentrations and the four different botanical origins assayed. The results were good not only in terms of extraction efficiency, or solvent consumption (21 mL per sample), but also regarding the clean nature of the chromatograms. It was not possible to make a comparison with other sample treatments as the authors did not provide any recovery/extraction efficiency data in the only study in which SFN had been determined in herbhoney (Świderski et al., 2003).

3.2. LC optimization

As mentioned previously, SFN determination has generally been performed in reverse phase mode by means of C_{18} based stationary phase columns with water and acetonitrile as the mobile phase components; such was the case of the only study in which SFN was determined in honey (Świderski et al., 2003). We recently published a paper concerning the analysis of SFN in broccoli (Ares et al., 2014), and we optimized the chromatographic conditions, selecting a Synergi™ Hydro-RP C_{18} 4 µm (150 × 4.6 mm) column and 0.02 M ammonium formate in water and acetonitrile (55:45, v/v) as the analytical column and mobile phase components, respectively. Therefore, we decided to start separation optimization by using the chromatographic conditions proposed in the latter study. In the previously reported LC conditions, it was not possible to completely separate SFN from certain matrix components. In addition, certain matrix-related compounds were firmly retained, and it was necessary to use long analysis times to elute them from the column. Several experiments were conducted by means of different mobile phase compositions and flow-rates in order to elute SFN and the highly retained matrix compounds in the shortest possible time. The best results were obtained with the chromatographic conditions described in Section 2.4. As can be observed in Fig. 1, SFN eluted at 4.9 with an isocratic mobile phase composition (0.02 M ammonium formate in water and acetonitrile, (52:48, v/v)) at a flow-rate of 0.5 mL/min. It was necessary to introduce several gradient steps (ii and iii) in order to elute certain remaining matrix components subsequent to SFN elution, and for this reason it was also necessary to include an equilibration time phase (iv and v) to obtain reproducible chromatograms.

Tests were carried out to study the influence of column temperature (between 20 and 45 °C at 5 °C intervals), which produced different retention times and peak symmetries. As expected, the former decreased slightly and the S/N ratio was also enhanced as the temperature increased, but a significant loss of symmetry, which did not compensate for the decrease in analysis time or improvement in the S/N ratio, was observed at temperatures over 25 °C. As a result, 25 °C was the working temperature chosen. The possibility of enhancing the sensitivity of the method by injecting higher sample volumes was also considered; this meant testing the injection of blank honey samples spiked with SFN in amounts ranging from 5 to 20 µL. The results showed an increase in S/N when up to 10 µL was injected, above which this ratio did not significantly improve and the chromatographic peaks began to appear somewhat deformed. Therefore, it was decided that 10 µL should be the corresponding injection volume.

With the chromatography conditions described it was possible to analyze SFN (a 4.9 min retention time (see Fig. 1)) in honey by

Table 1
Recovery data obtained for blank (SFN-free) honey samples treated accordingly to the procedures described in Sections 2.3, 3.1 and 3.3 ($n = 6$).

Quality control (QC) sample	Evaluation of the sample treatment Mean (%) ± RSD (%)				Evaluation of the matrix effect Mean (%) ± RSD (%)			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Rosemary	93 ± 4	92 ± 5	95 ± 4	97 ± 4	97 ± 5	99 ± 5	95 ± 5	93 ± 5
Multifloral	96 ± 4	99 ± 5	94 ± 4	98 ± 5	91 ± 6	93 ± 6	97 ± 5	92 ± 5
Orange blossom	99 ± 5	98 ± 4	93 ± 4	92 ± 4	89 ± 5	94 ± 5	96 ± 5	90 ± 6
Heather	98 ± 5	94 ± 5	99 ± 5	93 ± 5	48 ± 4	52 ± 5	70 ± 6	74 ± 6

QC1-4 µg/kg; medium QC2-40 µg/kg; QC3-400 µg/kg; QC4-1200 µg/kg.

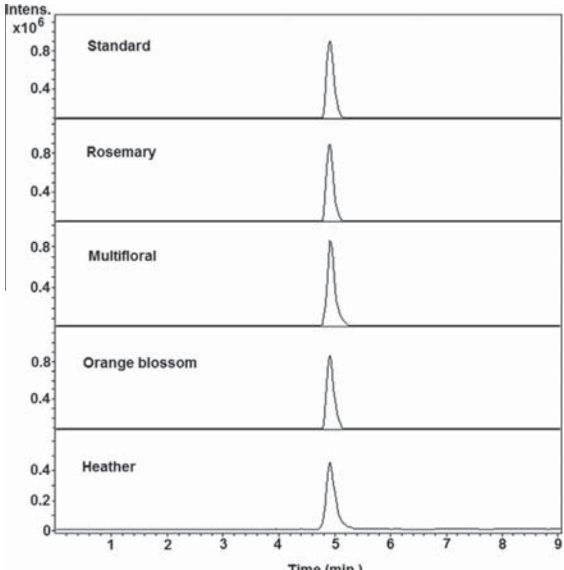

Fig. 1. Representative LC–ESI-MS/MS chromatograms (MRM (positive mode) using the quantification transition 178 > 114) obtained from a 50 μg/L standard solution of SFN and honey blank (SFN-free) samples of the four different botanical origins spiked at 40 μg/kg in SFN. It must be pointed out that the SFN concentrations were the same in the standard and spiked honey samples according to the proposed sample treatment and the unit conversion. The LC–ESI-MS/MS conditions are described in Section 2.4.

LC–ESI-MS/MS with an overall run time of 14 min; to our knowledge this is the most rapid proposal published for this matrix. It should be specified that SFN retention time was much higher (38.5 min) in the only method analyzing SFN in honey (Świderski et al., 2003).

3.3. MS optimization

SFN was previously analyzed in positive ESI mode (Ares et al., 2014; Dominguez-Perles et al., 2014; Hauder et al., 2011), the best results being obtained in terms of peak area and peak height. To establish optimal ESI-MS/MS conditions, several experiments (flow injection analysis) were conducted in order to choose these optimum ESI-MS/MS (positive mode) parameters and achieve maximum sensitivity by the infusion mode (5 μL/min) of standard and matrix-matched solutions (1000 μg/L) of SFN. The best results were found with the values described in Section 2.4. In ESI-MS, the compound exhibited intense $[M+H]^+$ (m/z 178, see proposed structure in Supplementary data, Fig. 1S) in full-scan spectra. This ion was selected as a precursor ion to obtain product ions for MS/MS analyses in MRM mode (see the MS/MS spectrum of the precursor ion in Supplementary data, Fig. 2S). The MRM transition that provided the highest signal was used for quantification (178 > 114 $[M-CH_3OS]^+$, see proposed structure in Supplementary data, Fig. 1S); meanwhile, the second MRM transition with a higher signal was used for confirmation (178 > 72 $[M-C_4H_9OS]^+$, see proposed structure in Supplementary data, Fig. 1S). To check how the matrix influenced ESI ionization, the peak areas of SFN in standard working solutions were compared with those acquired with blank honey samples spiked at four different concentrations (QC levels 1–4) following sample treatment (AF samples). SFN responses at the four concentrations tested in three of the botanical origins assayed (multifloral, rosemary and orange blossom) were higher than 89% for QC in all cases (see Table 1). Meanwhile, responses for heather honey samples were 70% and 74% for QC-3 and QC-4, respectively (see Table 1), and a much lower response was obtained for the QC-1 and QC-2 sample (~50%). If a comparison is made between SFN peaks in standard and matrix-matched solutions (see Fig. 1) at the same concentration levels, a slight (multifloral, rosemary and orange blossom) or a marked (heather) decrease in analyte signals can be observed due to the matrix effect. Hence, it was concluded that the matrix (honey) affected electrospray ionization of all the compounds, generating ion suppression; this is particularly noticeable in heather honey samples.

3.4. Validation of the LC–ESI-MS/MS method

To assess the selectivity of the method, extracts from blank honey samples of the four different botanical origins (SFN-free honey samples) along with honey samples spiked with SFN were injected (see Figs. 1 and 2). As can be seen, no chromatographic interference was observed at SFN retention time in any of the blank honey (SFN-free) samples analyzed. To identify the SFN peak in honey samples (spiked and with endogenous SFN content) from the four different botanical origins, a comparison was made of mass spectra of the SFN peak in standard solutions and honey samples with endogenous or spiked SFN content, at comparable concentrations and measured under the same conditions. Both mass spectra were quite similar (see Supplementary data, Fig. 2S), although some minor differences in ion intensity were observed and certain low intensity ions appeared only in honey spectra. The percentages relating to the intensity of quantification (178 > 114) and confirmation transitions (178 > 72) for SFN peak in standard solutions and honey samples were also compared. In all cases, the tolerances for relative transition intensities were below 10%, which is lower than the maximum tolerances permitted when mass spectrometric techniques are used (Commission Decision 2002/657/EC, 2002). Therefore, it can be concluded that the method was selective for determining SFN in honey. LODs and LOQs were determined experimentally for SFN in each botanical origin, as indicated in Section 2.5 (Table 2). As may be observed, the LOD and LOQ values were quite similar in all cases, ranging from 0.5 to 0.8 μg/kg (LOD) and 1.8 to 2.6 μg/kg (LOQ). To reiterate, no LOD or LOQ values were reported in the only publication analyzing SFN in honey (Świderski et al., 2003), so it was not

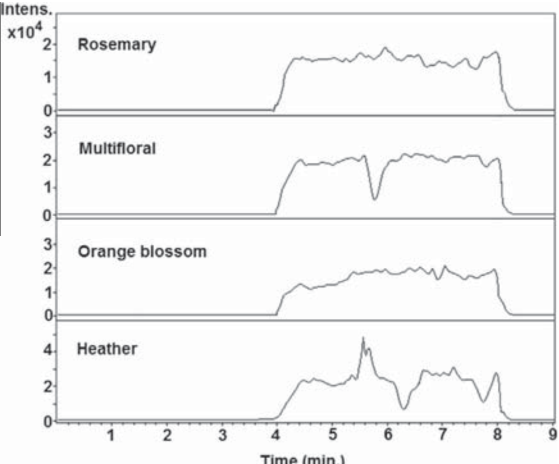

Fig. 2. Representative LC–ESI-MS/MS chromatograms (MRM (positive mode) using the quantification transition 178 > 114) obtained from honey blank (SFN-free) samples of the four different botanical origins assayed. The LC–ESI-MS/MS conditions are described in Section 2.4.

Table 2
Calibration curve data ($n = 6$) and LOD and LOQ values.

Compound	Calibration curve	Analytical range (μg/kg)	Slope confidence intervals	R^2	LOD[a] (μg/kg)	LOQ[a] (μg/kg)
Rosemary	Standard	1.8–1200	$1.6 \times 10^5 \pm 2.4 \times 10^4$	0.996	0.5	1.8
	Matrix-matched		$1.5 \times 10^5 \pm 2.6 \times 10^4$	0.995		
Multifloral	Standard	1.8–1200	$1.6 \times 10^5 \pm 2.4 \times 10^4$	0.996	0.5	1.8
	Matrix-matched		$1.4 \times 10^5 \pm 3.6 \times 10^4$	0.993		
Orange blossom	Standard	1.8–1200	$1.6 \times 10^5 \pm 2.4 \times 10^4$	0.996	0.5	1.8
	Matrix-matched		$1.4 \times 10^5 \pm 3.4 \times 10^4$	0.994		
Heather	Standard	2.6–1200	$1.6 \times 10^5 \pm 2.4 \times 10^4$	0.996	0.8	2.6
	Matrix-matched		$1.2 \times 10^5 \pm 1.1 \times 10^4$	0.999		

[a] LOD and LOQ values were calculated in matrix (honey).

Table 3
Summary of SFN detections in honey samples.

Botanical origin	Detects	Samples analyzed	Detections (μg/kg)	
			Low	High
Rosemary	5	10	<LOQ	16
Multifloral	6	10	<LOQ	18
Orange blossom	3	8	<LOQ	5
Heather	0	7	<LOD	<LOD

possible to make a proper comparison. As mentioned in Section 2.5, different calibration curves were used to quantify SFN in accordance with the botanical origin of the honey. In three cases (multifloral, rosemary and orange blossom), standard calibration curves could be employed as no significant matrix effect was observed (see Table 1 and Fig. 1); the slopes of the standard and matrix-matched calibration curves overlapped at the confidence intervals (see Table 2). However, matrix interference affected analyte ionization in heather honey samples, causing a suppression of the MS signal (>25%), while the slopes of the standard and matrix-matched calibration curves did not overlap at the confidence intervals (see Table 2). Consequently, SFN should be quantified only in honey from this botanical origin by means of matrix-matched calibration curves. This finding corroborated the need to evaluate the matrix effect prior to quantification of honey samples, which is frequently ignored, in order to correctly quantify the samples. The graphs obtained in all the calibration curves were straight lines, with linearity across the different concentration ranges studied, while the coefficient of the determination values (R^2) was above 0.99 in all cases (Table 2). The lack of bias was confirmed by a t test and a study of the distribution of residuals. It should also be pointed out that no peaks were observed at the retention times for the analyte when solvent or SFN-free honey samples were injected after running samples with high concentrations of the compound in question. This observation indicates a negligible carry-over effect when the proposed method is used to analyze SFN in honey. An evaluation of reinjection reproducibility revealed % RSD values <2% in all cases, demonstrating that the samples could be safely reanalyzed within 1 week. Finally, intra- and inter-day precision (% RSD values) were at all times lower than or equal to 7% (see Supplementary data, Table 2S). Accuracy (% RE values) ranged from 3% to 7% for the intra-day readings, and from 4% to 8% for the inter-day values (see Supplementary data, Table 2S). These results indicate that the present method is both precise and accurate, and it should be added that although an internal standard is recommended in most validation guidelines, it was not necessary in this study because of the degree of precision and accuracy.

3.5. Application of the method

With the proposed method honey samples from different botanical origins (multifloral, rosemary, heather and orange blossom) were analyzed to search for residues of SFN. All the samples were examined in triplicate. SFN was found in concentrations above the LOQs in several of the honey samples from multifloral, rosemary and orange blossom botanical origins at low concentration levels (5–18 μg/kg, see Table 3 and Fig. 3); in some cases it was possible to detect SFN but not quantify its presence (see Table 3). Meanwhile, no SFN residues were detected in any of the heather honey samples analyzed (see Table 3). These are quite interesting results, as SFN has never been reported in commercial honey samples. Moreover, the presence of SFN in several honey samples confirmed the suggestion by Truchado et al. (2010) relating to the potential presence of isothiocyanates in honey due to the degradation of their corresponding GLSs. It should also be pointed out that SFN was detected in herbal honey at 1.2 μM (Świderski et al., 2003), but in this case the authors used an SFN sugar extract to feed bees in a controlled beehive. Thus, on that occasion SFN content was directly related with the food given to the bees, and not with the activity of honey bees when collecting the pollen or nectar from plants containing this compound. Finally, it has been demonstrated that there is a need to develop an analytical method to determine SFN in honey; such a method should, as we have proposed, be very sensitive, as the content of SFN observed was lower than 20 μg/kg.

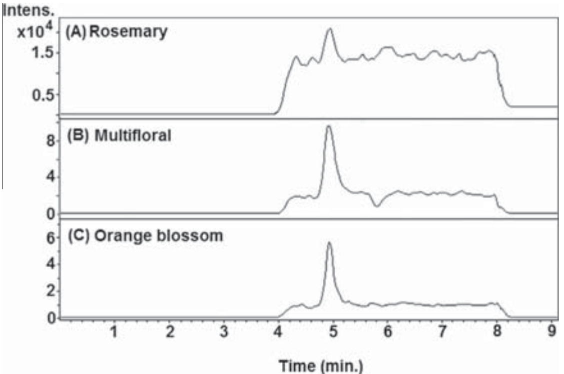

Fig. 3. Representative LC–ESI-MS/MS chromatograms (MRM (positive mode) using the quantification transition 178 > 114) obtained from honey blank (SFN-free) samples of a (**A**) commercial honey sample (rosemary) with a SFN content lower than the LOQ, a (**B**) commercial honey sample (multifloral) with a SFN content of 10 μg/kg, and a (**C**) commercial honey sample (orange blossom) with a SFN content of 5 μg/kg. The LC–ESI-MS/MS conditions are described in Section 2.4.

4. Conclusions

A new analytical method has been developed and optimized to simultaneously identify and quantify SFN in commercial honey samples from very different botanical origins (multifloral, rosemary, heather and orange blossom). The proposed extraction

method based on an SPE with polymeric sorbents has proven to be efficient with a relatively low consumption of solvents. Moreover, the LC–ESI-MS/MS method was fully validated and the data demonstrated that it is consistent and reliable, with a wide linear range of applicability; it was only necessary to employ matrix-matched standards to perform correct ESI-MS/MS quantification of SFN in heather honey samples, as significant ion suppression was observed. This finding should be taken into account in an analysis of honey samples from other different botanical origins, as the matrix effect need always be evaluated prior to quantification of honey samples. The usefulness of this method was proven by an analysis of honey samples from different botanical origins. SFN was detected in certain samples from multifloral, rosemary and orange blossom botanical origins at low concentration levels, while this was not found in any of the heather honey samples analyzed. Finally, the observed presence of SFN in honey samples from non-treated beehives is an interesting finding hitherto unreported.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.02.085.

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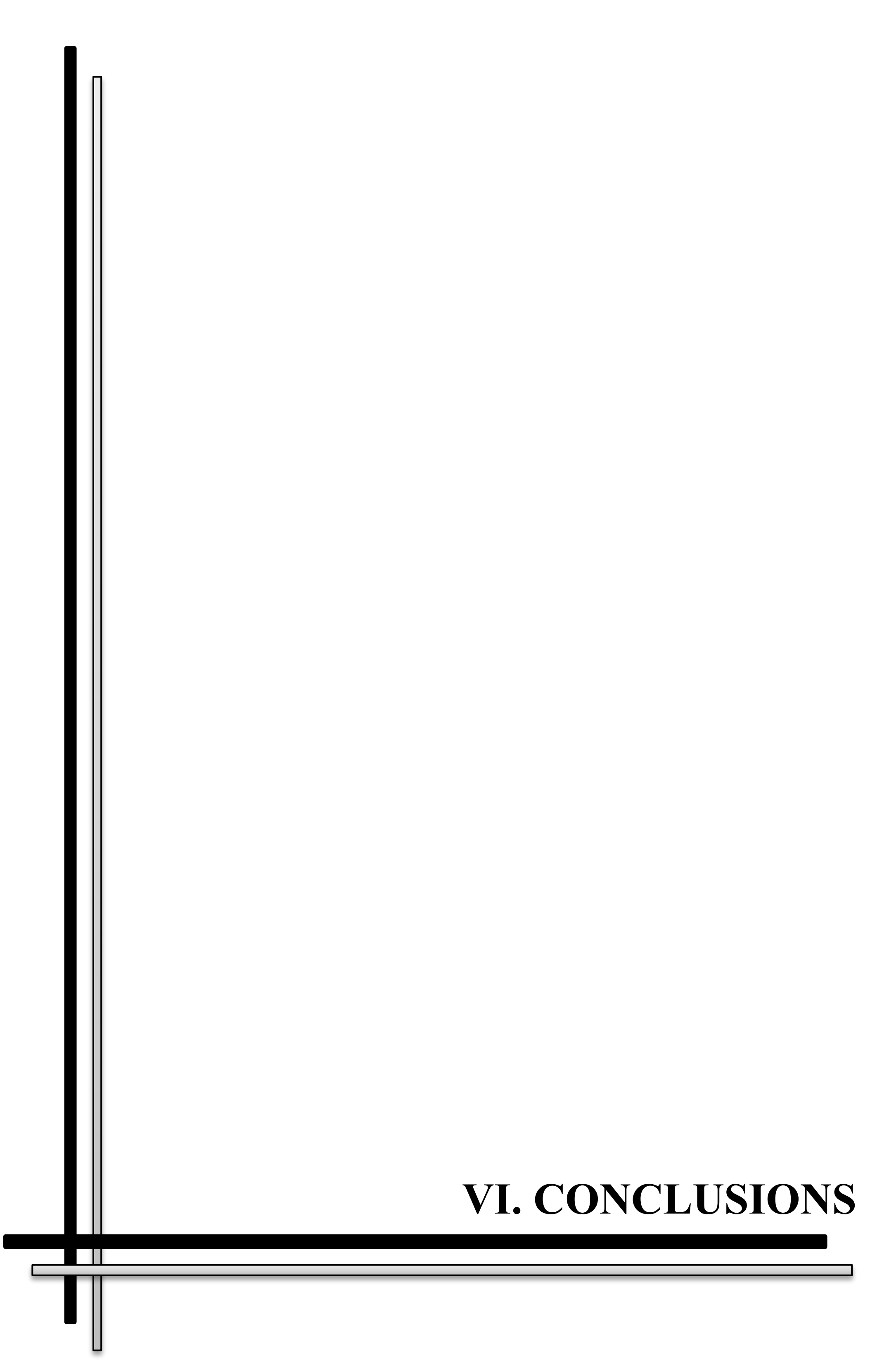

VI. CONCLUSIONS

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VI.1. GENERAL CONCLUSIONS

- This PhD Thesis has aimed to develop analytical methodologies based on LC coupled to several detectors (DAD, MS, MS/MS), as fast, simple, and environmentally friendly protocols, of maximum possible efficiency and sensitivity, to determine resveratrol isomers, GLSs and SFN in bee products (honey, beeswax and bee pollen), while investigating the potential of these compounds to control *Nosema* infection in honey bees (*Apis mellifera*). In addition, new methods with the same characteristics as those described above have been proposed to extract and determine GLSs and SFN from broccoli to obtain them in such a way that this is compatible with their potential administration to honey bees.

- It has been demonstrated that there is a need to produce specific analytical methods to determine resveratrol isomers, intact-GLSs and SFN in bee products; such methods should, as we have proposed, be very sensitive, as in most cases the content observed was at the µg/kg level. Moreover, the detection of resveratrol isomers, GLSs and SFN in bee products that would be consumed by humans, such as honey and bee pollen, implies that they could be positively affected by the beneficial health properties of these compounds, with the accompanying increase in the nutritional and bioactive value of the products in question.

VI.2. SPECIFIC CONCLUSIONS

In accordance with the data and results summarized in the corresponding papers, the present PhD Thesis has provided the following conclusions:

<u>Resveratrol and related compounds</u>

- Pioneer, selective, efficient and sensitive LC-MS analytical methods were proposed and validated to determine resveratrol and piceid isomers in beeswax (**Paper I**) and bee pollen (**Paper II**).

  The utility of the proposed methods was verified by an analysis of several samples from different origins (experimental, commercial and organic). The

observed presence of resveratrol isomers (*trans* and *cis*) in beeswax and bee pollen, or *cis*-piceid in one bee pollen sample, is an interesting finding, as this has hitherto not been reported; this is significant in commercial bee pollen samples as it would increase the nutritional value of this food supplement. In addition, the detection of the analytes in samples obtained from experimental apiaries has demonstrated that residues of these compounds could be found in bee products after their being supplied to the hive.

<u>Glucosinolates and sulforaphane</u>

Broccoli matrices

- Two different, rapid, efficient and environmentally friendly extraction procedures (heated water-**Paper IV** and PLE-**Paper VII**) to obtain intact-GLSs from broccoli leaves have been developed, optimized by means of experimental designs, and validated. In addition, new LC-DAD-MS (**Paper IV**) and UHPLC-MS/MS (**Paper VII**) conditions were also proposed; in both cases these were faster than previous proposals.

  Broccoli leaves from different cultivars were analyzed with both methodologies and differences in intact-GLSs content depended on the cultivar. Most of the studied compounds were identified in these samples, and the greatest total content of intact-GLSs was found in broccoli leaf samples from the *Viola* (**Paper IV**) and *Parthenon* (**Paper VII**) cultivars.

- A study of the effect of temperature and light exposure on the total intact-GLSs content (**Paper VI**) has demonstrated that a drying time of 12h at 60ºC is required in order to obtain a constant amount of dried material, but the heating procedure employed had no influence on total intact-GLSs content. Furthermore, results of stability studies in different storage conditions have shown that broccoli leaf extracts should be stored at lower temperatures (4ºC) and protected from light exposure in order to maintain maximum total intact-GLSs content in broccoli leaf extracts.

- A new rapid LC-DAD method has been created and validated to determine SFN in broccoli (florets, stems and leaves); this takes less than 5 min, which is the fastest LC proposal published (**Paper V**). Moreover, for the first time an optimization process has been devised for converting GRA to SFN for three different broccoli matrices; significant differences were observed in the action of most of the parameters examined depending on the matrix. The proposed extraction method based on a solvent extraction with MTBE followed by an SPE with silica cartridges has proven to be efficient and more environmentally friendly than previous proposals, due to use of MTBE instead of chlorinated solvents such as DCM.

  The proposed method was applied to determine SFN content in broccoli samples (florets, leaves, and stems) from two different cultivars (*Parthenon* and *Marathon*). SFN was detected in all the samples, and its content was quite different depending on the part and variety of the broccoli. Highest SFN content was found in broccoli floret samples, whilst the lowest amount of SFN was detected in broccoli leaf samples.

- Finally, it was demonstrated that broccoli by-products (leaves) possessed a significant GLS and SFN content. These by-products, which are usually discarded, could be used as complements in animal diets or as nutraceutical reservoirs that reduce their environmental impact and at the same time give them a certain economic value.

Bee products

- New and different analytical methods have been created, optimized and validated to identify and quantify intact-GLS in bee pollen (**Paper VIII**), honey (**Paper IX**) and beeswax (**Paper X**). The same LC conditions and slightly different MS/MS parameters were employed in all cases. It should be said that these compounds have never been investigated before in bee products, with the exception of honey.

  Intact-GLSs were efficiently extracted from bee pollen (**Paper VIII**) by an SLE with heated water, followed by SPE with an amino sorbent. This proposal is much faster than the overnight extraction procedure employed in the only study in which the compounds were analyzed in a similar matrix

(plant pollen). An analysis of different bee pollen samples proved the utility of this method. Several of the GLSs examined were identified in certain samples, which is an interesting finding hitherto unreported.

A similar extraction procedure was proposed for the analysis of intact-GLSs in honey (**Paper IX**). In this case honey was dissolved in heated water, followed by an SPE with amino SPE cartridges. This was the first time that intact-GLSs were quantified in honey from different botanical origins, which confirmed the results previously published relating to the presence of these compounds. It has been also demonstrated that there is a need to develop a specific analytical method to determine intact-GLSs in honey; such a method should, as we have proposed, be very sensitive, as the content observed was at the μg/kg level.

The proposed extraction method to obtain intact-GLSs from beeswax based on three steps (dissolution, liquid-liquid extraction, and concentration) has proven to be efficient, simple and relatively cheap (a low consumption of solvents). No residues, however, of the intact-GLSs studied were detected in any of the samples.

Finally, the proposed methods might also be of interest to other researchers as new analytical tools to be used in further investigative work on these compounds in bee matrices, for instance, as chemical markers for determining the botanical origin of honey, bee pollen and beeswax or as a bio-indicator of honey freshness.

- Determining SFN in honey (**Paper XI**) from different botanical origins was performed with a novel and validated LC-MS/MS method, which was much shorter and sensitive than the sole proposal previously published. The optimized extraction procedure based on an SPE with polymeric sorbents has proven to be efficient, with a relatively low consumption of solvents.

The usefulness of this method was proven by an analysis of honey samples from different botanical origins. SFN was detected in certain samples at low

concentration levels. Moreover, the observed presence of SFN in honey samples from non-treated hives is an interesting finding hitherto unreported.

- Finally, the detection of GLSs and SFN in bee products to be consumed by humans, such as honey and bee pollen, implies that they could be positively affected by the beneficial health properties of these compounds, with an accompanying increase in the nutritional and bioactive value of the products in question.