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AVANCES EN EL ESTUDIO DE LOS ÉSTERES DE CROCETINA, PICROCROCINA Y FLAVONOIDES DEL AZAFRÁN ESPECIA

ADVANCES IN THE STUDY OF CROCETIN ESTERS, PICROCROCIN AND FLAVONOIDS FROM SAFFRON SPICE

Memoria presentada por

Ana María Sánchez Gómez

para optar al grado de Doctor

Directores: Dr. Gonzalo L. Alonso Díaz-Marta

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Albacete, 2009

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CERTIFICA:

Que la presente memoria de investigación titulada: "Avances en el estudio de los ésteres de crocetina, picrocrocina y flavonoides del azafrán especia", que presenta Dña. Ana María Sánchez Gómez para optar al grado de Doctor, ha sido realizada bajo la dirección del Dr. Gonzalo L. Alonso Díaz-Marta y del Dr. Manuel Carmona Delgado en el Departamento de Ciencia y Tecnología Agroforestal y Genética de la Universidad de Castilla-La Mancha. Y para que conste, firma el presente certificado.

Albacete, 11 de noviembre de 2008

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CERTIFICAN:

Que la presente memoria de investigación titulada: "Avances en el estudio de los ésteres de crocetina, picrocrocina y flavonoides del azafrán especia", que presenta Dña. Ana María Sánchez Gómez para optar al grado de Doctor, ha sido realizada bajo su dirección, y a su juicio, cumple todos los requisitos para proceder a la lectura y defensa pública, por lo que autorizan su presentación en el Departamento de Ciencia y Tecnología Agroforestal y Genética de la Universidad de Castilla-La Mancha. Y para que así conste, firman el presente certificado en

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Fdo.: Dr. Gonzalo L. Alonso Díaz-Marta Dr. Manuel Carmona Delgado

A mis padres y a mi hermana,

por quererme y apoyarme en todo momento.

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PRÓLOGO

PROLOGUE

PRÓLOGO

Esta memoria de tesis es un compendio de siete artículos científicos sobre los principales componentes presentes en los extractos acuosos del azafrán especia. Cinco de estos artículos ya han sido publicados en revistas científicas internacionales de alto índice de impacto, mientras que los otros dos están en proceso de revisión por pares. Todos los trabajos se han planteado desde la Cátedra de Química Agrícola de la ETSI Agrónomos de Albacete y en ellos han colaborado otros miembros de las Universidades de Castilla-La Mancha, de Alcalá de Henares, Autónoma de Madrid, Politécnica de Valencia y Aristóteles de Tesalónica; así como, del CEBAS-CSIC de Murcia.

Parte del artículo de cinética de los ésteres de crocetina se llevó a cabo mediante una estancia en Grecia, en la Facultad de Químicas de la Universidad Aristóteles de Tesalónica, bajo la supervisión de la Profesora María Z. Tsimidou. Por ello, esta tesis opta a la mención de Doctorado Europeo.

De acuerdo a lo expuesto y con el deseo de proyectar los resultados tanto a nivel nacional como internacional, se ha combinado el español y el inglés en la redacción de este documento. Se ha intentado que los aspectos necesarios para la comprensión de la tesis y los resultados más importantes queden recogidos en ambos idiomas. En el índice, que se encuentra en español en la página xv y en inglés en la xvii, se detalla la página de cada apartado en su correspondiente idioma.

Así mismo, esta tesis incluye un CD que contiene esta memoria en formato digital, junto con las comunicaciones a congresos (seis internacionales y tres nacionales) originadas a partir de sus trabajos y el curriculum vitae de la doctoranda.

PROLOGUE

This thesis comprises seven articles on the principal components present in aqueous extracts of saffron. Five articles have already been published in international journals of high impact factor, while the other two are undergoing the process of peer review. All the studies on which these papers are based were planned in the group of Agricultural Chemistry of the ETSI Agrónomos of Albacete with the collaboration of the Universities of Castilla-La Mancha, Alcalá de Henares, Autonomous of Madrid, Polytechnic of Valencia and Aristotle of Thessalonica, together with the research institute CEBAS-CSIC of Murcia.

Part of the work for the article on the kinetics of crocetin esters was carried out in the Faculty of Chemistry of the Aristotle University of Thessalonica, under the aegis of Professor María Z. Tsimidou, which is why the thesis aims at being a European Doctorate.

Based on the above and in the hope that the results can be made known both at national and international level, the thesis is written in both Spanish and English. The aspects considered essential for understanding the thesis and the most important results will be presented in both languages. The index, page xv in Spanish and xvii in English, directs the reader to the page number of each section in the corresponding language.

The thesis is also presented in CD format which, in addition, contains communications to congresses (six international and three national) based on the contents, together with the curriculum vitae of the author.

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RESUMEN

Esta tesis pretende avanzar en el conocimiento de los ésteres de crocetina, picrocrocina y flavonoides de los extractos acuosos del azafrán especia.

Para ello, se ha trabajado en distintos ámbitos: 1) su aislamiento, identificación y cuantificación; 2) la cinética de los ésteres de crocetina y picrocrocina bajo el efecto de la temperatura; 3) el desarrollo de metodologías para el control de calidad de la especia en las empresas; y 4) futuras líneas de trabajo sobre estos compuestos, como su análisis sensorial y la aplicación de ultrafiltración en los extractos acuosos del azafrán.

Los estudios realizados se han recogido en siete artículos científicos para su publicación en revistas internacionales.

En el primer artículo, se identifican tentativamente los ésteres de crocetina, la picrocrocina y los compuestos glicosilados relacionados con ella de los extractos de azafrán y de gardenia.

En el segundo artículo, se identifican tentativamente los flavonoides de los extractos de azafrán y se compara su contenido en muestras de diferentes orígenes geográficos, observando que se produce la discriminación de las mismas según el contenido de kaempferol-3-*O*-soforósido.

El tercer artículo describe la cinética de deterioro de cada uno de los ésteres de crocetina en extractos acuosos sometidos a tratamiento térmico en oscuridad, abordando

los cambios que se producen en los espectros ultravioleta-visibles (UV-vis) y en los cromatogramas de los extractos, los parámetros cinéticos y los aspectos termodinámicos.

En el cuarto artículo, se realizan los mismos estudios cinéticos para la picrocrocina y además, se aborda el estudio de su umbral de percepción gustativa.

En el quinto artículo, se generan modelos multivariantes para la determinación rápida de los ésteres de crocetina y de la picrocrocina del azafrán especia mediante espectrofotometría UV-vis de sus extractos acuosos.

En el sexto artículo, se propone y valida de forma interna un método de extracción en fase sólida para la determinación del contenido de picrocrocina en el control de calidad en las empresas.

Por último, el séptimo artículo recoge los parámetros de rendimiento de varios dispositivos de ultrafiltración por centrifugación respecto a los principales componentes de los extractos acuosos del azafrán especia.

SUMMARY

With this thesis, we hope to advance our knowledge of the crocetin esters, picrocrocin and flavonoids found in the aqueous extracts of saffron spice.

To do this we have worked in different areas: 1) their isolation, identification and quantification; 2) the kinetics of crocetin esters and picrocrocin, as affected by temperature; 3) the development of methods to control the quality of saffron at a commercial level; and 4) the first steps of future work lines in these compounds, such as sensory analysis and the application of ultrafiltration to the aqueous extracts of saffron.

The studies carried out have been presented in seven papers in international scientific journals.

In the first article, we tentatively identified crocetin esters, picrocrocin and its related compounds in the extracts from saffron and gardenia.

In the second article, we tentatively identified the flavonoids in the aqueous extracts of saffron and compared the contents in samples from different geographical areas, observing that they could be differentiated by the kaempferol-3-*O*-sophoroside content.

The third article describes the degradation kinetics of individual crocetin esters in aqueous extracts upon thermal treatment in the dark, referring to the changes in the ultraviolet-visible (UV-vis) spectra and chromatograms of the extracts, and their kinetic and thermodynamic parameters.

The same kinetic studies were applied to picrocrocin in the fourth article and its taste detection threshold was studied.

In the fifth article, we generated multivariate models for the rapid determination of crocetin esters and picrocrocin of saffron spice by UV-vis spectrophotometry of aqueous extracts.

In the sixth article, we proposed and validated internally a solid phase extraction method for determining the picrocrocin content to be applied to quality control in commercial companies.

Lastly, the seventh article dealt with several centrifugal ultrafiltration devices and their performance with regard to the principal components of the aqueous extracts of saffron.

CAPÍTULO 1. JUSTIFICACIÓN

CHAPTER 1. JUSTIFICATION

1. JUSTIFICACIÓN

Europa ha tenido siempre un papel preponderante en la comercialización del azafrán (*Crocus sativus* L.) a nivel mundial. Existen vestigios arqueológicos que demuestran la tradición de este cultivo en la Cuenca Mediterránea desde hace miles de años. En la actualidad, el 90% de la comercialización mundial está en manos de empresas europeas y, aunque la producción en Europa se ha visto mermada, todos aquellos pueblos donde se cultiva o se cultivó en el pasado están tratando de mantener la rica cultura generada en torno a esta especia. Dentro de Europa, España se ha distinguido por alcanzar una calidad extraordinaria en sus producciones de azafrán especia y fue pionera en la creación de la Denominación de Origen "Azafrán de La Mancha".

Enmarcada en la Comunidad Autónoma que más azafrán produce de España, la Cátedra de Química Agrícola de la ETSI Agrónomos de Albacete viene realizando investigaciones sobre el azafrán especia desde el año 1986. Los trabajos inmediatamente anteriores a los de esta tesis doctoral profundizaban en el proceso de deshidratación y en su influencia sobre la calidad de la especia resultante, poniendo especial atención en el mecanismo de generación de su aroma.

En esta tesis doctoral, los trabajos se centran en los compuestos presentes en los extractos acuosos del azafrán especia que determinan o participan en su color y en su sabor: los ésteres de crocetina, la picrocrocina y los flavonoides.

La importancia del estudio de los extractos acuosos de azafrán radica en sus múltiples aplicaciones, tanto culinarias como en las industrias alimentaria y

farmacéutica, o incluso para tinción. Las aplicaciones relacionadas con el color se deben a la particularidad que tienen los carotenoides del azafrán, los ésteres glicosilados de la crocetina, de ser solubles en agua generando toda una gama de tonos amarillo-anaranjados según su concentración. Sin embargo, lo que distingue a esta especia de otras y de otros colorantes naturales o artificiales es su sabor y su aroma. En el sabor se piensa que intervienen la picrocrocina y los flavonoides, mientras que el safranal está directamente implicado en el aroma. Además, tanto la picrocrocina como los ésteres de crocetina estarían involucrados en la generación del safranal y en la de otros compuestos que participan en el aroma.

La disponibilidad de patrones comerciales de los ésteres de crocetina, de picrocrocina y de los flavonoides del azafrán es muy limitada y su pureza deja mucho que desear. Por tanto, antes de acometer cualquier estudio sobre estas sustancias, ha sido necesario proceder a su aislamiento y purificación, adaptando a nuestras necesidades y capacidades los métodos existentes o creando otros nuevos.

El punto de partida para el estudio de los principales compuestos de los extractos acuosos de azafrán ha sido su identificación por cromatografía de líquidos-espectrometría de masas. Respecto a los ésteres de crocetina, existe una amplia bibliografía referente a su análisis y elucidación estructural, tanto en azafrán como en gardenia (*Gardenia jasminoides* Ellis), de cuyos frutos se obtiene el colorante natural del mismo nombre. Sin embargo, la falta de consenso en cuanto a los compuestos presentes en cada especie ha hecho necesario un estudio detallado para identificar los distintos ésteres de crocetina y relacionarlos con los resultados descritos por otros autores. Además, cuando se inició esta tesis, los trabajos sobre generación de aromas realizados por el Dr. Carmona demostraban la obtención de safranal a partir de los ésteres de crocetina del azafrán, pero no de gardenia. Esos resultados requerían la investigación de las diferencias entre los ésteres de crocetina presentes en ambas especies y de otros posibles precursores del aroma tales como la picrocrocina y otros derivados glicosilados. A su vez, para los estudios de esta tesis resultaba muy

interesante la identificación de todos estos compuestos en el mismo análisis. Por ello, se decidió abordar de forma conjunta todos estos aspectos, dando lugar al primer artículo que aparece en esta memoria. En relación a los flavonoides, su identificación y la cuantificación de su contenido permitieron estudiar su contribución a la diferenciación geográfica del azafrán tal y como puede verse en el segundo artículo.

Una vez identificados los componentes, se ha estudiado la cinética de degradación de los ésteres de crocetina y de la picrocrocina para determinar cuestiones tan interesantes como la estabilidad de unos ésteres de crocetina frente a otros, el posible cambio conformacional de sus formas trans y cis y la evolución de la picrocrocina tras un tratamiento térmico. Muchos de los usos del azafrán implican un calentamiento y se ha querido evaluar su impacto en el sabor, mediante las variaciones que produce el calentamiento en el contenido de picrocrocina y su relación con el umbral de percepción gustativa. El conocimiento generado con estos estudios podría ser muy útil para la producción y estabilización de extractos acuosos de azafrán. Hasta el momento, los estudios descritos en la bibliografía trataban de establecer las condiciones para el almacenamiento de la especia y se centraban en su deterioro en estado sólido, a través de medidas del poder colorante o del poder amargo. Así mismo, los pocos trabajos existentes sobre extractos acuosos se basaban en los parámetros mencionados y en la evolución del espectro UV-vis. Por tanto, describían la evolución conjunta de todos los componentes de los extractos, pero en ningún caso se abordaba la cinética individualizada de cada compuesto.

Este estudio individualizado es de gran interés también para el control de calidad en las empresas que envasan y comercializan azafrán, siempre y cuando se pueda llevar a cabo con métodos rápidos, sencillos y sin grandes inversiones. Las técnicas de calibración multivariante se han aplicado en la resolución de muestras complejas a partir de datos espectroscópicos, sin la necesidad de un proceso de separación previo y han mostrado buenos resultados en otros productos agroalimentarios como aceites o vinos, en la industria alcoholera, en la determinación simultánea de

colorantes en mezclas e incluso en formulaciones farmacéuticas. Estas técnicas se presentan como una solución para conseguir métodos rápidos y de bajo coste, por lo que decidimos desarrollar modelos multivariantes para la determinación de los principales ésteres de crocetina y la picrocrocina, a partir del espectro UV-vis del extracto acuoso del azafrán.

Siguiendo en la línea de desarrollo de métodos rápidos y sencillos para el control de calidad, estudiamos la aplicación de cartuchos de extracción en fase sólida para aislar y determinar en el menor tiempo posible el contenido de picrocrocina por espectrofotometría UV-vis, evitando las interferencias que generan los ésteres de crocetina en su máximo de absorción.

En un nuevo campo de trabajo, hemos iniciado estudios sobre separación de los componentes hidrosolubles del azafrán mediante membranas de ultrafiltración. Hasta el momento, los procedimientos de separación y purificación para estos componentes se habían centrado en la cromatografía en columna, HPLC preparativa o analítica, cromatografía en contracorriente y TLC. La ultrafiltración es una atractiva alternativa a estos métodos debido a las condiciones suaves de operación, protección de la muestra de factores externos, separación sin adición de disolventes y a su alta selectividad. Este campo de estudio podría contribuir, desde el punto de vista analítico, a nuevas aplicaciones de separación, concentración o purificación; y desde el punto de vista de la Tecnología de Alimentos, a la introducción de modificaciones en el color, sabor o propiedades funcionales de los extractos de azafrán mediante cambios en la proporción de sus componentes.

En definitiva, todos nuestros trabajos están motivados por el deseo de avanzar en el conocimiento del azafrán especia para así contribuir, con bases científicas, a que siga siendo un producto muy valorado, a la defensa de su calidad y a la búsqueda de nuevas aplicaciones.

1. JUSTIFICATION

Europe has always been in the forefront of the saffron (*Crocus sativus* L.) trade. Archaeological evidence exists revealing the tradition that this crop has enjoyed in the Mediterranean Basin for thousands of years. At present, too, 90% of the world saffron trade is in European hands and, although production in Europe has fallen off, all the towns and areas where it has been grown traditionally are making great efforts to retain and maintain the customs and cultures associated with its growth. Within European countries, Spain is known for the extraordinary quality of its saffron and was pioneer in the creation of a Designation of Origin for the spice –"*Azafrán de La Mancha*".

Situated within the Autonomous Community that produces most of the saffron in Spain, the Group of Agricultural Chemistry of the ETSI Agrónomos of Albacete has been directing research into saffron since 1986. The studies carried out immediately previous to this thesis looked at the process of dehydration and its effect on product quality, with special attention on the mechanism of aroma generation.

In this thesis, the work focuses on the components present in the aqueous extracts of saffron that determine its colour and taste: crocetin esters, picrocrocin and flavonoids.

The importance of studying the aqueous extracts of saffron lies in their multiple applications in cookery, in food and pharmaceutical industries, or even as dyes. Colour-related applications are linked to the particular characteristic of the carotenoids contained in saffron, i.e. the glycosylated esters of crocetin, which are water soluble, generating a whole range of yellow-orange colours depending on their concentration.

However, what distinguishes this spice from others and from other natural or synthetic colorants is its taste and aroma. Picrocrocin and the flavonoids are thought to influence taste, while safranal is directly implicated in the aroma. Moreover, both picrocrocin and the esters of crocetin are probably involved in the generation of safranal and of the other compounds that contribute to the aroma.

The availability of commercial standards of the crocetin esters, picrocrocin and flavonoids from saffron is limited and their purity leaves much to be desired. Therefore, before undertaking a study of these substances, it has been necessary to isolate and purify them, adapting existing methods to our needs and developing new ones.

The starting point in the study of the principal components of saffron aqueous extracts was their identification by liquid chromatography-mass spectrometry. As regards crocetin esters, there is a generous bibliography concerning their analysis and structure elucidation, both in saffron and gardenia (*Gardenia jasminoides* Ellis), from whose fruits the colorant of the same name is obtained. However, the lack of consensus concerning the compounds present in each species has made it necessary to make a detailed study to identify the different esters of crocetin and to relate the findings with the results obtained by other authors. Furthermore, when we undertook this thesis, Dr. Carmona, in his studies of the generation of aromas, had demonstrated that safranal was obtained from crocetin esters of saffron but not from gardenia. These findings meant it was necessary to look into the differences between the crocetin esters of both species and study other possible precursors of aroma, such as picrocrocin and other glycosylated derivates. Similarly, for the purposes of this thesis, it was seemed important that such compounds could be identified in the same analysis. We therefore decided to treat all these aspects jointly and this is what gave rise to the first article that appears in this text. As regards the flavonoids of saffron, their identification and quantification have enabled us to study their contribution to the geographical differentiation of saffron –as can be seen in the second article.

After identifying the components, we studied the degradation kinetics of the crocetin esters and of picrocrocin to determine such interesting questions as the stability of some of the esters compared with the others, the possible conformational exchange of trans and cis forms and the evolution of picrocrocin after heat treatment. Many of saffron's uses involve heat and we evaluated the influence of such on its taste by looking at variations in the picrocrocin content brought about by heating and the relation with its taste detection threshold. The knowledge generated from these studies, it was thought, would be useful for the production and stabilisation of aqueous extracts of saffron. Until then, studies had been directed at establishing the best storage conditions and concentrated on the deterioration of the product in solid form through the colouring and the bitterness strength. The few studies on aqueous extracts were based on the above parameters and on the evolution of the UV-vis spectrum. In other words, they studied the overall behaviour of the components of the extracts but did not look at the individual kinetics of each component.

Such an individualised study is of great interest for quality control in packaging and marketing companies, as long as the methods involved were rapid, simple and inexpensive. Multivariate calibration techniques have been applied to the resolution of complex samples from the spectral data without the need for a prior separation step and good results have been achieved with other agro-food products such as oils and wines, in the alcohol industry, in the simultaneous determination of colorants in mixtures, and in pharmaceuticals. These techniques are considered a solution for obtaining rapid low-cost methods, and so we have developed multivariate models for determining the principal crocetin esters and picrocrocin from the UV-vis spectrum of the saffron extract.

Following the development of simple rapid methods for quality control, we studied the application of solid phase extraction (SPE) cartridges to isolate and determine the picrocrocin content by UV-vis spectrophotometry, avoiding the interferences generated by crocetin esters at its absorption maximum.

In a new field, we have undertaken studies on the separation of water-soluble components of saffron by membrane ultrafiltration. Until now, the separation and purification of these compounds have focused on column chromatography, preparative or analytical HPLC, countercurrent chromatography and TLC. Ultrafiltration is an attractive alternative to these methods due to the mild operation conditions, the protection of samples from external factors, solvent-free separation and relatively high selectivity. This field of study, from an analytical point of view, may contribute to new applications of separation, concentration or purification, and, from a food technology point of view, to the ability to modify the colour, taste or functional properties of aqueous extracts of saffron through changes in the proportion of the components.

Indeed, all our studies are motivated by the desire to deepen our knowledge of saffron and to contribute with solid scientific arguments to its continuation as a valuable product, to the defence of its quality and to possible new applications.

CAPÍTULO 2. OBJETIVOS

CHAPTER 2. OBJECTIVES

2. OBJETIVOS

El objetivo general de esta tesis doctoral es ampliar el conocimiento científico-técnico de los principales componentes de los extractos acuosos del azafrán especia: los ésteres de crocetina, la picrocrocina y los flavonoides. Así mismo, se pretende divulgar los avances en el estudio de estos compuestos a nivel internacional, mediante la publicación de artículos en foros de alto interés científico en el ámbito de la Química de los Productos Agrícolas y de la Tecnología de Alimentos.

Para conseguir estos objetivos generales, se han planteado cuatro líneas de acción. La primera de ellas es prácticamente común a todos los artículos y engloba las etapas de aislamiento, identificación y cuantificación de los compuestos a estudiar. Dentro de esta línea de acción, se han establecido los siguientes objetivos específicos:

▪ *Objetivo nº 1.* Aislamiento de los ésteres de crocetina, picrocrocina y flavonoides del azafrán en cantidades suficientes y con el grado de pureza necesario para llevar a cabo posteriores análisis físicos, químicos y/o sensoriales.

▪ *Objetivo nº 2.* Identificación de los ésteres de crocetina, picrocrocina y flavonoides que están presentes en el azafrán especia.

▪ *Objetivo nº 3.* Cuantificación de los ésteres de crocetina, de la picrocrocina y de los principales flavonoides de los extractos acuosos de azafrán, estudiando en este último caso la posibilidad de diferenciar azafranes de distinto origen geográfico por su contenido en flavonoides.

La segunda línea de acción está dedicada a los estudios cinéticos de los principales compuestos y su objetivo específico puede formularse como:

▪ *Objetivo nº 4.* Estudio de la cinética de deterioro de los ésteres de crocetina y de la picrocrocina en extractos acuosos de azafrán y en disoluciones acuosas de los compuestos aislados, mantenidos en oscuridad y sometidos a tratamiento térmico.

La tercera línea de acción tiene un carácter eminentemente práctico, pues está orientada al control de calidad del azafrán en las empresas envasadoras y comercializadoras. Su objetivo específico ha sido el siguiente:

▪ *Objetivo nº 5.* Desarrollo de metodologías rápidas, de baja manipulación y de bajo coste para el análisis de los ésteres de crocetina y/o picrocrocina en muestras de azafrán, que puedan ser aplicadas de forma rutinaria en las empresas envasadoras y comercializadoras del azafrán para el control de calidad de las materias primas y de los productos terminados.

La última línea de acción abre nuevos campos de trabajo como el análisis sensorial de forma individualizada de los compuestos de los extractos, o la separación por membranas. Como objetivos específicos de esta línea se han planteado los siguientes:

▪ *Objetivo nº 6.* Desarrollar estudios sensoriales para establecer el umbral de percepción gustativa de la picrocrocina.

▪ *Objetivo nº 7.* Estudiar, a pequeña escala, la aplicación de técnicas de ultrafiltración con membranas semipermeables para separar o concentrar los principales compuestos de los extractos acuosos del azafrán.

2. OBJECTIVES

The general objective of this doctoral thesis is to deepen our scientific-technical knowledge of the main components of the aqueous extracts of saffron: crocetin esters, picrocrocin and flavonoids. We intend to make the results available internationally by publishing them in forums of general scientific knowledge in the field of Agricultural Product Chemistry and Food Technology.

To this end, we have set four lines of action. The first is common to practically all the articles and involves the isolation, identification and quantification of the compounds to be studied. Within this line of action, we have established the following specific objectives:

- *Objective nº 1.* Isolation of the crocetin esters, picrocrocin and flavonoids of saffron on sufficient quantities and with sufficient purity to carry out the planned physical, chemical and/or sensorial analyses.

- *Objective nº 2.* Identification of the crocetin esters, picrocrocin and flavonoids present in the spice.

- *Objective nº 3.* Quantification of the crocetin esters, picrocrocin and principal flavonoids in aqueous extracts of saffron, studying, in this last case, the possibility of differentiating saffron samples from different geographical areas.

The second line of action is dedicated to kinetic studies of the main components and its specific objective might be described as:

▪ *Objective nº 4.* Study of the degradation kinetics of crocetin esters and picrocrocin in aqueous extracts of saffron and in aqueous solutions of the isolated compounds, kept in darkness and submitted to heat treatment.

The third line of action is of an eminently practical nature, since it is directed at the quality control of saffron in commercial companies. Its specific objective is the following:

▪ *Objective nº 5*. To develop rapid, low-cost methods involving minimal manipulation to analyse the crocetin esters and/or picrocrocin in saffron samples, which can be applied as a matter of routine by saffron packaging and sales companies for the quality control of the raw materials and final products.

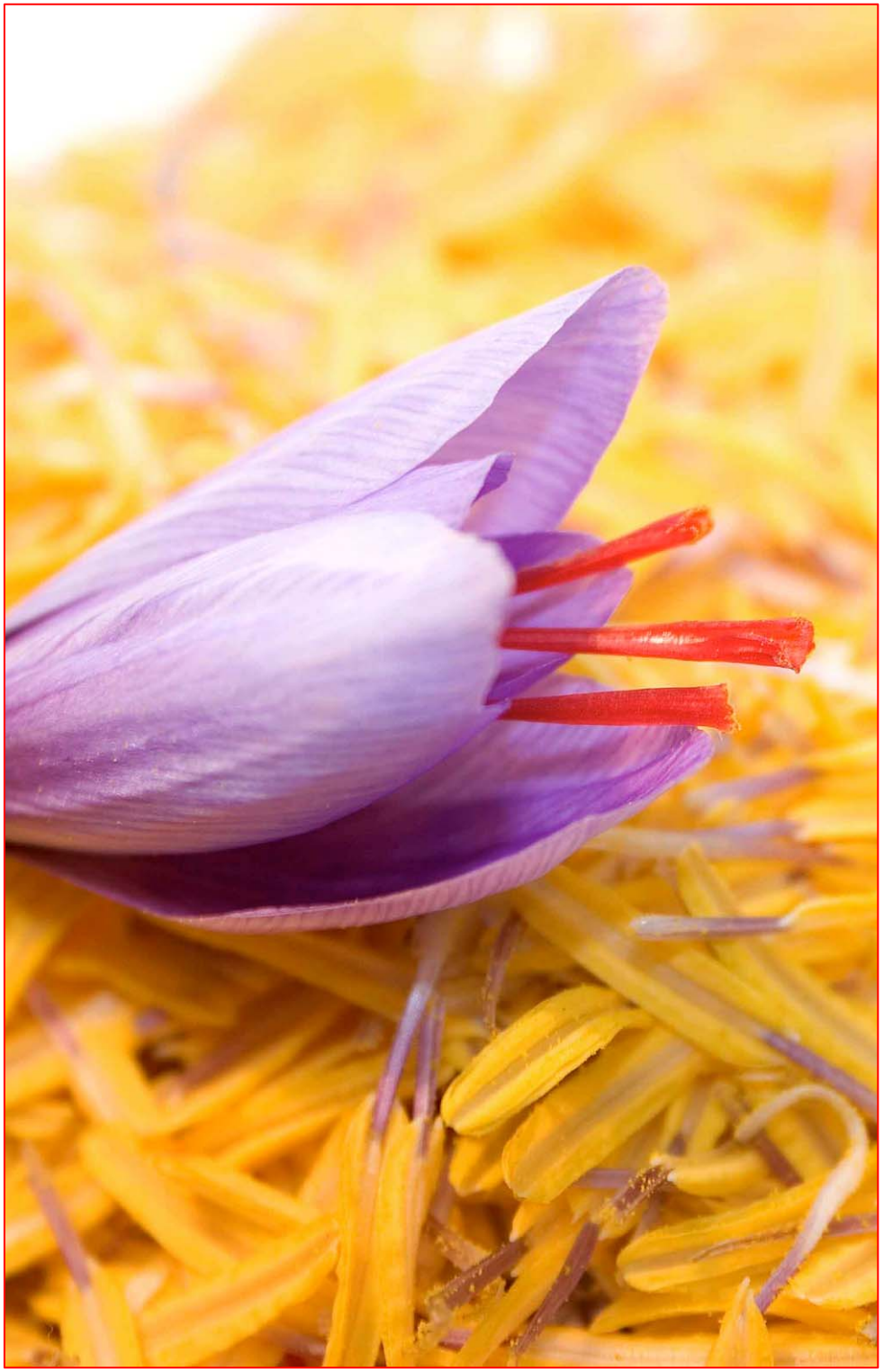
The last line of action opens up new fields such as the sensory analysis of individual compounds of aqueous extracts or their membrane separation. As specific objectives of this line we propose the following:

▪ *Objective nº 6.* To develop sensory studies to establish the taste detection threshold of picrocrocin.

▪ *Objective nº 7.* To study, at small scale, the application of semipermeable membrane ultrafiltration techniques to the separation or concentration of the main compounds in aqueous extracts of saffron.

CAPÍTULO 3. INTRODUCCIÓN

CHAPTER 3. INTRODUCTION



3. INTRODUCCIÓN

3.1. Definición y obtención del azafrán especia

Según el Código Alimentario Español (1967), el azafrán especia se define como "los estigmas desecados de *Crocus sativus* L. con las tolerancias máximas de estilos y restos florales que se especifiquen para las diversas calidades en la reglamentación correspondiente". A nivel internacional, la normativa de referencia aceptada en el control de calidad del azafrán es la especificación técnica ISO/TS 3632 (2003) que incluye en su alcance el azafrán en filamentos, en filamentos cortados y en polvo. Además, de su definición de azafrán en filamentos como estigmas de entre 20 y 40 mm de la parte aérea de los pistilos desecados de la flor de *Crocus sativus* L., se deduce que presentaciones como, por ejemplo, las madejas iraníes no pueden considerarse azafrán hasta que no se les elimina parte del estilo (Figura 3.1).

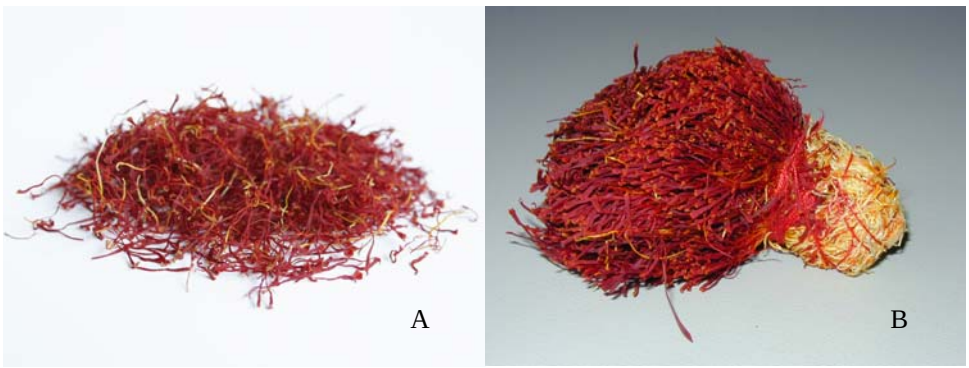


Figura 3.1. Azafrán especia (A) y madeja de estigmas y estilos de *Crocus sativus* L. producida de forma tradicional en Irán (B).

Tal y como indican las definiciones anteriores, una vez que se han recogido las flores y se han separado sus estigmas en el proceso de monda, para obtener la especia es necesario proceder a su deshidratación. Dicho proceso de deshidratación se conoce tradicionalmente en Castilla-La Mancha con el nombre de tostado y es una operación muy delicada de la que depende, en gran parte, la calidad y la conservación del producto (Pérez-Bueno, 1995; Carmona y col., 2005).

En países como Irán, India o Marruecos la deshidratación se produce generalmente a temperatura ambiente, extendiendo los estigmas en grandes superficies expuestas al sol o situadas a la sombra en un lugar ventilado.

En India, los estigmas se desecan al sol durante 3-5 días hasta que el contenido de humedad se reduce al 8-10% (Nauriyal y col., 1997; Sampathu y col., 1984). En este mismo país, en el área de Kashmir, el procedimiento habitual es secar la flor completa al sol y separar después su estigma (Hassnain, 1998).

En el caso de Marruecos, los estigmas se colocan sobre telas en capas muy finas y son secados durante dos horas al sol o durante 7-10 días a la sombra (Ait-Oubahou y El-Otmani, 1999).

En otros países como Italia, Grecia y España el azafrán fresco es sometido a temperaturas elevadas mediante flujos de aire caliente o colocándolo sobre una fuente de calor.

En Italia, la deshidratación se lleva a cabo situando los estigmas frescos en un cedazo a unos 20 cm sobre ascuas de roble. A la mitad del proceso, que dura entre 15 y 20 minutos, se les da la vuelta para asegurar un secado homogéneo. La deshidratación termina cuando los estigmas tienen una humedad entre el 5 y el 20%, momento en el que poseen cierta elasticidad al presionarlos entre los dedos (Tammaro, 1999). En la región de Cerdeña, antes de la deshidratación se realiza la "*feidatura*" que consiste en impregnar ligeramente los estigmas con aceite de oliva virgen para mejorar su aspecto y

prolongar su conservación. En el proceso de deshidratación propiamente dicho, se utilizan tablas de madera donde se extienden los estigmas. Éstos se ponen al sol, o se dejan en la chimenea por la noche durante el tiempo que permanezcan las ascuas. Actualmente, también se utilizan secadores eléctricos equipados con un termostato a una temperatura aproximada de 45 ºC (Alonso y col., 2007).

En Grecia, la mezcla de estigmas y estambres frescos se extiende en una fina capa de 4-5 mm sobre bandejas de 40 × 50 cm con el fondo de tela de seda. Estas bandejas se colocan sobre soportes verticales, separadas entre ellas 25-30 cm, en habitaciones habilitadas para la deshidratación. Durante las primeras horas la temperatura se mantiene a 20 ºC y posteriormente se eleva a 30-35 ºC. El proceso de secado termina cuando la humedad se reduce hasta el 10-11%, habitualmente tras unas 12 horas (Goliaris, 1999). Recientemente, se han hecho grandes esfuerzos para homogeneizar las condiciones que aplican los productores del área de *Kozani*. Se ha propuesto que la separación entre bandejas sea de 50 cm, la temperatura de la habitación se mantenga entre 35-45 ºC y la humedad relativa nunca supere el 50% (Ordoudi y Tsimidou, 2004).

En España, la deshidratación se realiza poniendo los estigmas en capas de menos de 2 cm sobre cedazos de tela metálica o seda y sometiéndolos a fuentes de calor como la cocina de gas butano, brasas de sarmiento y braseros eléctricos o a carbón. En Castilla-La Mancha la fuente de calor más habitual es la cocina de gas, seguida de las ascuas de carrasca y sarmiento y, en menor medida, se utilizan fuentes eléctricas (Alonso y col., 1998d). Aproximadamente a la mitad del proceso, transcurridos 10-15 minutos, se le da la vuelta a toda la masa con ayuda de otro cedazo del mismo tipo, que se vuelve a colocar sobre la fuente de calor hasta terminar el secado. Es preferible que el tiempo de deshidratación sea corto, en torno a media hora, y a temperaturas superiores a 70 ºC (Carmona y col., 2005).

En países como Inglaterra y Francia donde en el pasado se cultivó azafrán y en los que hoy su cultivo es anecdótico, también se empleaba alta temperatura durante el proceso de deshidratación (Pellowe, 1989). En Francia, el proceso tradicional en la región de *Gâtinais* se llevaba a cabo repartiendo los estigmas sobre un tamiz que se colocaba a unos 40 cm de un brasero, alcanzándose una temperatura entre 50 y 60 ºC. Transcurrida aproximadamente media hora se le daba la vuelta y se continuaba el proceso 15 minutos más por la otra cara. El final de la operación se evaluaba por el tacto y no por el aspecto o el aroma (Aucante, 2000).

Otra posibilidad menos tradicional, es la liofilización. Este método mantiene la estructura inicial del producto en cuanto a tamaño, forma y color de los estigmas, pero su aroma se ve limitado, por lo que requiere un proceso posterior de generación del mismo (Loskutov y col., 2000: Carreres y col., 2005).

La humedad del azafrán no sólo determina el final del proceso de deshidratación, sino que también es de gran importancia durante el almacenamiento. El azafrán, como muchos productos desecados, es higroscópico tendiendo a alcanzar un equilibrio con la humedad del ambiente (Kaminski y Kudra, 2000). Cuando la humedad es superior al 20%, el azafrán comienza a absorber agua de forma proporcional e independientemente del tamaño de sus partículas (Alonso y col., 1997). Por ello, después de deshidratar el azafrán, éste se guarda tratando de preservarlo de la humedad y de la luz, factores que afectan de manera muy directa a su color y aroma (Alarcón y Sánchez, 1968).

3.2. Importancia económica y social del azafrán

Tradicionalmente se ha aceptado que la introducción del azafrán en la Península Ibérica se debió a los árabes (Alarcón y Sánchez, 1968), aunque algunos autores proponen que fueron los romanos (Alonso y col., 1988). En cualquier caso, los árabes reglamentaron, difundieron su cultivo e impulsaron su consumo.

Hacia finales de la Edad Media, cuando comenzó la exportación de azafrán español a otras partes del mundo, el cultivo se había generalizado por todo el país y el azafrán de Cataluña gozaba de renombre. Su comercio principal radicaba en Barcelona, desde donde era enviado a Nápoles, ciudad que controlaba el mercado europeo del azafrán tras haber desplazado a Génova, y ésta anteriormente a Venecia. Más tarde, la excelente calidad del azafrán manchego lo llevó a ocupar el primer lugar en cuanto a demanda comercial y los centros de comercialización se desplazaron a las ciudades de Alicante y Valencia, funcionando en esta última ciudad una Lonja del Azafrán.

Como puede verse en la Figura 3.2, a principios del siglo XX se cultivaban en España algo más de 11000 ha y se producían casi 82 toneladas de azafrán (datos del MAPA correspondientes al año 1930). A mediados de ese siglo, su cultivo estaba muy extendido por las provincias de Albacete, Alicante, Baleares, Ciudad Real, Cuenca, Guadalajara, Murcia, Navarra, Soria, Teruel, Toledo, Valencia y Zaragoza (Morales, 1945). Fue entonces cuando se produjo un importante descenso en la superficie cultivada y, por tanto, en la producción. El cultivo ofrecía a los agricultores pobres con pequeñas parcelas de terreno las ventajas de su rusticidad y excelente rendimiento económico por unidad de superficie. Además, el azafrán no se consideraba un producto perecedero y se convertía en el capital de ahorro de las familias. Esta concepción ejercía un cierto control en las oscilaciones del precio de mercado y evitaba, en parte, la especulación. La población rural era muy importante, se disponía de mano de obra y al no necesitarse infraestructura alguna, su cultivo se recuperó oscilando en torno a las 4000 ha desde el año 1960 hasta 1989.

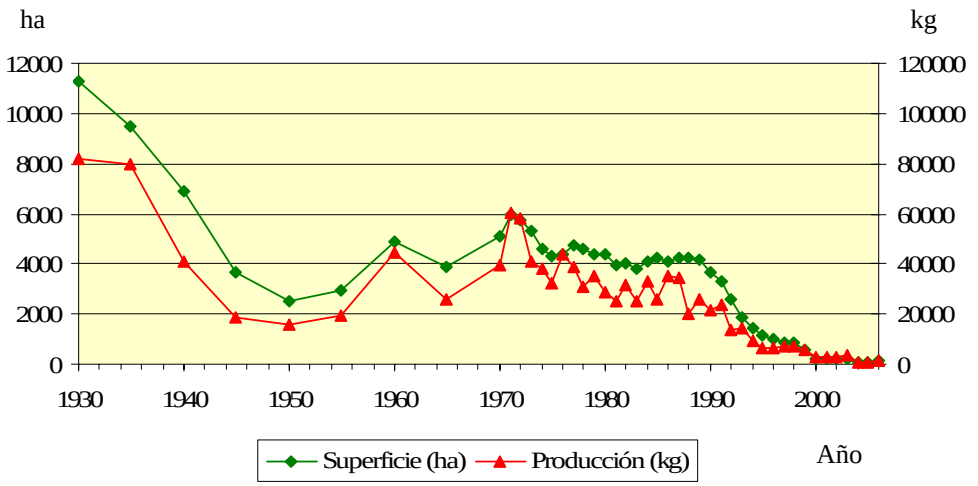


Figura 3.2. Evolución de la superficie de cultivo de azafrán y de la producción de estigmas tostados en España (MAPA, 2007).

En 1990 comenzó un continuo descenso de la superficie de cultivo del azafrán que duraría hasta el año 2005. Este descenso se explica por el aumento del nivel de vida y la gran demanda de mano de obra que este cultivo requiere en un corto periodo de tiempo. Los últimos datos del MAPA (2007) cifran la superficie de cultivo en España en el año 2006 en 116 ha, lo que supone un aumento respecto al año anterior, con una producción de 1330 kg que está distribuida en las provincias que muestra la Figura 3.3. Esta pequeña recuperación del cultivo refleja la labor de políticas de desarrollo rural y del Consejo Regulador de la Denominación de Origen "Azafrán de La Mancha".

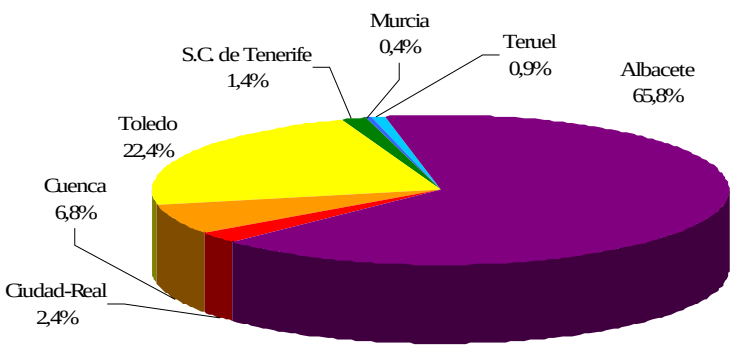


Figura 3.3. Distribución provincial de la producción de azafrán en España en el año 2006 (MAPA, 2007).

A nivel mundial, según datos del 1er Simposio Internacional sobre Biología y Biotecnología del azafrán la producción se ha desplazado hacia oriente (Tabla 3.1).

Tabla 3.1. Datos de producción de azafrán a nivel mundial (1er Simposio Internacional sobre Biología y Biotecnología del Azafrán, Octubre 2003).

PAÍS	**PRODUCCIÓN (kg)**
Irán	150000 - 170000
India	8000 - 10000
Grecia	4000 - 6000
Marruecos	800 - 1000
España	300 - 500
Italia	100
Turquía	10
Francia	4 - 5
Suiza	1

En la Tabla 3.1 no aparecen recogidos los datos para países como Inglaterra, Austria o Alemania que mantienen una producción testimonial para satisfacer la demanda de algunas tradiciones, ni de otros cultivadores más recientes como Israel, Japón, Azerbaiyán, China o Méjico. Tampoco se recoge el establecimiento de pequeñas plantaciones que diferentes migraciones europeas llevaron consigo, británicos a Estados Unidos y Nueva Zelanda, los holandeses a Sudáfrica y los españoles a Perú, Argentina y Chile (Aucante, 2000).

En el año 2004, la producción de azafrán especia en la Unión Europea fue aproximadamente de 6800 kg, lo que suponía alrededor del 4% de la producción mundial estimada en 170 toneladas. A pesar de la reducción del cultivo de azafrán en Europa, su repercusión socioeconómica es grande al corresponder, tal y como se ha dicho en el *Capítulo 1. Justificación*, aproximadamente el 90% de la comercialización mundial de azafrán a empresas europeas (Alonso y col., 2007).

3.3. Usos y propiedades del azafrán especia

Por sus propiedades colorantes, aromatizantes y saborizantes, el uso más importante del azafrán en la actualidad es el alimentario. Esta especia forma parte de platos tradicionales mediterráneos muy conocidos: el *risotto alla milanése* en Italia, la *bouillabaisse* en Francia o la paella en España. También es un ingrediente imprescindible en algunos pasteles y panes, en ocasiones conmemorativos de una fiesta laica o religiosa, como el *gugelhupf* alemán, los bollos de Santa Lucía en Suecia, bollos y panes en Cornwall (Inglaterra), panes navideños en Estonia, dulces de las islas griegas, pudines de arroz en las celebraciones chiítas iraníes, o el pan sabático *challah* de los judíos (Pellowe, 1989; Pérez-Bueno, 1995; Winterhalter y Straubinger, 2000). En la industria alimentaria se utiliza además en la producción de diversos licores y vermú, salsas, chocolates y productos lácteos (Attri y col., 1993; Sen y Rajorhia, 1994).

La preciosa gama de tonos amarillo-anaranjados que proporciona el azafrán ha sido a lo largo de la historia símbolo de luz, sabiduría y espiritualidad. Líderes políticos y religiosos, como reyes asirios, babilónicos, persas, medas, egipcios e irlandeses vistieron túnicas de color amarillo azafrán (Singer y col., 1958). Los especialistas en tintes antiguos afirman que el azafrán fue conocido como "el gran tinte" que no necesitaba mordiente, duradero y permanente por fermentación (Cannon y Cannon, 2003). Su interés como tinte había desparecido prácticamente con el desarrollo de los colorantes sintéticos, pero está resurgiendo al aumentar el interés por los productos naturales. El azafrán se emplea para teñir prendas textiles de alta calidad fabricadas con seda, algodón o lana (Takaoka y col., 1992; Tsatsaroni y Eleftheriadis, 1994; Liakopoulou-Kyriakides y col., 1998; Tsatsaroni y col., 1998); para teñir alfombras, sombreros y ropas tradicionales en Cerdeña (Campanelli, 1990); y para realizar tinciones histológicas humanas y animales, en combinación con hematoxilina, eritrosina y otros (Rostoker y col., 2001; Desmettre y col., 2001; Alyahya y col., 2002; Edston y col., 2002).

Su fascinante color también atrajo la atención de las mujeres que lo empleaban como cosmético, para teñir las uñas, el cabello y los labios (Roia, 1966; Cannon y Cannon, 2003). Por su aroma las jóvenes persas lo utilizaban como desodorante, untando su cuerpo con él y los romanos lo empleaban en la composición de numerosos perfumes. Hoy día, para los usos cosméticos también se están considerando otras partes de la planta, lo que supondría un valor añadido al cultivo (Betti y Schmidt, 2007).

Los usos terapéuticos del azafrán se remontan también a la antigüedad. En general, el azafrán, sus extractos y tinturas, han sido empleados en la medicina tradicional por ser antiespasmódico, eupéptico, sedante, carminativo, diaforético, expectorante, estomacal, estimulante, afrodisíaco, emenagogo y abortivo (Basker y Negbi, 1983; Ríos y col., 1996). Así mismo, se ha empleado para el tratamiento de afecciones oculares y cutáneas; para heridas, fracturas y dolores articulares; para prevenir la peste y otras epidemias; para curar la anemia, las cefaleas o el insomnio; para los males de garganta, la tisis, la ictericia, las llagas o como cardiotónico (Fernández y Escribano, 2000). Plinio “el viejo” en su obra *Naturae Historiarum XXXVII libri* lo describe como una especie de panacea (Alonso y col., 1988). Entre los siglos XVII y XIX, el empleo del azafrán por sus características medicinales conoció un declive progresivo, hasta prácticamente dejar de usarse. Sin embargo, gracias a los estudios farmacológicos de los últimos años, su actividad biológica está siendo nuevamente considerada. Se ha propuesto que el azafrán interviene en la asimilación de los lípidos (Hänsel, 1977) y se han evidenciado también sus efectos sobre el sistema cardiovascular (Grisolía, 1974; Seyde y col., 1986; Xuan y col., 1999). La crocetina, núcleo central de los carotenoides del azafrán, incrementa la difusión de oxígeno en el plasma sanguíneo, compensando la disminución causada por un elevado nivel de colesterol, por lo que ha mostrado tener efecto contra la arteriosclerosis y como reductor de los niveles de colesterol en sangre (Gainer y Chisolm, 1974; Gainer y Jones, 1975; Miller y col., 1982).

De forma paralela al estudio del aumento de la oxigenación de diversos tejidos (Gainer y Nugent, 1976; DiLuccio y Gainer, 1980; Seyde y col., 1986; Holloway y Gainer, 1988; Gainer y col., 1993), se comenzó a ensayar la actividad de estas sustancias sobre diversos tumores (Wilkins y col., 1977; Wilkins y Wilkins, 1977; Wilkins y Gainer, 1979). Muchos científicos han concentrado desde entonces su interés en la actividad antitumoral y anticarcinogénica del azafrán y sus efectos citotóxicos y antimutagénicos (Panikkar, 1990; Nair y col., 1995; Abdullaev, 1993, 2002; Abdullaev y Frenkel, 1999). Se han llevado a cabo ensayos tanto *in vivo*, sobre tumores de ratón, como *in vitro* sobre líneas celulares establecidas (Abdullaev y Frenkel, 1992 a y b; Escribano y col. 1996, 1999a y b, 2000; Jagedeeswaran y col., 2000; Konoshima y col., 1998; Mathews, 1982; Molnar y col., 2000; Morjani y col., 1990, 1993; Nair y col., 1991; Tarantilis y col., 1990, 1994a). El núcleo central de los pigmentos del azafrán, la crocetina, no actúa como precursor de la vitamina A, por lo que su toxicidad sería reducida (Winterhalter y Straubinger, 2000). Efectivamente, los ensayos de toxicidad celular *in vitro* demostraron que los extractos de estigmas de azafrán inhibían el crecimiento y la síntesis de ácidos nucleicos en células tumorales, mientras que las células normales eran menos sensibles e incluso totalmente insensibles (Nair y col., 1991; Abdullaev y Frenkel, 1992a y b). Se comprobó que las concentraciones que inducían la inhibición del 50% del crecimiento celular tumoral eran sólo ligeramente superiores a las observadas para el ácido retinoico todo-trans pero sin sus efectos secundarios (Tarantilis y col. 1992, 1994a). La menor toxicidad de la crocetina con respecto al ácido retinoico (Martín y col., 2002), junto con la ausencia de casos de sensibilización a la ingesta de azafrán (Moneret-Vautrin y col., 2002; Lucas y col., 2001), han fomentado el interés de su uso farmacológico. De hecho, los carotenoides del azafrán se han propuesto como agentes antitumorales alternativos que podrían alcanzar, solos o en combinación con otras sustancias químicas, cierta relevancia en el futuro para el tratamiento de algunos cánceres (Winterhalter y Straubinger, 2000; Abdullaev, 2004).

Los extractos de azafrán tienen una capacidad antioxidante similar a la del hidroxibutilanisol y el hidroxibutiltolueno, aditivos alimentarios comunes que se

emplean para evitar la oxidación de los alimentos (Martínez-Tomé y col., 2001; Pham y col., 2000). Incluso se ha llegado a comprobar su actividad antioxidante en humanos a los que se les administraban 50 mg de azafrán en 100 ml de leche dos veces diarias (Verma y Bordia, 1998). Este modo de acción es el que ha tomado mayor fuerza para explicar la actividad antitumoral del azafrán con varios estudios relativamente recientes (Hsu y col., 1999; Premkumar y col., 2001, 2003).

Además, se ha demostrado que el azafrán posee actividad antidepresiva con una eficacia comparable a la de la fluoxetina (Noorbala y col., 2005) o la imipramina (Akhondzadeh y col., 2004), con superioridad sobre un placebo (Akhondzadeh y col., 2005) y, por lo que se conoce hasta el momento, con menores efectos secundarios que los antidepresivos convencionales.

Por otra parte, la capacidad de inhibir las reacciones en cadena de los radicales libres se ha esgrimido para usar el azafrán en la crioconservación de esperma (Paramonova y col., 1989) y también podría estar implicada en el efecto hepatoprotector que tiene la crocetina sobre el hígado cuando se induce daño con algún producto tóxico (Lin y Wang, 1986; Wang y col, 1991a y b; Tseng y col., 1995). Con la misma idea, al irse acumulando evidencias de que el estrés celular causado por radicales libres podría ser responsable de varias enfermedades neurodegenerativas tales como el Alzheimer, el Parkinson o la esclerosis amiotrófica lateral, algunos autores han muostrado interés hacia la evaluación de los efectos del azafrán sobre este tipo de patologías (Abe y Saito, 2000; Ahmad y col., 2005). Resulta llamativo que de los pocos estudios que existen de su actividad sobre las funciones cerebrales, los más importantes sean aquellos en los que se demuestra el efecto antagonista de los extractos de azafrán frente al deterioro del aprendizaje y la memoria causada por el etanol (Zhang y col., 1994; Sugiura y col., 1994, 1995a b y c; Abe y Saito, 2000). A la vista de las conclusiones de éstos, podría pensarse que los romanos estaban en lo cierto cuando consumían azafrán junto con el vino para aminorar sus efectos secundarios.

De la misma manera, otras de las actividades farmacológicas clásicas citadas por Hipócrates o Dioscórides, han recibido respaldo científico (Schmidt y col., 2007). Se ha demostrado que los extractos de azafrán tienen un efecto mitigador de las convulsiones y que el azafrán es antinociceptivo, antiinflamatorio, ansiolítico, antiestrés y sedante (Benigni y col., 1964; Ghosal y col., 1989; Hosseinzadeh y Khosravan, 2002; Hosseinzadeh y Younesi, 2002; Hosseinzadeh y col., 2003).

3.4. Principales componentes de los extractos acuosos del azafrán

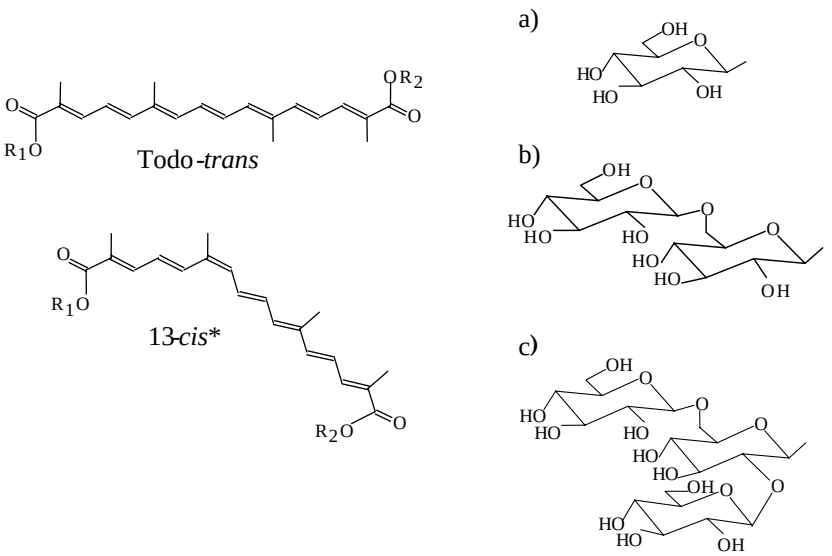
Los metabolitos secundarios de mayor interés en el azafrán son los ésteres de crocetina, debido principalmente al papel que desempeñan en el color y las propiedades medicinales; la picrocrocina, por su aportación al sabor amargo; y el safranal, compuesto mayoritario en la fracción aromática de la especia.

Debido a la poca solubilidad en agua del safranal, en los extractos acuosos son los ésteres de crocetina y la picrocrocina los compuestos más destacables. Además, en los extractos acuosos se encuentran flavonoides del grupo de los kaempferoles que son interesantes por su posible contribución a las propiedades y atributos del azafrán especia, a pesar de encontrarse en el azafrán en menores proporciones que el resto de los compuestos mencionados.

A continuación, se expondrán algunos aspectos particulares de cada uno de estos grupos de compuestos.

3.4.1. Ésteres de crocetina

Las sustancias responsables del color que tiene y que proporciona el azafrán especia a soluciones acuosas pertenecen al grupo de los pigmentos carotenoides. En los extractos acuosos de azafrán se encuentra una mezcla de diferentes ésteres glicosilados del ácido 8,8'-diapo-Ψ,Ψ'-carotenedioico, denominado crocetina (Figura 3.4).



$R_1 = R_2 = H$; Crocetina (todo-*trans*); $C_{20}H_{24}O_4$; Mr = 328.
$R_1 = H$; $R_2 = a$; (β-D-glucosil) éster de crocetina; $C_{26}H_{34}O_9$; Mr = 490.
$R_1 = R_2 = a$; Di-(β-D-glucosil) éster de crocetina; $C_{32}H_{44}O_{14}$; Mr = 652.
$R_1 = H$; $R_2 = b$; Mono-(β-D-gentiobiosil) éster de crocetina; $C_{32}H_{44}O_{14}$; Mr = 652.
$R_1 = a$; $R_2 = b$; (β-D-glucosil)-(β-D-gentiobiosil) éster de crocetina; $C_{38}H_{54}O_{19}$; Mr = 814.
$R_1 = R_2 = b$; Di-(β-D-gentiobiosil) éster de crocetina; $C_{44}H_{64}O_{24}$; Mr = 976.
$R_1 = b$; $R_2 = c$; (β-D-gentiobiosil)-(β-D-neapolitanosil) éster de crocetina; $C_{50}H_{74}O_{29}$; Mr = 1138.

(*) En el caso de las crocinas con conformación cis no se ha podido precisar la posición de los sustituyentes R_1 y R_2 con respecto al enlace $C_{13\text{-}14}$.

Figura 3.4. Estructura de los ésteres de crocetina encontrados en azafrán por diferentes autores.

Los azúcares que esterifican los extremos de la crocetina son glucosa, gentiobiosa y neapolitanosa. A diferencia de la mayoría de los carotenoides, estos ésteres tienen la particularidad de ser hidrosolubles y se les conoce también con el nombre de crocinas. Este nombre proviene del di-(β-D-gentiobiosil) éster de crocetina, compuesto mayoritario en el azafrán al que se llamó crocina cuando fue estudiado por primera vez por Aschoff en 1818. Este éster de crocetina cristalizado en metanol se presenta en agujas rojo brillantes que funden a 186 ºC y con ácido sulfúrico adquiere progresivamente diferentes coloraciones tomando al final un tono azul, de aquí su antigua denominación de policroita (Morales, 1945). Su coeficiente de extinción molar, ε, en agua a 440 nm es de 132200 L cm^{-1} mol^{-1} según Basker y col. (1985), de 133750 L

cm^{-1} mol^{-1} según Friend y col. (1960), de 133500 L cm^{-1} mol^{-1} según Weber y col. (1974) y de 89000 L cm^{-1} mol^{-1} según Speranza y col. (1984). En metanol su coeficiente de extinción molar a 443 nm es 133000 L cm^{-1} mol^{-1} (Weber y Grosch, 1976).

Los ésteres de crocetina, además de encontrarse en el azafrán, también se encuentran en los frutos de gardenia (*Gardenia jasminoides* Ellis), de los que se obtiene un colorante natural que, a parte de sus usos culinarios, se utiliza en la medicina oriental.

Se han propuesto dos vías diferentes para la biosíntesis de la crocetina y, a partir de ella, de los ésteres de crocetina y de la picrocrocina: la degradación oxidativa de un carotenoide C_{40} y la dimerización de dos compuestos C_{10}. Los trabajos de Pfander y Schurtenberger (1982) descartaban la segunda hipótesis. Dichos autores sintetizaron los posibles precursores C_{20} y comprobaron que no se encontraban en el azafrán. Además, pudieron aislar fitoflueno, tetrahidrolicopeno y fitoeno, tres conocidos precursores del β,β-caroteno y el zeaxanteno que también fueron aislados, aunque no fueron capaces de aislar β,ψ-caroteno o licopeno, tal y como habían encontrado Kuhn y Winterstein (1934), ni tampoco el complejo xantona-carotenoide encontrado por Ghosal y col. (1989). Se consolidó así la primera hipótesis mencionada, en la que la sustancia considerada precursora era el zeaxanteno, sobre todo desde que Buchecker y Eugster (1973) confirmaran que la configuración R del carbono que soporta el grupo hidroxilo en la picrocrocina es la misma que en el zeaxanteno (Figura 3.5). El zeaxanteno sufre una ruptura de los dos extremos para generar el crocetindialdehido, éste se oxida y da lugar a la crocetina que, posteriormente, se glicosila por acción de una glucosiltransferasa (Coté y col., 2000; Moraga y col., 2004). El respaldo más firme a esta teoría proviene del descubrimiento de una enzima denominada *CsZCD* (*Crocus sativus* zeaxanthin cleavage dioxygenase) (Bouvier y col., 2003). Esta enzima expresada en *Escherichia coli* y posteriormente purificada se ha mostrado capaz de transformar el zeaxanteno en crocetindialdehido.

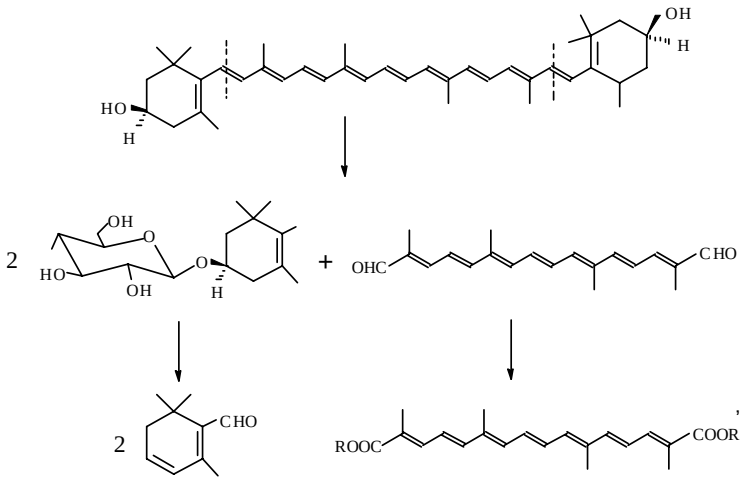


Figura 3.5. Hipótesis aceptada para la generación de los ésteres de crocetina, picrocrocina y safranal en *Crocus sativus* L. R, R' = H, glucosa, gentiobiosa o neapolitanosa.

Otro aspecto importante de este grupo de carotenoides del azafrán es su posible intervención en la formación del aroma, tal y como propusieron Carmona y col. (2006b) (Figura 3.6). Según estos autores, la formación del aroma tendría lugar a partir de los ésteres de crocetina por la acción de una carotenasa. De esta forma, por la presencia de una enzima, se explicaría por qué los compuestos C_9 y C_{10} odorantes no se encuentran en gardenia y sí en azafrán. Esta hipótesis es compatible con el conocimiento que existe sobre la formación de anillos de seis miembros en extremos lineales de carotenoides (Britton, 1998). Además de simple, cumple el principio de economía metabólica, una única ruta que dotada de enzimas adicionales puede progresar más adelante y es coherente con los resultados obtenidos por Himeno y Sano (1987). Ellos encontraron que en el momento de la antesis se producía, además de la disminución del contenido de picrocrocina, un descenso muy importante del contenido de crocina al que no encontraron una explicación adecuada.

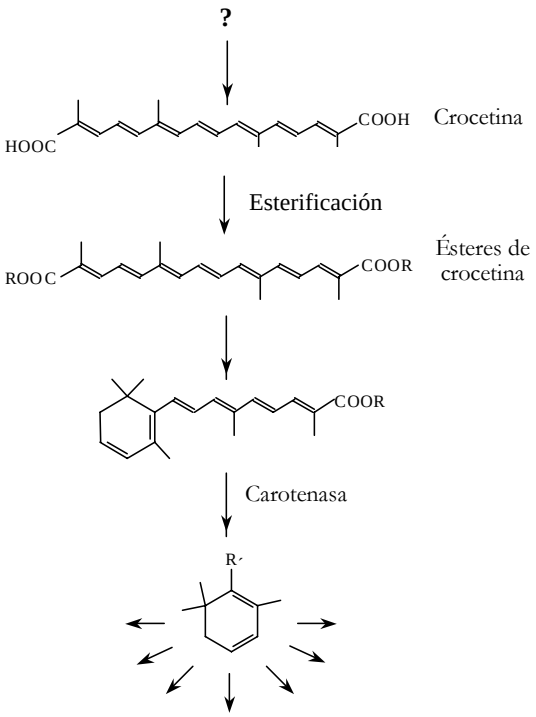


Figura 3.6. Hipótesis para la formación de compuestos volátiles presentes en el aroma del azafrán a partir de los ésteres de crocetina (Carmona y col., 2006b).

En esta memoria y en los artículos de esta tesis, con el fin de abreviar, se ha utilizado la siguiente nomenclatura para designar los ésteres de crocetina:

En primer lugar, se hace referencia a la conformación cis o trans, seguida por un guión que la separa del número de residuos de glucosa en la molécula. Posteriormente, se indica la distribución de los residuos como (t) triglucósido, (n) neapolitanósido, (G) gentiobiosiósido o (g) glucósido. El nombre de la estructura base, éster de crocetina, se omite por tratarse del mismo en todos los compuestos.

Las crocinas cis con respecto a sus homónimos trans tienen un punto de fusión más bajo, presentan una banda de absorción adicional alrededor de 324 nm en su espectro UV-vis y el máximo de absorción a 440 nm presenta un efecto hipsocrómico de unos 5 nm (Figura 3.7) (Speranza y col., 1984; Pfister y col., 1996; Van Calsteren y col., 1997; Assimiadis y col., 1998).

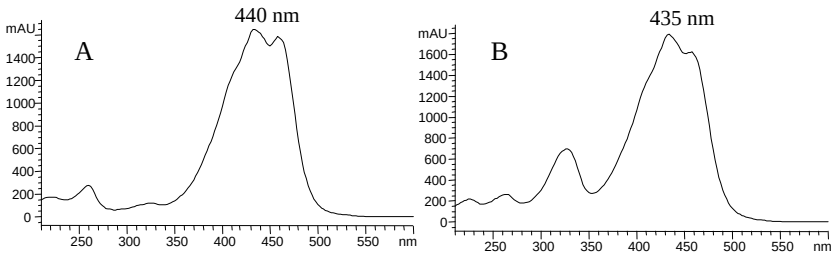


Figura 3.7. Espectros UV-vis característicos de las crocinas con conformación trans (A) y cis (B).

Los ésteres de crocetina son los responsables del poder colorante del azafrán que se define como el valor de $E_{1\text{cm}}^{1\%}$ 440 nm para los extractos acuosos de la especia. Son numerosos los trabajos sobre el color del azafrán y los ésteres de crocetina centrados en desarrollar métodos analíticos basados en espectrofotometría UV-vis, TLC (Sujata y col., 1992; Visvanath y col., 1990) y HPLC para separar, identificar y/o cuantificar estos carotenoides y proponer su estructura (Speranza y col., 1984; Himeno y Sano, 1987; Tarantilis y col., 1994; Morimoto y col., 1994; Tarantilis y col., 1995; Pfister y col., 1996; Straubinger y col., 1997; Van Calsteren y col., 1997; Li y col., 1999; Lozano y col., 1999; Winterhalter y Straubinger, 2000; Alonso y col., 2001a).

La heterogeneidad de las muestras de azafrán (de diferentes orígenes, con distintos procesos de deshidratación y calidades) junto con la falta de patrones comerciales de cada uno de los ésteres de crocetina y las distintas técnicas de análisis, han dado lugar a que aparezca en la bibliografía un amplio rango de resultados sobre el contenido en ésteres de crocetina del azafrán especia (Tabla 3.2).

Tabla 3.2. Contenidos de ésteres de crocetina en azafrán especia (% en masa) según diversos autores.

CONTENIDOS DE ÉSTERES DE CROCETINA EN AZAFRÁN ESPECIA (% EN MASA)[a]							
Autores	Caballero-Ortega y col., 2007[b]	Zougagh y col., 2005[b]	Alonso y col., 2001a[c]	Li y col., 1999[c]	Raina y col., 1996[c]	Iborra y col., 1992[c]	Corradi y Micheli 1979b[b]
trans-4-GG	3,84 ± 0,06	7,5 - 1,81	12,12 - 0,46	9,00 ± 0,71	11 - 17,05	8,0 - 7,9	9,46 - 6,87
trans-3-Gg	2,44 ± 0,01	0,86 - 0,10	9,44 - 0,01	4,60 ± 0,35			
trans-2-G	0,21 ± 0,00	0,06 - 0,05		1,30 ± 0,12			
cis-4-GG	0,58 ± 0,00	0,03 - 0,02	8,53 - 0,04				
cis-3-Gg			2,26 - 0,01				
cis-2-G	0,22 ± 0,00						
trans-1-g				0,36 ± 0,06			
Total	7,38		32,25 - 0,52	15,26			
Análisis	HPLC	Electroforesis capilar	HPLC/ UV-vis	HPLC	UV-vis	HPLC	UV-vis
Patrón interno	2-nitroanilina			ácido 13-*cis* retinoico			

[a] Media ± desviación estándar o bien, máximo - mínimo.
[b] Los autores no especifican si se trata de resultados en base seca o húmeda.
[c] % en base seca.

Teniendo en cuenta el gran valor del azafrán especia, son numerosos los estudios existentes para establecer las mejores condiciones en su almacenamiento (Mannino y Amelotti, 1977; Alonso y col., 1990; Tsimidou y Biliaderis, 1997; Selim y col., 2000). La mayoría de estos trabajos utilizan la pérdida de poder colorante ($E_{1cm}^{1\%}$ 440 nm) para valorar los cambios sufridos por la especia al ser sometida a distintas condiciones de almacenamiento y tras extraer las crocinas justo antes de realizar las medidas. Sin embargo, la literatura sobre degradación de los extractos acuosos de azafrán es bastante escasa. En los trabajos de Alonso y col., 1993; Tsimidou y Tsatsaroni, 1993; y Orfanou y Tsimidou, 1995, se concluye que la pérdida de poder colorante sigue una cinética de primer orden que se ve influenciada por la luz, la

temperatura, el pH y la presencia de aditivos como el ácido ascórbico en combinación con el ácido etilendiaminotetraacético (EDTA). Gran parte de estos trabajos están basados en medidas que reflejan el resultado de una degradación conjunta de todos los ésteres de crocetina pero no indican nada sobre los cambios que sufre cada uno de ellos.

Vickackaite y col. (2004) estudiaron los procesos térmicos y fotoquímicos que degradan el azafrán en extractos metanólicos. Recientemente, Manzocco y col. (2008) han estudiado modelos para establecer el periodo de conservación antes de la venta de productos sensibles a la luz. Para ello, han tomado como ejemplo la decoloración de bebidas coloreadas mediante un sistema modelo formado por una disolución de azafrán en metanol/agua. En este trabajo, se describe una cinética de primer orden para dicha decoloración y se pone de manifiesto tanto el efecto acelerador de la luz en la misma, como la relevancia de las reacciones de oxidación producidas.

3.4.2. Picrocrocina

La picrocrocina ($C_{16}H_{26}O_7$, Mr = 330) es el 4-(β-D-glucopiranosiloxi)-2,6,6-trimetil-1-ciclohexen-1-carboxaldehido y es considerada la sustancia responsable del sabor amargo del azafrán. Su estructura fue establecida por Kuhn y Winterstein en 1934 (Figura 3.8). En el apartado anterior ya se habló de la estereoisomería de su centro quiral, configuración R determinada por Buchecker y Eugster (1973), cuando se exponía la ruta biosintética generalmente aceptada que comparten los ésteres de crocetina y la picrocrocina.

Los cristales de picrocrocina son incoloros con un punto de fusión de 155 ºC, solubles en agua y en etanol, poco solubles en cloroformo y éter etílico, y prácticamente insolubles en éter de petróleo y benceno. Su espectro UV-vis en disolución acuosa presenta un máximo a 250 nm (Figura 3.9), siendo su coeficiente de extinción molar en disolución acuosa de 10100 L cm^{-1} mol^{-1} (Buchecker y Eugster, 1973).

Como se indica en la Figura 3.8, a la picrocrocina se le considera el precursor del safranal, 2,6,6-trimetil-1,3-ciclohexadien-1-carboxaldehido ($C_{10}H_{14}O$, Mr = 150). Durante el proceso de deshidratación esta transformación tiene lugar bien por la temperatura que se alcanza o por la acción de glicosidasas, (Himeno y Sano, 1987; Iborra y col., 1992; Lozano y col., 1999).

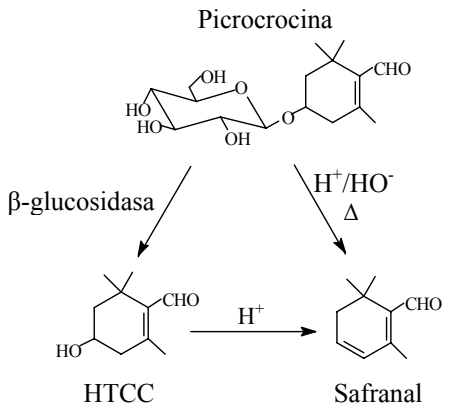


Figura 3.8. Esquema de la formación química o enzimática del safranal a partir de la picrocrocina (Himeno y Sano, 1987).

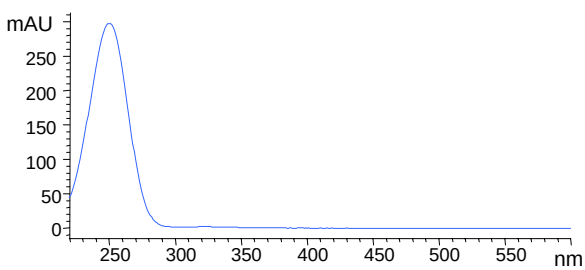


Figura 3.9. Espectro de absorción UV-vis de la picrocrocina.

Tomando como referencia los trabajos de Himeno y Sano (1987), la nueva hipótesis de generación del aroma a partir de los ésteres de crocetina, propuesta por Carmona y col. (2006b) que describiría lo que sucede *in vivo*, se formuló de forma

esquemática como se recoge en la Figura 3.10. Durante el desarrollo del estigma, crocinas y picrocrocina mantendrían una proporción más o menos constante debido a que el HTCC (4-hidroxi-2,6,6-trimetil-1,3-ciclohexadien-1-carbaldehido) formado por la acción de una carotenasa se glucosidaría transformándose en picrocrocina. Este mecanismo empleado habitualmente por las plantas para acumular metabolitos en grandes cantidades, evitaría su oxidación. En el momento de la antesis una o varias glicosidasas, especificas o no, actuarían sobre la picrocrocina convirtiéndola de nuevo en HTCC. Su concentración crecería mucho y no sólo por la transformación de la picrocrocina, también porque la carotenasa actuaría activamente sobre las crocinas.

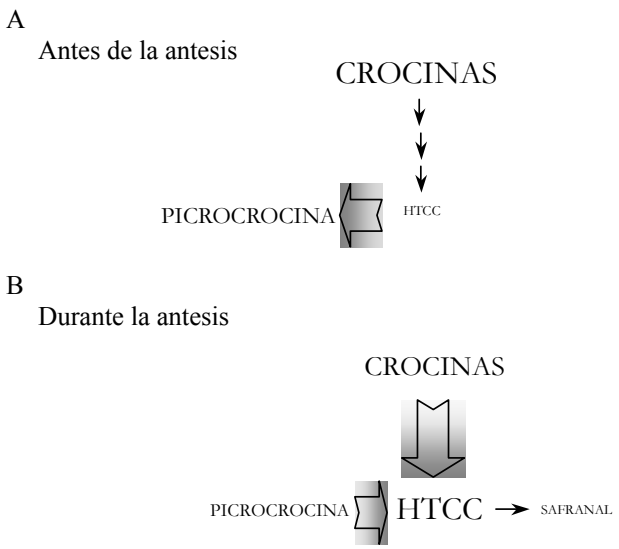


Figura 3.10. Hipótesis para la formación de HTCC y safranal antes (A) y durante la antesis del *Crocus sativus* L. (B) según Carmona y col. 2006b.

En el caso de los azafranes desecados a alta temperatura la actividad de estas enzimas, glicosidasas y carotenasas, sería corta, pero en el caso de azafranes deshidratados a temperatura ambiente podrían mantenerse activas durante muchas horas a lo largo de los varios días que dura el proceso de secado. Este periodo podría ser tan importante como lo es para otros productos, tales como el pimentón cuando se somete al tradicional proceso de deshidratación lento de La Vera en Extremadura, donde el alto

contenido de humedad permite a las frutos mantener un largo periodo de actividad metabólica y seguir acumulando carotenoides (Pérez-Gálvez y col., 2004; Mínguez-Mosquera y col., 2000). Esto también se ha puesto de manifiesto en otros países productores de pimentón como Turquía, al comparar métodos tradicionales de secado al sol durante 5-7 días con métodos de secado rápido con aire caliente. Al contrario de lo que sucedería con el azafrán, la deshidratación lenta bajo el sol produce una menor pérdida de los pigmentos presentes en el fruto fresco que una deshidratación rápida con aire a 70 ºC durante 90 minutos (Topuz y Ozdemir, 2003).

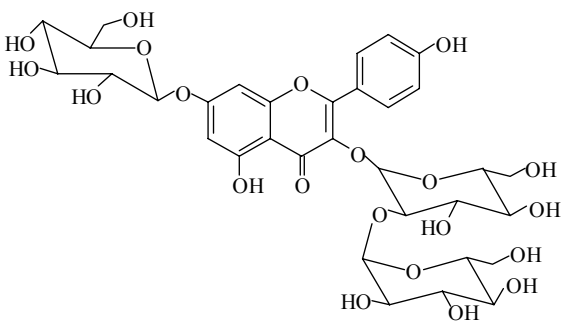
3.4.3. Flavonoides

Tarantilis y col. (1995) identificaron por primera vez mediante espectrometría de masas un flavonoide en los extractos de azafrán. Propusieron que se trataba de un kaempferol unido a un disacárido formado por dos unidades de glucosa.

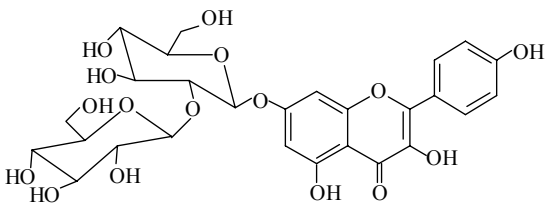
Straubinger y col. (1998b) empleando cromatografía en contracorriente aislaron dos flavonoides (Figura 3.11 A, B) e indicaron que ambos contenían el disacárido soforosa, dos glucosas unidas mediante enlace glicosídico (1→2). Basándose en los resultados, estos mismos autores consideraron errónea la adjudicación estructural llevada a cabo por Lozano y col. (1999) (Figura 3.11 C) donde la unión entre las dos glucosas (1→6) conformaba el disacárido llamado gentiobiosa (Winterhalter y Straubinger, 2000).

No se descartaba que existiesen más compuestos similares en el azafrán especia. De hecho, en los pétalos de otros *Crocus* se ha descrito un grupo muy diverso de kaempferoles asociados a rhamnosa y glucosa (Nørbæk y Kondo, 1999).

A



B



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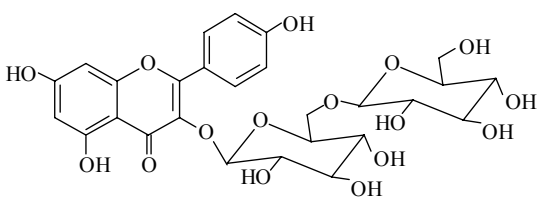

Figura 3.11. Estructura de los flavonoides encontrados en azafrán por diferentes autores. (A) Kaempferol 3-*O*-soforósido-7-*O*-glucósido; (B) kaempferol 7-*O*-soforósido; (C) kaempferol 3-*O*-gentiobiósido.

3.5. Calidad del azafrán especia

Como ya se mencionó en el apartado 3.1, la normativa de referencia aceptada a nivel internacional en el control de calidad del azafrán es la especificación técnica ISO/TS 3632 (2003). La Norma ISO 3632 para el azafrán se creó en 1975 y ha sufrido tres revisiones, la primera en 1980, otra en 1993 y la última se inició en 2003 y todavía se está debatiendo.

ISO/TS 3632 (2003) establece una clasificación del azafrán en tres categorías en relación a los parámetros y características que se muestran en la Tabla 3.3.

Tabla 3.3. Requerimientos para el azafrán en hebra o molido según ISO/TS 3632-1 (2003).

CARACTERÍSTICAS	ESPECIFICACIONES		
	Categoría I	Categoría II	Categoría III
Restos Florales, % máximo	0,5	3	5
Materia Extraña, % máximo	0,1	0,5	1,0
Humedad y materia volátil, % máximo Azafrán en hebra Azafrán molido	 12 10	 12 10	 12 10
Cenizas totales, % máximo sobre materia seca	8	8	8
Cenizas insolubles en ácido, % máximo sobre materia seca.	1,0	1,0	1,5
Solubilidad en agua fría, % máximo, sobre materia seca.	65	65	65
$E_{1cm}^{1\%}$ 257 nm (a esta longitud de onda tiene su máximo la picrocrocina). Mínimo	70	55	40
$E_{1cm}^{1\%}$ 330 nm (a esta longitud de onda tiene su máximo el safranal). Mínimo Máximo	 20 50	 20 50	 20 50
Poder colorante, $E_{1cm}^{1\%}$ 440 nm (a esta longitud de onda tienen su máximo las crocinas)	190	150	100
Colorantes ácidos artificiales hidrosolubles	Ausencia	Ausencia	Ausencia

Tras todas estas revisiones, ISO/TS 3632 (2003) se ha convertido en una normativa muy completa con respecto a los parámetros exigidos al azafrán, por lo que, aunque no es de obligado cumplimiento, es la empleada para certificar el azafrán en la mayoría de las transacciones comerciales internacionales, facilitando la desaparición de azafranes de muy baja calidad y la detección del azafrán adulterado.

El problema de la adulteración ha estado históricamente presente en el azafrán (Tabla 3.4), pues es y ha sido una de las especias más valoradas en el mercado mundial, alcanzando en muchas épocas precios realmente altos. Así por ejemplo, en tiempos de Marco Polo (siglo XIII) su precio llegó a ser superior al del oro.

Tabla 3.4. Adulteraciones más frecuentes sufridas por el azafrán a lo largo de la historia.

	FORMA DE ADULTERAR
A	▪ *Sin adición de sustancias extrañas:* - Mezcla con azafrán extractado o viejo.
B	▪ *Adición de otras partes de la planta de azafrán:* - Adición de estambres o del perigonio cortado en tiras y teñido.
C	▪ *Adición de sustancias que aumentan la masa:* - Incremento de la humedad. - Impregnación con jarabes, miel o glicerina. - Adición a los jarabes anteriores de sulfato de bario, sulfato de sodio, sulfato de calcio, carbonato de calcio, hidróxido de potasio, nitrato de potasio, tartrato (doble) de potasio y sodio, borato de sodio, lactosa, almidón o glucosa.
D	▪ *Adición de partes de otras plantas:* - Flores de *Carthamus tinctorius, Calendula officinalis.* - Estigmas de otras especies de *Crocus*, generalmente más cortos y sin propiedades tintóreas (*Crocus vernus*, *Crocus speciosus*, etc.). - Flores de *Papaver rhoeas*, *Punica granatunm*, *Arnica montana* y *Scolimus hispanicus* cortadas en tiras. - Estambres de algunas especies de clavel. - Pimiento rojo molido. - Plantas herbáceas cortadas en trozos y coloreadas con un colorante azoico. - Raicillas de *Allium porrum.* - Curcuma.
E	▪ *Adición de sustancias animales:* - Fibras de carne salada y desecada.
F	▪ *Adición de productos artificiales:* - Hilos de gelatina coloreados.
G	▪ *Adición de colorantes orgánicos:* - Amarillo de martins, tropeolina, fucsina, ácido pícrico, tartracina, eritrosina, escarlata o ponceau 4R, azorrubina, amarillo anaranjado, etc.

Se han desarrollado numerosos métodos para detectar las adulteraciones y se sigue trabajando para evitar las nuevas formas de fraude. En cada país existen normas propias de control de calidad tanto de los parámetros intrínsecos del azafrán (humedad, composición, etc.) como de otros extrínsecos (adulteraciones, carga microbiológica y residuos de pesticidas). Por ejemplo, en España están vigentes la Reglamentación Técnico Sanitaria (BOE, 1984), la Norma de Calidad del Comercio Exterior del azafrán (NCCEA, 1988 y 1999), el Pliego de Condiciones del "Azafrán de La Mancha" (DOCM, 1999) y el Reglamento técnico para la utilización de la marca "Calidad Alimentaria" para el "Azafrán de Aragón" (RT, 2003). Sin embargo, recientemente se ha derogado la Norma Española UNE 34013 h1 y h2 (1965).

Los factores que afectan a la calidad del azafrán están presentes en todos los pasos de su producción, desde su cultivo hasta el producto final puesto a disposición del consumidor. Influyen las condiciones de cultivo (suelo, agua, temperatura, estado sanitario, etc.), las prácticas culturales, la recogida de la flor y su monda, el proceso de deshidratación, la manipulación, el almacenamiento y el envasado.

Los extractos de azafrán deben cumplir ciertos criterios de calidad, según la finalidad o aplicación que se les vaya a dar. Por ejemplo, extractos con aplicaciones alimentarias requieren un color, aroma y sabor definidos y a un precio asequible. Esto implica trabajar en torno a la composición en los principales compuestos del azafrán, sin olvidar su evolución y estabilidad en las condiciones de manejo y almacenamiento. Hay que tener en cuenta que más del 95% de los estigmas de azafrán que se producen en la actualidad van destinados a este uso. En el caso de extractos para usos farmacéuticos, surge la necesidad de producir extractos estandarizados de determinados componentes del azafrán. Fruto del renovado interés por las propiedades medicinales del azafrán, este tipo de extractos está tomando importancia. Al igual que para el uso alimentario, este tipo de aplicación requiere trazabilidad y reproducibilidad de la materia prima, así como una calidad y composición química constante. Además, en usos farmacéuticos es obligatorio demostrar la estabilidad de los extractos (Betti y Schmidt, 2007).

En los últimos años, ha aumentado también el interés por garantizar y defender la calidad del azafrán producido históricamente en determinadas regiones. Como resultado, existen cuatro Denominaciones de Origen Protegidas, DOP, en Europa: "Azafrán de La Mancha" en España (DOCE, 2001), "*Krokos Kozani*" en Grecia (DOCE, 1999), "*Zafferano dell'Aquila*" y "*Zafferano di San Gimignano*" en Italia (OJUE, 2005). Además, están reconocidos otros distintivos de calidad como la marca "C Calidad Alimentaria" para el "Azafrán de Aragón" (BOA, 2003), en Suiza "*Munder Safran*" está registrado como Denominación de Origen Controlada (FOSC, 2004) y en Italia la denominación "*Zafferano delle Colline Fiorentine*" está temporalmente protegida a nivel nacional (GU, 2005a) y se ha publicado la propuesta de reconocimiento para la DOP "*Zafferano di Sardegna*" (GU, 2005b).

En Castilla-La Mancha, el Consejo Regulador de la Denominación de Origen "Azafrán de La Mancha" utiliza el análisis sensorial para completar la caracterización de los azafranes y verificar su calidad. Este Consejo Regulador cuenta con un Comité de Cata especializado que participa en la elección del mejor azafrán producido cada año y posee una ficha (Figura 3.12) y un manual de cata. Como puede verse en la Figura 3.12, la ficha está dividida en tres apartados: aspecto, sensación olfato-gustativa y textura. Los descriptores del aspecto, de la textura y del olor se determinan sobre azafrán en hebra y el resto sobre una disolución acuosa.

Figura 3.12. Ficha de cata del azafrán de la Denominación de Origen “Azafrán de La Mancha”.

			Excelente	Muy Bueno	Bueno	Deficiente	Rechazable	Total puntuación	OBSERVACIONES
ASPECTO	Color	Uniformidad	5	4	3	2	1		
		Tono	7	6	5	4	3		
		Intensidad	7	6	5	4	3		
	Materias extrañas		6	5	4	3	2		
	Apariencia		10	8	6	4	2		
SENSACION OLFATO-GUSTATIVA	Olor	Intensidad	12	10	8	6	4		
		Frescura	8	7	6	5	4		
		Olores atípicos	7	6	5	4	3		
	Aroma		8	7	6	5	4		
	Amargor		8	7	6	5	4		
	Persistencia de sabores		7	6	5	4	3		
TEXTURA	Fragilidad		8	7	6	5	4		
	Humedad		7	6	5	4	3		
	TOTALES								

Excelente: Mayor o igual de 86 puntos
Muy bueno: Entre 71 y 85 puntos
Bueno: Entre 56 y 70 puntos
Deficiente: Entre 41 y 55 puntos
Rechazable: Menor o igual de 40 puntos

Lugar/ Fecha
Firma del Catador

CAPÍTULO 4. PLAN DE TRABAJO

CHAPTER 4. WORK PLAN

4. PLAN DE TRABAJO

En este capítulo se presenta, en forma de cuadro sinóptico, el plan de trabajo que se ha seguido a lo largo de esta tesis, permitiendo una visión global de todos los estudios realizados.

Cada fila del cuadro está dedicada a un artículo y en cada columna se indica, según su encabezado, un aspecto referente al planteamiento y a la metodología seguida. El color del fondo señala la línea de acción (véase *Capítulo 2. Objetivos*) donde está integrado cada artículo: naranja para la primera, amarillo para la segunda, morado para la tercera y azul para la cuarta. Cuando un artículo incluye estudios de más de una línea de acción, su fondo está representado por la mezcla de sus respectivos colores. Es el caso del artículo que recoge los estudios cinéticos de la picrocrocina y también la determinación de su umbral de percepción gustativa, cuyo fondo es verde.

Por columnas, de izquierda a derecha, se incluyen de forma muy resumida: la línea de acción (en letras mayúsculas); los compuestos estudiados (en letras minúsculas); los objetivos abordados numerados según se indicó en el *Capítulo 2*; las muestras utilizadas; los patrones; la preparación de los extractos; las técnicas de separación de los compuestos; el tratamiento dado a los extractos y su forma de análisis; la cuantificación; el tratamiento estadístico de los resultados; y por último, la revista y, en su caso la página, donde se ha publicado o se prevé la publicación del artículo.

Se puede encontrar de forma más detallada el procedimiento experimental y los materiales utilizados en el *Capítulo 5. Artículos Científicos*, en el apartado "*Materials and Methods*" de cada uno de los artículos.

LÍNEA DE ACCIÓN		OBJETIVOS	MUESTRAS	PATRONES	PREPARACIÓN DE EXTRACTOS
IDENTIFICACIÓN	Ésteres de crocetina, picrocrocina y derivados glicosilados	▪ Objetivo nº 1 ▪ Objetivo nº 2	▪ Azafrán español secado de la forma tradicional en Castilla-La Mancha: 2 × cosecha 2003 2 × cosecha 2004 ▪ Gardenia, extracto comercial	▪ Gardenia (Chromadex)	Maceración de 20 mg de muestra en 8 mL de agua, en oscuridad durante 1 h
	Flavonoides	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3	▪ Azafranes de distintos orígenes: 3 × españoles 3 × griegos 3 × iraníes 3 × marroquíes	▪ Kaempferol-3-O-soforósido -7-O-glucósido ▪ Kaempferol-3-O-soforósido ▪ Rutina (Sigma-Aldrich)	Extracción de 200 mg de azafrán en polvo en 200 mL de agua, en oscuridad y con agitación magnética durante 1 h
ESTUDIOS CINÉTICOS	Ésteres de crocetina	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3 ▪ Objetivo nº 4	▪ Azafrán español de la D.O. "Azafrán de La Mancha" 1 × cosecha 2004	▪ Ácido gálico (Sigma-Aldrich)	Según ISO 3632/TS (2003) modificando a 500 mg L^{-1} Extractos con y sin materia vegetal Solución de ésteres de crocetina purificados
NUEVOS CAMPOS DE TRABAJO	ANÁLISIS SENSORIAL Picrocrocina	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3 ▪ Objetivo nº 4 ▪ Objetivo nº 6	▪ Azafrán español de la D.O. "Azafrán de La Mancha" 1 × cosecha 2004	▪ Ácido gálico (Sigma-Aldrich) ▪ Safranal (Sigma-Aldrich)	Según ISO/TS 3632 (2003) modificando a 500 mg L^{-1} Extractos con y sin materia vegetal Solución de picrocrocina purificada
APLICACIONES PARA CONTROL DE CALIDAD	ANÁLISIS MULTIVARIANTE Ésteres de crocetina y picrocrocina	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3 ▪ Objetivo nº 4 ▪ Objetivo nº 6	▪ Azafranes españoles: 45 × de D.O. "Azafrán de La Mancha" 16 × no acogidos a D.O.	▪ di-(β-D-gentiobiosil) éster de crocetina (Fluka)	Según ISO/TS 3632 (2003)
	SPE Picrocrocina	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3 ▪ Objetivo nº 5	▪ Azafranes de distintos orígenes, 2004, 2005 y 2006: 7 × españoles 5 × griegos 5 × iraníes 4 × italianos		Según ISO/TS 3632 (2003) modificando a 2500 mg L^{-1} y 500 mg L^{-1}
NUEVOS CAMPOS DE TRABAJO	ULTRAFILTRACIÓN Ésteres de crocetina, picrocrocina y flavonoides	▪ Objetivo nº 1 ▪ Objetivo nº 2 ▪ Objetivo nº 3 ▪ Objetivo nº 7	▪ Azafrán español de la D.O. "Azafrán de La Mancha" 1 × cosecha 2006	▪ Rutina (Sigma-Aldrich)	Extracción previa con ciclohexano 500 mg L^{-1} Clarificación por centifugación y filtración

SEPARACIÓN	TRATAMIENTO Y ANÁLISIS	CUANTIFICACIÓN Y CÁLCULOS	ESTADÍSTICA	PUBLICACIÓN
	▪ LC/ DAD/ MS ESI			JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2006**, *54*, 973-979
▪ Extracción en fase sólida (SPE) con cartuchos C_{18}	▪ Hidrólisis ácida ▪ LC/ DAD/ MS/ MS ESI	▪ Contenido absoluto (mg rutina / g azafrán) ▪ Contenido relativo expresado como % del total de flavonoides	▪ Comparación de los contenidos medios según los países de origen, ANOVA ▪ Análisis discriminante	Food Chemistry **2007**, *100*, 445-450
▪ Cromatografía en columna, C_{18}	▪ Tratamiento térmico (5-70 ºC) ▪ UV-vis (190-700 nm) ▪ HPLC/ DAD	▪ Ésteres de crocetina (% en base seca) ▪ Parámetros cinéticos y termodinámicos (orden, k, $t_{1/2}$, E_a, ΔH^*, ΔS^*, T_{isok}, ΔG_{isok})	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 1627-1637
Cromatografía en columna, C_{18}	▪ Tratamiento térmico (5-100 ºC) ▪ UV-vis (190-700 nm), HPLC/ DAD ▪ SBSE/ GC/ MS ▪ Prueba triangular ISO 4120 (2004)	▪ Picrocrocina ▪ Parámetros cinéticos y termodinámicos (orden, k, $t_{1/2}$, E_a, ΔH^*, ΔS^*) ▪ Umbral de percepción gustativa	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY En revision por pares
	▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Humedad y materia volátil ▪ mg éster crocetina /100 g azafrán en base seca ▪ mg picrocrocina /100 g azafrán en base seca	▪ Calibración multivariante ▪ Modificación del algoritmo de Kennard-Stone, ▪ algoritmo SPXY	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 3167-3175
▪ Cromatografía en columna SPE con cartuchos C_{18}	▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Contenido de picrocrocina (% en base seca), ΔEpic, $E^{1\%}_{1cm}$ 257 nm y 250 nm ▪ Parámetros de validación	▪ ANOVA ▪ t de Student	Food Chemistry En revision por pares
▪ Picrocrocina por cromatografía en columna, C_{18}	▪ Ultrafiltración: 3220 g a 20 ºC entre 10 y 60 min ▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Perfil de filtración de distintos dispositivos ▪ Parámetros de rendimiento de las membranas ensayadas	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 7293-7301

4. WORK PLAN

In this chapter we summarise the work plan followed during the thesis, providing an overall view of the studies carried out.

Each row is dedicated to an article and each column indicates, according to its heading, a part of the approach or method followed. The background colour refers to the line of action (see *Chapter 2. Objectives*), in which each article is integrated: orange for the first, yellow for the second, mauve for the third and blue for the fourth line. When the article includes studies from more than one line of action, the background is composed of a mixture of the relevant colours. This is the case with the article describing the kinetic studies of picrocrocin and the determination of its taste perception threshold, whose background is green (blue + yellow).

From left to right, the columns refer to: the line of action (in upper case letters); the components studied (in lower case); the objectives according to the index of *Chapter 2*; the samples used; standards; method of preparing the extracts; the techniques used to separate the compounds; the treatment of the extracts and the method used to analyse them; their quantification; the statistical treatment of the results; and, lastly, the journal and page number where the article has been published or, in which journal its publication is foreseen.

The experimental procedure and materials used can be found in more detail in *Chapter 5. Scientific Papers*, in the section "*Materials and Methods*" of each article.

LINE OF ACTION		OBJECTIVES	SAMPLES	STANDARDS	EXTRACTS PREPARATION
IDENTIFICATION	Crocetin esters, picrocrocin and glycosylated compounds	▪ Objective 1 ▪ Objective 2	▪ Spanish saffron dried in the traditional way of Castilla-La Mancha: 2 × harvest 2003 2 × harvest 2004 ▪ Gardenia, commercial extract	▪ Gardenia (Chromadex)	Maceration of 20 mg sample in 8 mL of water for 1 h, in the dark
	Flavonoids	▪ Objective 1 ▪ Objective 2 ▪ Objective 3	▪ Saffron from different origins: 3 × Spanish 3 × Greek 3 × Iranian 3 × Moroccan	▪ Kaempferol-3-O-sophoroside -7-O-glucoside ▪ Kaempferol-3-O-sophoroside ▪ Rutin (Sigma-Aldrich)	Extraction of 200 mg powdered saffron in 200 mL of water, magnetically stirred for 1 h in the dark
KINETIC STUDIES	Crocetin esters	▪ Objective 1 ▪ Objective 2 ▪ Objective 3 ▪ Objective 4	▪ Spanish saffron from the PDO "Azafrán de La Mancha" 1 × harvest 2004	▪ Gallic acid (Sigma-Aldrich)	According to ISO/TS 3632 modified to 500 mg L^{-1} Extracts with and without vegetal matter Solution of purified crocetin esters
NEW FIELDS OF WORK	SENSORY ANALYSIS Picrocrocin	▪ Objective 1 ▪ Objective 2 ▪ Objective 3 ▪ Objective 4 ▪ Objective 6	▪ Spanish saffron from the PDO "Azafrán de La Mancha" 1 × harvest 2004	▪ Gallic acid (Sigma-Aldrich) ▪ Safranal (Sigma-Aldrich)	According to ISO/TS 3632 modified to 500 mg L^{-1} Extracts with and without vegetal matter Solution of purified crocetin esters
APPLICATIONS TO QUALITY CONTROL	ANÁLISIS MULTIVARIANTE Crocetin esters and picrocrocin	▪ Objective 1 ▪ Objective 2 ▪ Objective 3 ▪ Objective 4 ▪ Objective 6	▪ Spanish saffron: 45 × PDO "Azafrán de La Mancha" 16 × not belonging to this PDO	▪ di-(β-D-gentiobiosil) ester of crocetin (Fluka)	According to ISO/TS 3632
	SPE Picrocrocin	▪ Objective 1 ▪ Objective 2 ▪ Objective 3 ▪ Objective 5	▪ Saffron from different origins:, 2004, 2005 y 2006: 7 × Spanish 5 × Greek 5 × Iranian 4 × Italian		According to ISO/TS 3632 modified to 2500 mg L^{-1} and 500 mg L^{-1}
NEW FIELDS OF WORK	ULTRAFILTRATION Crocetin esters, picrocrocin and flavonoids	▪ Objective 1 ▪ Objective 2 ▪ Objective 3 ▪ Objective 7	▪ Spanish saffron from the PDO "Azafrán de La Mancha" 1 × harvest 2006	▪ Rutin (Sigma-Aldrich)	Previous extraction with cyclohexane 500 mg L^{-1} Clarification by centrifugation and filtration

SEPARATION	TREATMENT & ANALYSIS	CUANTIFICATION & CALCULATIONS	STATISTIC	PUBLICATION
	▪ LC/ DAD/ MS ESI			JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2006**, *54*, 973-979
▪ Solid phase extraction (SPE) with C_{18} cartridges	▪ Acid hydrolysis ▪ LC/ DAD/ MS/ MS ESI	▪ Content (mg rutin / g saffron) ▪ Relative content expressed as % of total flavonoids	▪ Comparison of mean contents according to the saffron origin, ANOVA ▪ Discriminant analysis	Food Chemistry **2007**, *100*, 445-450
▪ Column chromatography, C_{18}	▪ Thermal treatment (5-70 ºC) ▪ UV-vis (190-700 nm) ▪ HPLC/ DAD	▪ Crocetin esters (% dry basis) ▪ Kinetics and thermodynamics (order, k, $t_{1/2}$, E_a, ΔH^*, ΔS^*, T_{isok}, ΔG_{isok})	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 1627-1637
▪ Column chromatography, C_{18}	▪ Thermal treatment (5-100 ºC) ▪ UV-vis (190-700 nm), HPLC/ DAD ▪ SBSE/ GC/ MS ▪ Triangle test ISO 4120 (2004)	▪ Picrocrocin ▪ Kinetics and thermodynamics (order, k, $t_{1/2}$, E_a, ΔH^*, ΔS^*) ▪ Taste perception threshold	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY In peer review
	▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Moisture and volatile matter content ▪ mg crocetin ester /100 g saffron dry basis ▪ mg picrocrocin /100 g saffron dry basis	▪ Multivariant calibration ▪ Kennard-Stone algorithm modified, ▪ SPXY algorithm	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 3167-3175
▪ Column chromatography, C_{18}SPE with C_{18} cartridges	▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Picrocrocin content (% dry basis), Δ*E*pic, $E^{1\%}_{1cm}$257 nm y 250 nm ▪ Parámetros de validación	▪ ANOVA ▪ Student t test	Food Chemistry In peer review
▪ Picrocrocin by column chromatography, C_{18}	▪ Ultrafiltration: 3220 g at 20 ºC between 10 and 60 min ▪ UV-vis (190-700 nm) ▪ HPLC/DAD	▪ Filtration profile of different centrifugal filter devices ▪ Membrane performance parameters	▪ ANOVA	JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY **2008**, *56*, 7293-7301

CAPÍTULO 5. ARTÍCULOS CIENTÍFICOS

CHAPTER 5. SCIENTIFIC PAPERS


80
60
40
20
30
MBL
ENGLAND
B100

5. ARTÍCULOS CIENTÍFICOS

Esta tesis doctoral ha dado lugar a los artículos científicos referenciados a continuación.

- Carmona, M.; Zalacain, A.; Sánchez, A. M.; Novella, J. L.; Alonso, G. L. Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS. *J. Agric. Food Chem.* **2006**, *54*, 973-979.

ISSN: 0021-8561 Índice de impacto $_{SCI\ 2006}$: 2,322 Puesto $_{SCI}$: 1/31 (*Agriculture, Multidisciplinary*)

- Carmona, M.; Sánchez, A. M.; Ferreres, F.; Zalacain, A.; Tomás-Barberán, F.; Alonso, G. L. Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins. *Food Chem.* **2007**, *100*, 445-450.

ISSN: 0308-8146 Índice de impacto $_{SCI\ 2007}$: 3,052 Puesto $_{SCI}$: 4/103 (*Food Science and Technology*)

- Sánchez, A. M.; Carmona, M.; Ordoudi, S. A.; Tsimidou, M. Z.; Alonso, G. L. Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark. *J. Agric. Food Chem.* **2008**, *56*, 1627-1637.

ISSN: 0021-9673 Índice de impacto $_{SCI\ 2007}$: 2,532 Puesto $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; Pardo, J. E.; Alonso, G. L. Picrocrocin: kinetics of its degradation in aqueous extracts of saffron (*Crocus sativus* L.) spice upon thermal treatment and taste detection threshold. Enviado al *J. Agric. Food Chem.*

ISSN: 0021-9673 Índice de impacto $_{SCI\ 2007}$: 2,532 Puesto $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; Zalacain, A.; Carot, J. M.; Jabaloyes, J. M.; Alonso, G. L. Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV-Visible spectrophotometry for quality control. *J. Agric. Food Chem.* **2008**, *56*, 3167-3175.

ISSN: 0021-9673 Índice de impacto $_{SCI\ 2007}$: 2,532 Puesto $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; del Campo, C. P.; Alonso, G. L. Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.). Enviado a *Food Chem*.

ISSN: 0308-8146 Índice de impacto $_{SCI\ 2007}$: 3,052 Puesto $_{SCI}$: 4/103 (*Food Science and Technology*)

- Sánchez, A. M.; Carmona, M.; Prodanov, M.; Alonso, G. L. Effect of centrifugal ultrafiltration on the composition of aqueous extracts of saffron spice (*Crocus sativus* L.). *J. Agric. Food Chem.* **2008**, *56*, 7293-7301.

ISSN: 0021-9673 Índice de impacto $_{SCI\ 2007}$: 2,532 Puesto $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

5. SCIENTIFIC PAPERS

This doctoral thesis has given rise to the articles indicated below.

- Carmona, M.; Zalacain, A.; Sánchez, A. M.; Novella, J. L.; Alonso, G. L. Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS. *J. Agric. Food Chem.* **2006**, *54*, 973-979.

ISSN: 0021-8561 Impact factor $_{SCI\ 2006}$: 2,322 Place $_{SCI}$: 1/31 (*Agriculture, Multidisciplinary*)

- Carmona, M.; Sánchez, A. M.; Ferreres, F.; Zalacain, A.; Tomás-Barberán, F.; Alonso, G. L. Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins. *Food Chem.* **2007**, *100*, 445-450.

ISSN: 0308-8146 Impact factor $_{SCI\ 2007}$: 3,052 Place $_{SCI}$: 4/103 (*Food Science and Technology*)

- Sánchez, A. M.; Carmona, M.; Ordoudi, S. A.; Tsimidou, M. Z.; Alonso, G. L. Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark. *J. Agric. Food Chem.* **2008**, *56*, 1627-1637.

ISSN: 0021-9673 Impact factor $_{SCI\ 2007}$: 2,532 Place $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; Pardo, J. E.; Alonso, G. L. Picrocrocin: kinetics of its degradation in aqueous extracts of saffron (*Crocus sativus* L.) spice upon thermal treatment and taste detection threshold. Sent to *J. Agric. Food Chem.*

ISSN: 0021-9673 Impact factor $_{SCI\ 2007}$: 2,532 Place $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; Zalacain, A.; Carot, J. M.; Jabaloyes, J. M.; Alonso, G. L. Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV-Visible spectrophotometry for quality control. *J. Agric. Food Chem.* **2008**, *56*, 3167-3175.

ISSN: 0021-9673 Impact factor $_{SCI\ 2007}$: 2,532 Place $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

- Sánchez, A. M.; Carmona, M.; del Campo, C. P.; Alonso, G. L. Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.). Sent to *Food Chem.*

ISSN: 0308-8146 Impact factor $_{SCI\ 2007}$: 3,052 Place $_{SCI}$: 4/103 (*Food Science and Technology*)

- Sánchez, A. M.; Carmona, M.; Prodanov, M.; Alonso, G. L. Effect of centrifugal ultrafiltration on the composition of aqueous extracts of saffron spice (*Crocus sativus* L.). *J. Agric. Food Chem.* **2008**, *56*, 7293-7301.

ISSN: 0021-9673 Impact factor $_{SCI\ 2007}$: 2,532 Place $_{SCI}$: 1/35 (*Agriculture, Multidisciplinary*)

5.1. Ésteres de crocetina, picrocrocina y compuestos relacionados con ella que se encuentran en los estigmas de *Crocus sativus* y en los frutos de *Gardenia jasminoides*. Identificación tentativa de siete nuevos compuestos mediante LC-ESI-MS

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J. Agric. Food Chem. **2006,** *54,* 973–979

Crocetin Esters, Picrocrocin and Its Related Compounds Present in *Crocus sativus* Stigmas and *Gardenia jasminoides* Fruits. Tentative Identification of Seven New Compounds by LC-ESI-MS

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Este primer artículo se centró en la identificación de los ésteres de crocetina, la picrocrocina y otros compuestos glicosilados relacionados con la picrocrocina. Uno de sus objetivos principales fue la determinación de los ésteres de crocetina que se encuentran en el azafrán especia y en los frutos de gardenia, y su comparación con la información que aparecía en la bibliografía. El otro objetivo principal fue estudiar la posibilidad de detectar e identificar en el mismo análisis la picrocrocina y otros precursores glicosilados del aroma citados en la bibliografía para ambas especies.

Ésteres de crocetina

Con las condiciones cromatográficas establecidas se identificaron tentativamente 15 ésteres de crocetina. Aunque se ha descrito en la bibliografía (Dufresne et al., 1999) la existencia del éster de crocetina di-(β-D-neapolitanósido), en nuestro trabajo los ésteres de mayor masa molecular poseían cinco unidades de glucosa y los de menor una, habiéndose encontrado tanto la conformación trans como la cis en todos ellos excepto para el monoglucósido que sólo se encontró en conformación trans.

Se pudo considerar la identificación por primera vez de cinco ésteres de crocetina (Figura 5.1), pues la estructura que propusieron Tarantilis y col. (1995) como *trans*-5-tG, parecía corresponder al *trans*-5-nG propuesta por Pfister y col. (1996) y corroborada posteriormente por Carmona y col. (2005).

Siguiendo el orden de elución en los cromatogramas a 440 nm, el primer pico, a un tiempo de retención de 8,2 minutos, correspondía al *trans*-5-tG, siendo así identificado tentativamente por primera vez en este trabajo. En su patrón de fragmentación de masas aparecía la señal m/z 1161 que correspondía a $[M + Na]^+$, m/z 592 correspondiente a $[M + 2Na]^+/2$, y las señales m/z 837 y 675 que procedían de la pérdida de dos y tres unidades de glucosa, respectivamente. Además, su menor tiempo de retención indicaba una estructura menos plegada que el resto de los *trans*-ésteres de crocetina. También fue posible identificar tentativamente el *cis*-5-tG a un tiempo de retención de 10,1 minutos, aunque no fue posible determinar la posición de las moléculas de glucosa en relación al C_{13}. En los espectros UV-vis, los isómeros cis presentaban una banda de absorción adicional en torno a los 324 nm respecto a los trans y el máximo a 440 nm presentaba un efecto hipsocrómico de unos 5 nm, tal y como ha sido descrito por otros autores (Speranza y col., 1984).

El siguiente compuesto identificado por primera vez fue el *trans*-4-ng. Este compuesto con cuatro unidades de glucosa distribuidas como neapolitanosa en un extremo y glucosa en el otro, eluyó a los 10,8 minutos y presentaba la señal m/z 999 correspondiente a $[M + Na]^+$, m/z 635 debida a la pérdida de un residuo de glucosa (162 u) y un residuo de glucosa en posición 2 (179 u) de la parte del trisacárido [M - glucosa (162 u) - glucosa (179 u)]$^+$. Las semejanzas de su espectro UV-vis con el de otros ésteres de crocetina conocidos que tienen un residuo de neapolitanosa, como el *trans*-5-nG, y sus diferencias con el espectro de otros ésteres de crocetina que tienen residuos de glucosa o gentiobiosa, apuntaban también hacia la estructura propuesta. Su correspondiente isómero cis, el *cis*-4-ng, con un tiempo de retención de 12,9 minutos, también fue identificado tentativamente por primera vez. Además de las señales m/z

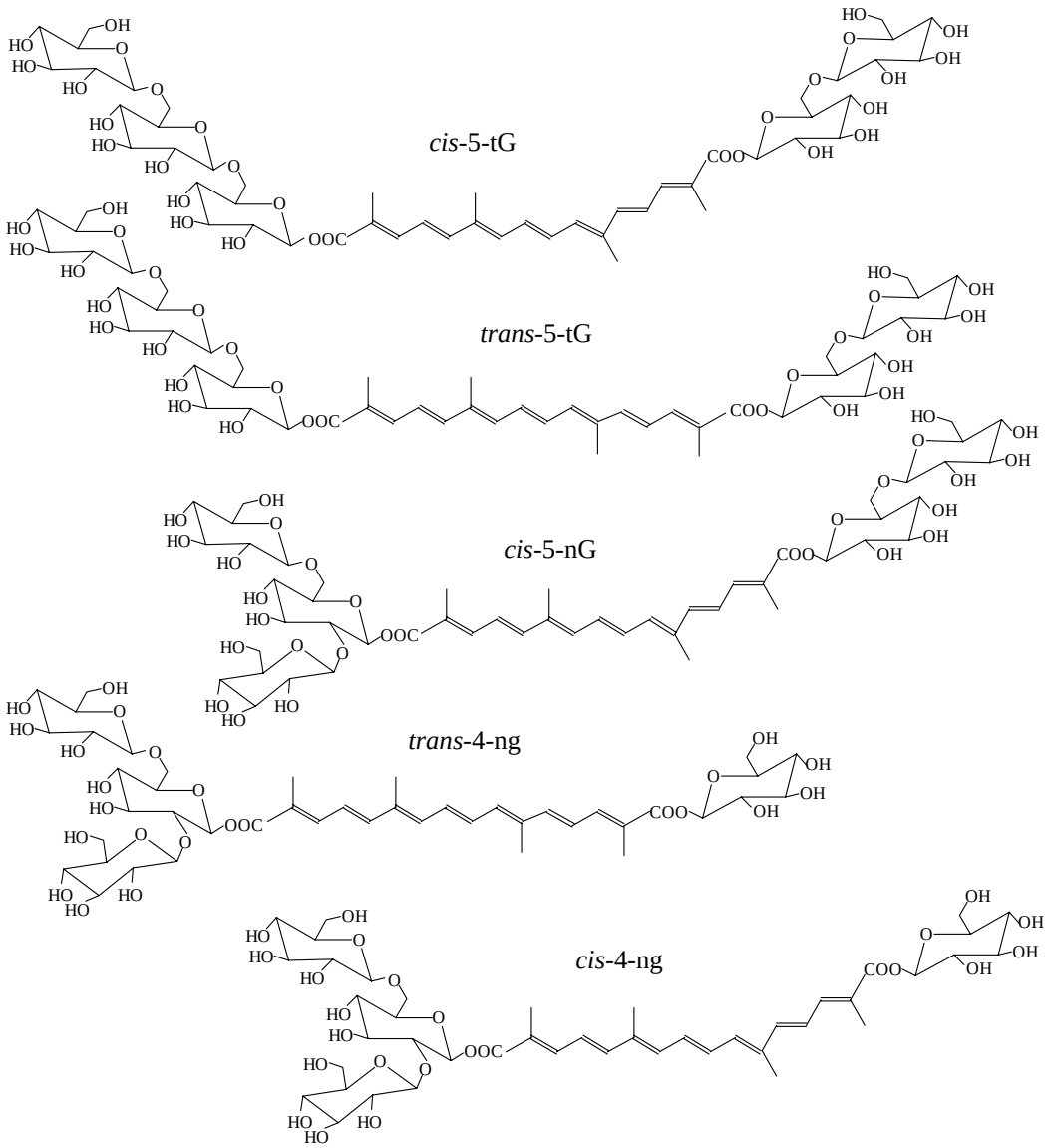

Figura 5.1. Estructuras propuestas para las crocinas identificadas por primera vez.

indicadas para el isómero trans, éste presentaba la señal m/z 797 que junto a la de 635 nos permitió diferenciarlo fácilmente del *cis*-4-GG que eluyó próximo a él (con un

tiempo de retención de 12,6 minutos), a pesar de que sus diferencias espectrales con otros ésteres de crocetina no eran tan evidentes como en el caso anterior.

El último de los ésteres de crocetina identificados por primera vez fue el *cis*-5-nG a los 12,2 minutos. Su patrón de fragmentación estaba formado por las señales m/z 1161, 797 y 592, correspondientes a $[M + Na]^+$, $[M - gentiobiosa]^+$ y $[M + 2Na]^+/2$, respectivamente. También se observó la señal m/z 635 que correspondía a la pérdida de una glucosa (162 u) en la estructura responsable de la señal m/z 797, posiblemente debido a la pérdida de una gentiobiosa de un extremo y de un residuo de glucosa en posición 2 (179 u) de la parte del trisacárido.

En cuanto a la comparación de los ésteres de crocetina presentes en azafrán y en gardenia, otros autores (Pfister y col., 1996; Van Calsteren y col., 1997; Li y col., 1999) que han abordado el tema han identificado siempre un mayor número de los mismos en azafrán. Sin embargo, en nuestros estudios observamos prácticamente los mismos en ambas, con la excepción del *trans*-2-gg que no fue encontrado en gardenia, mientras que en azafrán eluyó a 11,8 minutos (m/z 675, $[M + Na]^+$). Además, los resultados indicaban que en ambas especies el *trans*-4-GG era el que mayor área presentaba en los cromatogramas a 440 nm. Su tiempo de retención era de 10,3 minutos y las principales señales en el patrón de fragmentación de masas eran m/z 999 y 511 correspondientes a $[M + Na]^+$ y $[M + 2Na]^+/2$, respectivamente.

Por otro lado, entre las diferencias más relevantes se observó una mayor proporción del *trans*-5-nG y del *trans*-3-Gg en azafrán que en gardenia con respecto al área de *trans*-4-GG. El *trans*-5-nG presentó un tiempo de retención de 9,7 minutos y las señales m/z 1161, 797 y 592 $[M + Na]^+$, $[M - gentiobiosa]^+$ y $[M + 2Na]^+/2$, respectivamente; mientras que el *trans*-3-Gg eluyó a los 11,0 minutos y su patrón de fragmentación, al igual que el de su isómero cis de tiempo de retención 13,4 minutos, mostraba las señales m/z 837 procedente de $[M + Na]^+$ y 675 de $[M - glucosa + Na]^+$. Sin embargo, la proporción del *cis*-4-GG (tiempo de retención 12,6 minutos) y del *trans*-2-G (tiempo de retención 13,7 minutos) fue menor en azafrán que en gardenia. La

identificación tentativa del *trans*-2-G fue posible gracias a su elución más rápida cuando se acidificaba la fase móvil con ácido fórmico debido a la protonación de su término ácido, y a la señal m/z 513 que, a diferencia del *trans*-2-gg, mostraba junto a la señal m/z 675, y que correspondía a la pérdida de una de las glucosas de la gentiobiosa. La misma señal m/z 513 se utilizó para identificar el *trans*-1-g a los 14,4 minutos. Como se ha mencionado anteriormente, no se encontró el *trans*-2-gg en gardenia pero muy próximo al tiempo de retención del mismo, a los 11,9 minutos aparecía un pico correspondiente a un compuesto con el siguiente patrón de fragmentación de masas: 828 (100), 635 (22), 386 (54) y 289 (61). La señal m/z 635 y su espectro UV-vis eran similares a los del *trans*-5-nG y *trans*-4-ng, indicando la presencia de un residuo de neapolitanosa. La señal m/z 828 podría indicar la presencia de un grupo metilo en la estructura $[M + H]^+$. Es posible que el uso de metanol en la preparación de los extractos comerciales haya podido producir la reesterificación del *trans*-5-nG y el *trans*-4-ng (Carmona y col., 2004). Con los tiempos de retención más avanzados, 14,9 minutos y 15,1 minutos, fue posible identificar el *cis*-2-gg y el *cis*-2-G.

Las diferentes muestras de azafrán español analizadas presentaron muy pocas diferencias, probablemente debido a que habían sido deshidratadas de la misma forma. Además, la coincidencia de los resultados con los descritos por Tarantilis y col. (1995) para azafrán griego indicó que el tipo de ésteres de crocetina del azafrán era independiente del origen geográfico, encontrándose más bien las diferencias entre muestras de distinto origen en la proporción de tales ésteres.

De la misma forma, el extracto de gardenia usado como colorante y comprado en el mercado internacional y el patrón comercial presentaban los mismos compuestos y en la misma proporción.

Gracias a estos estudios de identificación tentativa empleando, además de los espectros UV-vis y tiempos de retención, los patrones de fragmentación de masas y gracias a la comparación de sus resultados con los de otros autores que habían utilizado herramientas espectroscópicas para la identificación de estos compuestos u otros

similares, pudimos establecer la base para los siguientes trabajos sobre los ésteres de crocetina del azafrán.

Picrocrocina y compuestos glicosilados

Los cromatogramas a 250 nm procedentes de los mismos análisis realizados para los ésteres de crocetina reflejaban la composición en picrocrocina y otros compuestos glicosilados precursores del aroma.

La identificación de la picrocrocina de los extractos acuosos de azafrán tras la separación cromatográfica no fue difícil al ser el pico mayoritario. Su mayor polaridad respecto a los ésteres de crocetina hacía que eluyera antes, su espectro UV-vis presentaba un máximo de absorción a 250 nm y las señales m/z 353 y 169 correspondientes a $[M + Na]^+$ y $[M - glucosa]^+$ respectivamente, confirmaban su identificación. Pero en estos extractos acuosos, junto a la picrocrocina, aparecían otros ocho compuestos de los cuales siete presentaban espectros UV-vis muy parecidos al suyo. Se procedió a su elucidación estructural y se identificaron tentativamente por primera vez dos de ellos en azafrán (Figura 5.2). Uno fue el 5-hidroxi-7,7-dimetil-4,5,6,7-tetrahidro-3H-isobenzo-furanona 5-*O*-β-D-gentiobiósido, el otro fue el 4-hidroximetil-3,5,5-trimetil-2-ciclohexenona 4-*O*-β-D-gentiobiósido.

A diferencia de lo que ocurría con los ésteres de crocetina, los perfiles cromatográficos a 250 nm de gardenia diferían mucho respecto a los de azafrán presentando sólo dos picos. El primero de ellos, con un tiempo de retención de 2,6 minutos se identificó como 2-metil 6-oxo-2,4-heptenato de *O*-β-D-gentiobiósido. Mientras que el segundo, que eluyó aproximadamente un minuto más tarde, mostraba la señal m/z 411 en su patrón de fragmentación de masas correspondiéndose con $[M + Na]^+$ y fue tentativamente identificado como el compuesto conocido con el nombre de genipósido. En el artículo se remarcó la importancia de esta última identificación, pues la bibliografía pone de manifiesto la dificultad de su ionización mediante *electrospray* (Hamerskia y col., 2003; Wang y col., 2004). La capacidad del método para detectar

este compuesto permite que sea empleado en la detección de adulteraciones con gardenia en el azafrán.

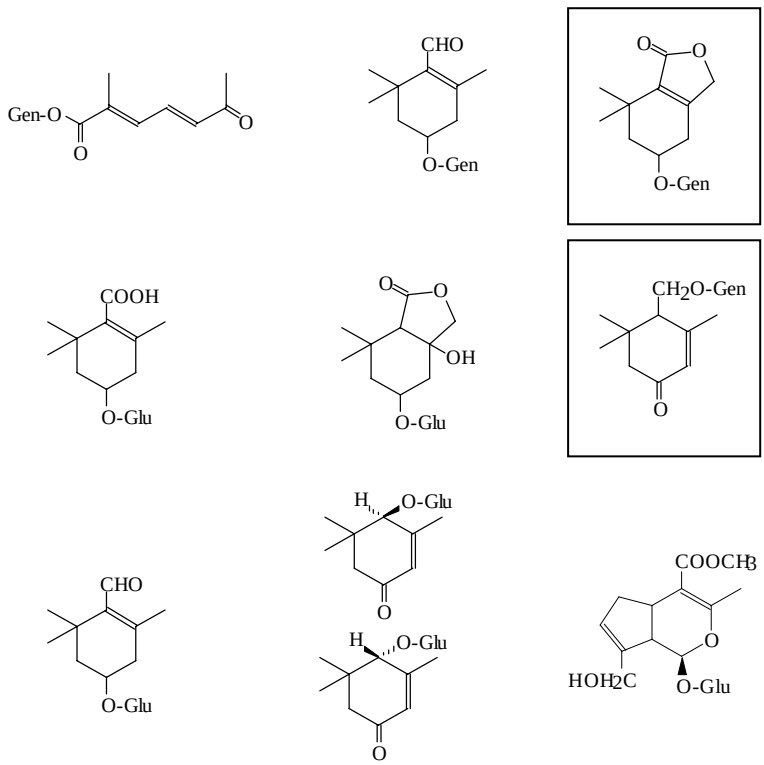

Figura 5.2. Estructuras propuestas para los compuestos glicosilados detectados a 250 nm en un extracto acuoso de azafrán. En los recuadros, estructuras identificadas por primera vez. Glu = glucosa, Gen = gentiobiosa.

La diferencia con resultados anteriores descritos en la bibliografía (Straubinger y col., 1997, 1998a, 1998b) fue fundamentalmente el mayor grado de glicosidación de los compuestos encontrados en nuestros resultados, posiblemente por las condiciones suaves empleadas, tanto en la extracción como en la inyección directa y en la ionización proporcionada por la interfase ESI. Respecto a estos compuestos glicosilados son necesarios más estudios que traten de determinar la relación entre su contenido y el potencial aromático del azafrán determinado por GC.

5.1. Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and Gardenia jasminoides fruits. Tentative identification of seven new compounds by LC-ESI-MS

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Crocetin esters present in saffron (*Crocus sativus* L.) stigmas and in *Gardenia jasminoides* Ellis fruit are the compounds responsible for their color. Of the fifteen crocetin esters identified in this study, five new compounds were tentatively identified: *trans* and *cis* isomers of crocetin (β-D-triglucoside)-(β-D-gentibiosyl) ester, *trans* and *cis* isomers of crocetin (β-D-neapolitanose)-(β-D-glucosyl) ester, and *cis* crocetin (β-D-neapolitanose)-(β-D-gentibiosyl) ester. The most relevant differences between both species were a low content of the *trans* crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester, the absence of *trans* crocetin di-(β-D-glucosyl) ester in gardenia, and its higher content of *trans* crocetin (β-D-gentibiosyl) ester and *cis* crocetin di-(β-D-gentibiosyl) ester. With the same chromatographic method it was possible to identify, in a single run, ten glycosidic compounds in saffron extracts with a UV/vis pattern similar to that of picrocrocin; among them, 5-hydroxy-7,7-dimethyl-4,5,6,7-tetrahydro-3*H*-isobenzofuranone 5-*O*-β-D-gentibioside and 4-hydroxymethyl-3,5,5-trimethyl-cyclohexen-2-one 4-*O*-β-D-gentibioside were tentatively identified for the first time in saffron. Of these ten glycosides, only the *O*-β-D-gentibiosyl ester of 2-methyl-6-oxo-2,4-hepta-2,4-dienoic acid was found in gardenia samples, but it was possible to identify the iridoid glycoside, geniposide.

KEYWORDS: Crocetin esters; gardenia; geniposide; glycosidic aroma precursors; picrocrocin; saffron

INTRODUCTION

An unusual apocarotenoid, called crocetin ($C_{44}H_{64}0_{24}$, 8,8'-diapo-Ψ,Ψ'-carotenedioic acid), which is esterified with one or two glucose, gentibiose, or neapolitanose sugar moieties, is present in *Crocus sativus* L. stigmas and *Gardenia jasminoides* Ellis fruit. These compounds are known for their coloring properties owing to their peculiar water soluble behavior, in contrast to most families of carotenoids.

The bibliography on crocetin ester elucidation and analysis for both spices is quite abundant (*1*−*5*), but there is no consensus as to which compounds are present in each species (**Table 1**). Accurate knowledge about the presence of these compounds may provide important information on their generation pathway and will clarify if the presence of one of them in saffron and not in gardenia may be related to the glycoside and volatile compounds present in saffron only. It is known that, by thermal treatment applied to fresh saffron stigmas to obtain the spice, volatile compounds are generated from its precursors (*6*-*8*). Although several authors have proposed analytical methods such as liquid chromatography to simultaneously detect in saffron the compounds responsible for its color (crocetin glycosides), bitterness (picrocrocin), and aroma (mainly safranal) (*9*−*12*), recently Gregory *et al.* (*7*) have demonstrated that this is not possible with a single-polarity extraction method and only one detection technique. Safranal and other volatiles should be extracted with nonpolar solvents and analyzed by gas chromatography. The glycosidic compounds related to picrocrocin have been studied by countercurrent chromatography (MLCCC) (*13*−*15*). However, the acid form of picrocrocin was detected in saffron when crocetin ester identification was carried out by RP-chromatography (*11*, *12*). Evidently, it is of interest to use a unique methodology to determine both families of compounds, crocetin esters and glycosides related to picrocrocin, and to determine if the quantification of the glycosides could be used to estimate the aromatic potential of the samples. The aroma precursors described in gardenia are linalool and borneol, 1-linalyl 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranoside and bornyl 6-*O*-β-D-xylopyranosyl-β-D-glucopyranoside (*16*), which are different from the ones found in saffron. Iridoid glycosides, such as geniposide, gardenoside, gardoside, and scandoside methyl ester, among others, are also present in gardenia (*17*−*19*). Geniposide, the major component, is reported to be present

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Table 1. UV/Vis and Mass Fragmentation Data for Crocetin Esters and Comparison with Those Found in the Literature[a]

Crocins structure				Crocins identified in										Results				
				Ichi *et al.* (9)	Tarantilis *et al.* (23)	Pfister *et al.* (12)		Straubinger *et al.* (21)	Van Calsteren *et al.* (13)		Knapp *et al.* (10)	Carmona *et al.* (14)	Tentatively identified		Chromatographic and spectrometric behavior		
nº glucose moieties (R + R´)	moiety distribution (R/R´)	isomer	named as	*Gj*	*Cs*	*Gj*	*Cs*	*Cs*	*Gj*	*Cs*	*Gj*	*Cs*	*Cs*	*Gj*	RT (min)	Fragmentation pattern ES+	UV/Vis (λmax)
5	trig/gen	*trans*	*trans*-5-tG										X	X	8.2	592(100), 675(35), 837(21),1161(40)	258,444, 467
		cis	*cis*-5-tG										X	X	10.0	592(100), 675(12), 1161(25)	263, 349, 444, 468
	nea/gen	*trans*	*trans*-5-nG		X		X					X	X	X	9.7	592(62), 797(100), 1161(30)	264, 422sh, 467sh, 440
		cis	*cis*-5-nG		X								X	X	12.2	592(65), 635(100), 1161(15)	263,327, 412, 434
4	nea/glu	*trans*	*trans*-4-ng										X	X	10.8	635 (100), 817(21), 999(28)	252, 422sh, 440
		cis	*cis*-4-ng										X	X	12.9	635(100), 797(18), 999(52)	246, 326, 424, 437sh
	gen/gen	*trans*	*trans*-4-GG		X	X		X	X	X	X	X	X	X	10.3	511(22), 999(100)	262, 442, 465
		cis	*cis*-4-GG		X	X				X	X	X	X	X	12.6	511(32), 999(100)	264, 327,434, 457
3	gen/glu	*trans*	*trans*-3-Gg	X	X			X	X	X		X	X	X	11.0	329(17), 675(14), 837(100)	262, 442, 465
		cis	*cis*-3-Gg		X					X		X	X	X	13.4	543(24), 675(15), 837(100)	259, 327,434, 457
2	gen/-	*trans*	*trans*-2-G	X	X	X		X	X	X	X	X	X	X	13.7	513(25), 675(100)	260, 434, 457
		cis	*cis*-2-G	X				X					X	X	15.9	513(100)	260, 324,432, 452
	glu/glu	*trans*	*trans*-2-gg	X	X			X		X		X	X		11.8	675(100)	262, 44, 464
		cis	*cis*-2-gg		X			X					X	X	15.7	675(100)	260, 326, 430, 452
1	glu	*trans*	*trans*-1-g	X	X			X	X				X	X	15.0	675(100)	261,433, 458
		cis	*cis*-1-g	X													

[a] Abbreviations: (trig) triglucoside; (nea) neapolitanoside; (gen) gentibioside; (glu) glucoside; (*Cs*) *Crocus sativus* L.; (*Gj*) *Gardenia jasminoides* Ellis; (RT) retention time.

up to 35.9% in commercial gardenia preparations (*20*, *21*) and can be hydrolyzed to its aglycone genipin, which possesses genotoxicity. With this in mind, re-evaluation of the safety of gardenia must be carried out before using it as a food colorant (*22*).

The aim of this study was to establish which crocetin esters are present in saffron and in gardenia, comparing the results with those found in the literature, and to determine if it is possible to detect and identify aroma glycoside precursors reported in both species in the same run.

MATERIALS AND METHODS

Plant Materials. *Saffron (Crocus sativus L.).* Harvesting, removal, and dehydration of the stigmas took place in Motilla del Palancar (Cuenca, Spain) using traditional procedures which adhered to the trade standard of the Protected Denomination of Origin "Azafrán de la Mancha" (*23*, *24*). Two samples from the 2003 harvest plus two samples from 2004 were analyzed.

Gardenia (Gardenia jasminoides Ellis). Two samples of gardenia were characterized. One sample was a commercial standard supplied by Chromadex (Santa Ana, CA), and the second one was a commercial extract supplied by Verdú-Cantó Saffron Spain (Novelda, Alicante, Spain).

LC-DAD-MS Conditions. Twenty milligrams of the sample was macerated for 1 h in 8 mL of milliQ water previously bubbled with helium. The entire process was carried out in darkness and at room temperature. Twenty microliters of the extract filtered through a PVDF filter of 0.45 μm (Millipore) was injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex Luna C18 column thermostated at 30 °C. Two elution systems were assayed, the unique difference being that the aqueous phase was acidified. The solvents were water (or acidified with 0.25% formic acid) (A) and acetonitrile (B), using the following gradient: 80% A for 5 min to 20% A in 15 min, at a flow rate of 0.8 mL/min. Dual on-line detection was carried out by a diode array spectrophotometer and a quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 1100). The probe of the mass spectrometer was connected to the UV cell outlet. The DAD detector was set at 250, 330, and 440 nm. Both the auxiliary and the sheath gases were nitrogen with a flow rate of 12 L/min. The drying gas temperature was set at 350 °C, and the nebulizer pressure, at 30 psi. The capillary voltage was ±2500 V, and the capillary temperature, 195 °C. Spectra were recorded in positive and negative ion modes between *m*/*z* 100 and 1500. Identification was carried out with Agilent Chemstation software for LC/MS.

Nomenclature for Crocetin Esters. To abbreviate the names of crocetin esters in this paper, they have been labeled as follows: first, the nomenclature which refers to the isomeric *cis* and *trans* forms has been written with a hyphen separating the total number of glucose moieties at both extremes of the base molecule. Then, the glucose moiety distribution was indicated as (t) triglucoside, (n) neapolitanoside, (G) gentibioside, or (g) glucoside. The name of the base structure, crocetin esters, was removed, since it is the same in all compounds.

RESULTS AND DISCUSSION

Crocetin Ester Identification. During routine assays with the mobile phase, it was observed that the addition of formic acid to the aqueous phase to facilitate compound ionization scarcely improved the sensitivity. However, on the contrary, it produced wider peaks and a different chromatographic behavior in the case of the *trans* crocetin (β-D-gentibiosyl) ester (*trans*-2-G), since it was eluted earlier due to the protonation of its acidic terminus. This elution behavior made the identification of the preceding *cis* isomers difficult. Next, all samples were analyzed by both methods, taking advantage of these acidic properties plus a better chromatographic resolution and higher fragmentation degree.

Although Pfander and Schurtenberger (*25*) were the first to isolate six glycosides of crocetin in saffron, Tarantilis *et al.* (*11*, *12*) identified a greater number of crocetin esters and their *trans* and *cis* isomers (**Table 1**), by high performance liquid chromatography with UV/vis photodiode array detection coupled to mass spectrometry. The compounds with higher molecular

weight found were the crocetin esters with five glucose units. Unexpectedly, it seems that a crocetin di-(β-D-neapolitanose) ester exists, but it has not been possible to detect its presence in saffron spice extracts. Dufresne *et al.* (*26*) found that this was the pigment preponderantly produced by a *Crocus sativus* L. culture in suspension when crocetin was the substrate.

The results present in this paper confirmed what has been found by these authors, except for *trans*-5-tG, whose structure was not in accordance with the one proposed by Pfister (*4*) as *trans*-5-nG and corroborated later by Carmona *et al.* (*6*). In this study, its corresponding *cis* isomer (*cis*-5-nG), which eluted at 12.2 min, was tentatively identified for the first time, with a fragmentation pattern of *m*/*z* 1161, 797, and 592, corresponding to $[M + Na]^+$, $[M -$ gentibiose$]^+$, and $[M + 2Na]^+/2$, respectively. A signal at *m*/*z* 635 was also observed and corresponded to the loss of an additional glucose moiety (162 amu) from the structure responsible for *m*/*z* 797. This situation was probably due to the gentibiose loss of one extreme and the glucose molecule in position 2 (179 amu) from the trisaccharide end. Furthermore, in relation to their *trans* isomers, *cis*-crocetin esters present an additional absorption band around 324 nm in their UV/vis spectra and their maximum absorption at 440 nm presents a hypsochromic effect of some 5 nm (*27*) that facilitates their identification.

A new crocetin ester present in both species, which would correspond to the structure proposed by Tarantilis *et al.* (*12*), *trans*-5-tG, was identified at $t_R = 8.2$ min (**Figure 1**). The mass spectrum of this new crocetin ester denoted the presence of five glucose residues, *m*/*z* 1161, corresponding to $[M + Na]^+$, as well as the previous one, but with a different disposition. Its shorter retention time implied a more unfolded structure, as crocetin esters of a larger size are the first to elute from the chromatographic column. Its fragmentation pattern coincided with this structure proposal with *m*/*z* 1161 and 592 and two other signals 837 and 675 which correspond to the loss of two and three glucoses, respectively. Also, a tentative identification of its corresponding *cis* isomer (*cis*-5-tG) at 10.1 min was possible. Using LC-MS, it is not possible to determine the positions of the sugar moieties in relation to the C_{13} bond.

The higher crocetin ester content in both spices was *trans*-4-GG at $t_R = 10.3$ min (**Figure 1**), whose signals at *m*/*z* 511 and 999 correspond to $[M + Na]^+$ and $[M + 2Na]^+/2$, respectively. Its *cis* isomer (*cis*-4-GG) at $t_R = 12.6$ min was found in higher proportion in gardenia than in saffron (**Figure 1**). In addition, for the first time, it was possible to tentatively identify two new compounds ($t_R = 10.8$ and 12.9 min) with four glucose molecules, neapolitanose at one end and a glucose at the other end of the molecule, the *trans*-4-ng and *cis*-4-ng. Next to the *m*/*z* 999 signal corresponding to $[M + Na]^+$, a predominant *m*/*z* 635 peak appears in both cases that again corresponds to $[M -$ glucose (162 uma) $-$ glucose (179 uma)$]^+$. Its UV/vis spectrum also revealed the structure proposed, as it was identical to that of *trans*-5-nG (**Figure 2a**) and different from the characteristic spectrum of the other crocetin esters (**Figure 2b**). Nevertheless, in its *cis* isomer (*cis*-4-ng), spectroscopic differences were not so evident, but the signal at *m*/*z* 797 and 635 allowed us to differentiate it easily from the crocetin ester (*cis*-4-GG) that eluted next to it (**Figure 1**).

The crocetin structure with three glucoses had a gentibiose moiety in one extreme and glucose in the other (*trans*-3-Gg, *cis*-3-Gg). Both isomers, *trans* ($t_R = 11.0$ min) and *cis* ($t_R = 13.4$ min) showed signals at *m*/*z* 837 and 675 corresponding to $[M + Na]^+$ and $[M -$ glucose $+ Na]^+$, respectively. As can be observed in **Figure 1**, the content of *trans*-3-Gg was much higher in saffron than in gardenia in relation to the *trans*-4-GG content, which in both cases is the most important compound.

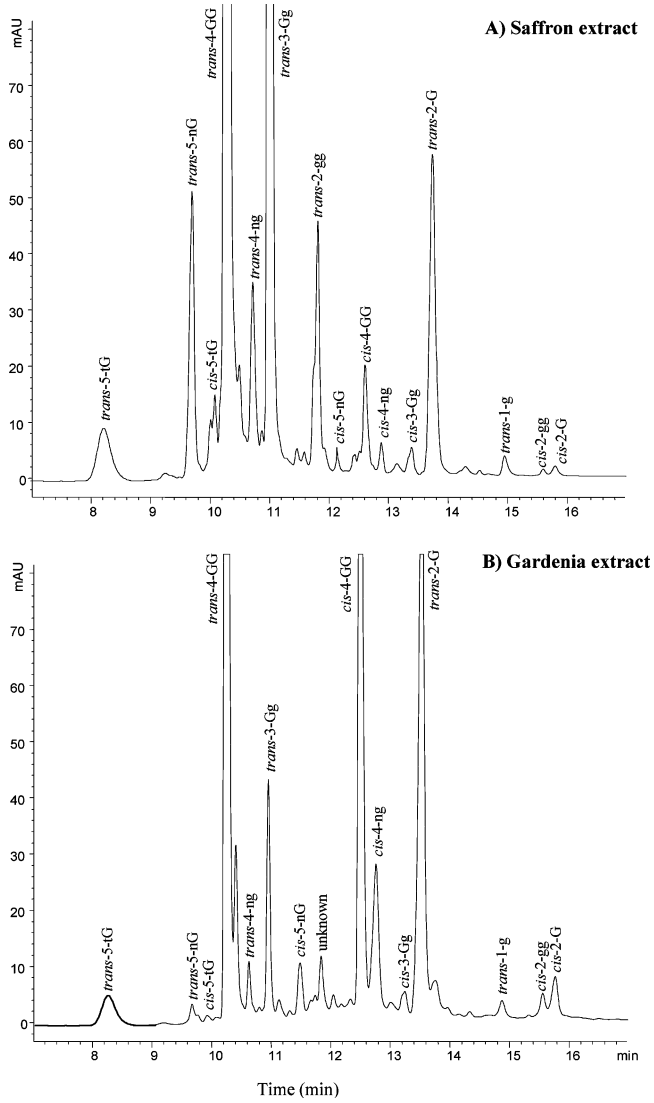

Figure 1. Crocetin esters identified in saffron and gardenia extracts, based on UV/vis spectra and mass fragmentation patterns.

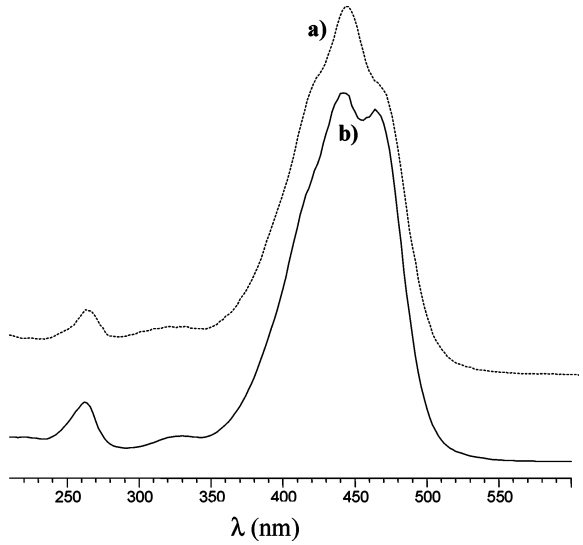

Figure 2. Characteristic UV/vis spectra of (a) a *trans*-crocetin ester including the neapolitanose moiety and (b) *trans*-crocetin esters including glucose and gentibiose but without neapolitanose.

The crocetin ester with two glucose units (*m*/*z* 675, $[M + Na]^+$) had two possible compositions, one with one glucose at each extreme (*trans*-2-gg, $t_R = 11.8$ min; *cis*-2-gg, $t_R = 14.9$ min) and one with both as a gentiobiose unit in one extreme (*trans*-2-G, $t_R = 13.7$ min; *cis*-2-G, $t_R = 15.1$ min). The *trans*-2-G ester was possible to identify since its chromatographic behavior changed when formic acid was added to the mobile phase, showing an additional *m*/*z* 513 peak which corresponded to the loss of one of the two glucoses that form the gentibiose unit and which was not observed for *trans*-2-gg. This *m*/*z* 513 ion was the one which allowed us to identify *trans*-1-g ($t_R = 14.4$ min). In the case of gardenia, *trans*-2-gg was not detected and the peak at retention time 11.9 min (**Figure 1**) was a compound with a fragmentation pattern at *m*/*z* 828 (100), 635 (22), 386 (54), and 289 (61). Its *m*/*z* 635 ion and UV/vis spectra were similar to the ones found in *trans*-5-nG and *trans*-4-ng (**Figure 2b**), confirming the presence of a neapolitanose moiety. The *m*/*z* 828 fragment may indicate an additional methyl group $[M + H]^+$ in the structure. It is possible, as happens in commercial food grade extracts, that the use of methanol might re-esterify *trans*-5-nG and *trans*-4-ng, as there is evidence that methanol in the chromatographic system or during extraction produces this effect (*25*, *28*). It is also important to point out that, in relation to saffron, crocetin esters *cis*-2-G, *cis*-2-gg, and *trans*-1-g were found in higher proportions in old saffron samples, probably due to the degradation of higher structures over time, as several authors suggest that this happens during storage (*29*−*33*).

In general, the differences found between the saffron samples were few, probably due to the fact that the dehydration process used was the same. In addition, the coincidence of the results with those reported when Greek saffron was analyzed (*12*) indicates that it is possible to identify the same crocetin ester structures independently of their geographical origin, while the important differences would be the ratio between them. Nowadays, it is assumed that the differences found are due to the plant. Since it is a sterile triploid, all the world's vegetable material could come from a unique starting point (*34*), although some genetic variations could occur in diverse populations and they could not be genetically identical.

In relation to the crocetin ester analysis in gardenia extracts, we noted the similar behavior of both gardenia samples, an extract acquired on the international market as a coloring material, and a commercial standard. They contained the same compounds, and the relationship between them hardly varied. How these pigments are extracted from the fruit and whether the extraction techniques used may affect the crocetin ester profile remain to be studied. Previous papers that have compared both profiles (*4*, *5*, *35*) always identified a larger number of crocetin esters in saffron than in gardenia, which has not been corroborated in this paper, with the exception of *trans*-2-gg. To sum up, the most relevant differences between both species were the absence of *trans*-2-gg in gardenia, the low presence of *trans*-3-Gg, and the high content of *trans*-2-G and *cis*-4-GG.

Glycosidic Compounds: Aroma Precursors. In contrast to what occurred with crocetin esters, when the detector was set at 250 nm, the differences found between both species were very important (**Figure 3**). In the case of saffron, seven different compounds with a maximum wavelength at 250 nm, characteristic of picrocrocin, were found. Only peak **a** showed a UV spectrum significantly different from that of picrocrocin, showing a λ_{max} at 290 nm, probably due to the linear configuration chain of this compound. The signals at *m*/*z* 501 and 367 of peak **a** corresponded respectively to $[M + Na]^+$ and the loss of a linear fragment of the molecule, $[M - C_7OH_{10}]^+$ (**Figure 3a**). The compound was identified as the *O*-*β*-D-gentibiosyl ester of 2-methyl-6-oxo-2,4-hepta-2,4-dienoic acid, previously described in saffron (*13*−*15*). When the aqueous mobile phase was acidified with formic acid to facilitate the substances ionization, peak **a** was observed to move from a retention time of 2.5 min to 3.5 min. It would be the same compound in an ionic form but with a glucose residue loss (*m*/*z* 361 $[M + 2Na]^+$ in the positive ion mode and *m*/*z* 337 $[M - H + Na]^-$ in the negative ion mode), confirming this structure with *m*/*z* 177 [M − glucose + Na]$^+$ and the UV spectrum previously described.

Peak **b** was identified as 4-hydroxy-2,6,6-trimethyl-cyclohexen-1-carbaldehyde 4-*O*-*β*-D-gentibioside, where the *m*/*z* 515 and 339 values corresponded to $[M + Na]^+$ and the loss of the cyclohexene moiety $[M - C_{10}H_{16}O]^+$ (**Figure 3b**). Peak **c** was identified as 5-hydroxy-7,7-dimethyl-4,5,6,7-tetrahydro-3*H*-isobenzo-furanone5-*O*-*β*-D-gentibioside, after assigning the signals at *m*/*z* 527, 369, and 185 to their respective ions $[M + Na]^+$, $[M - C_5H_{12}O_4]^+$, and $[M - C_7H_{12}O_7]^+$ (**Figure 3c**). The signals 369 and 185 corresponded to different sugar losses.

The compound assigned to peak **d** matched one found by Tarantilis *et al.* (*12*) and Winterhalter (*36*), which was identified as the 4-hydroxy-2,6,6-trimethyl-1-cyclohexene carboxylic acid 4-*O*-*β*-D-glucopyranoside. The mass fragmentation signals at *m*/*z* 369 and 167 corresponded to $[M + Na]^+$ and the loss of a sugar moiety $[M - C_6H_{11}O_5]^+$, respectively (**Figure 3d**). The mass fragmentation pattern of peak **e** (**Figure 3e**) was consistent with a compound whose molecular formula was $C_{10}O_4H_{14}$ plus a glucose moiety. Careful examination of the signals at *m*/*z* 383 and 367, which corresponded to $[M + Na]^+$ and $[M - OH + Na]^+$, identified the peak as 5-hydroxy-7,7-dimethyl-4,5,6,7-tetrahydro-3*H*-isobenzo-furanone 5-*O*-*β*-D-glucopyranoside, which is the hydrated structure of the compound assigned to peak **c** but with a glucose residue loss. Peak **f** showed *m*/*z* 515 $[M + Na]^+$ and 357 $[M - C_5H_{12}O_4]^+$, which means that a part of the gentibiose had been cleaved. This compound was identified as 4-hydroxymethyl-3,5,5-trimethyl-cyclohex-2-en-1-one 4-*O*-*β*-D-gentibioside (**Figure 3f**).

The most abundant compound, peak **g**, found at 250 nm, had *m*/*z* 353, corresponding to $[M + Na]^+$, while *m*/*z* 169 represented a glucose residue loss [M − glucose + Na]$^+$ which confirmed the picrocrocin identification (**Figure 3g**). Finally, peak **h**, which showed two shoulders, was assigned to the two isomers, *R* and *S*, of 4-hydroxy-3,5,5-trimethyl-cyclohex-2-en-1-one 4-*O*-*β*-D-glucopyranoside (*m*/*z* 337 $[M + Na]^+$) (**Figure 3h**).

Besides the clear benefit of using the LC technique to identify at the same time crocetin esters and glycoside aroma precursors, it should be pointed out that this methodology could reproduce the real composition of saffron more precisely. The important differences from the results found by other authors are that when they employed MLCCC, compounds **c**, **e**, and **f** were not found (*14*, *15*), even though they were able to identify similar ones but with one glucose molecule less in the structure, as happened here when the mobile phase was acidified and peak **a** determined. This might have been the result of using an exhaustive Soxhlet extraction instead of the water extraction and direct injection as proposed in this paper.

In the case of gardenia, only two compounds were detected, the first with the same chromographic behavior, fragmentation pattern, and UV/vis spectra as compound **a** (**Figure 3**), previously identified in saffron. The second compound, peak **i** (**Figure 3i**), showed a fragmentation pattern (*m*/*z* 411) corre-

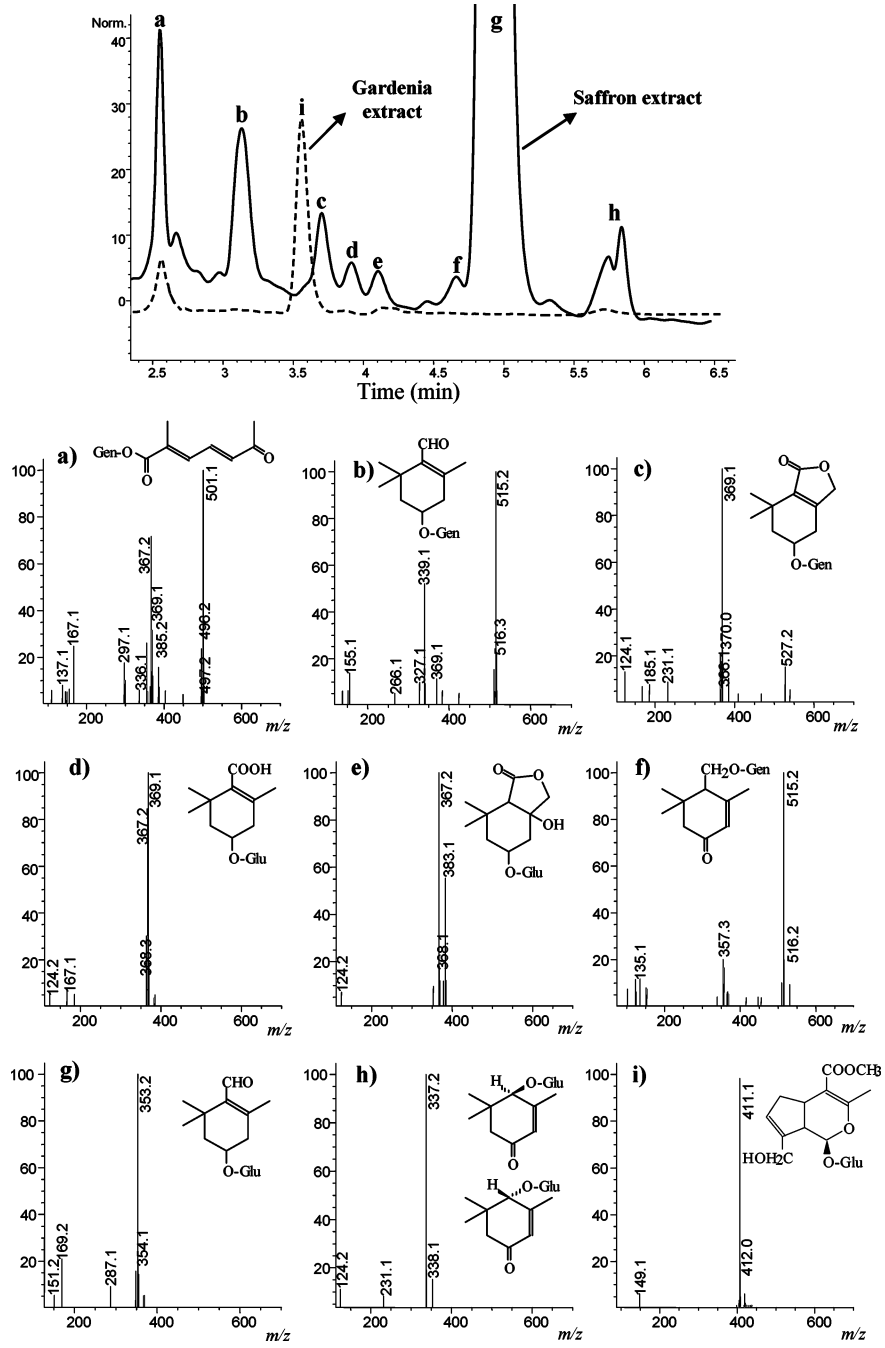

Figure 3. Chromatogram (250 nm) for saffron and gardenia extracts with their respective mass fragmentation patterns.

sponding to $[M + Na]^+$ and tentatively identified as 1,4,5,7-tetrahydro-7-(acetomethyl)-cyclopentapyran-4-carboxylic acid methyl ester, commonly known as geniposide (*20*). This last identification is of great importance, because the ionization of this iridoid glycoside via electrospray is difficult (*37*). According to Wang and co-workers (*19*), this identification is an analytical challenge since there are no LC-MS methods published. The absence of picrocrocin in gardenia extract makes the authors doubt about the biosynthesis of crocetin and its glycosylated forms, related with the oxidative degradation of zeaxanthin (*25*). This oxidative degradation takes place at both ends of zeaxanthin, (7, 8) and (7′, 8′), by means of a carotenase action liberating crocetinaldehyde and picrocrocin (*38*). The crocetindialdehyde is oxidized to crocetin and subsequently glycosylated by different glycosyltransferases (*39, 40*), generating the different crocetin glycosides, mainly crocin, *trans*-crocetin di-(β-D-gentibiosyl) ester. This hypothesis explains what happens with saffron, as both zeaxanthin extremes produce picrocrocin (4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), the compound responsible for saffron bitterness as described in the bibliography (*9, 41, 42*). Degradation of picrocrocin and other glycosidic compounds, which results from the different chain breaks due to the action of other carotenases, produces different volatile saffron compounds (*36*). In contrast, in gardenia fruits, none of these glycosidic compounds have been identified. If the biosynthetic pathway described for crocetin esters in both cases is assumed to be the same, then, due to the economy principle for unusual compounds, gardenia should have an extraordinarily efficient enzymatic system in order to eliminate both chain extremes that are produced in considerable amounts, e.g. picrocrocin. To our knowledge, this mechanism has not yet been described.

The chromatographic method proposed in this study enabled us to identify the crocetin esters present in saffron and gardenia.

The differences between both species are, except for *trans*-2gg, a question of proportions, as the same compounds are identified. At the same time, it is possible to identify the glycosides present in both species that would permit the authentification of saffron powder, avoiding the adulterations with cheaper gardenia extracts. Further studies could determine the possibility of relating the glycosidic compound content to the aromatic potential determined by gas chromatography.

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5.2. Identificación de la fracción de flavonoides del azafrán especia mediante LC/DAD/MS/MS: Estudio comparativo de muestras con distintos orígenes geográficos

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Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins

Manuel Carmona [a,*], Ana M[a] Sánchez [a], Federico Ferreres [b], Amaya Zalacain [a], Francisco Tomás-Barberán [b], Gonzalo Luis Alonso [a]

Una vez identificados los ésteres de crocetina, la picrocrocina y los derivados glicosilados, dedicamos nuestros esfuerzos a la identificación, cuantificación y comparación de los flavonoides de los extractos acuosos del azafrán. Así, los objetivos del segundo artículo que conforma esta tesis fueron la identificación de la fracción de flavonoides de los extractos acuosos del azafrán mediante LC/DAD/MS/MS y el estudio de las diferencias en sus contenidos en muestras procedentes de España, Grecia, Irán y Marruecos. Este último planteamiento trataba de revelar si los flavonoides podrían utilizarse como marcadores biológicos en la determinación del origen del azafrán.

Para obtener la fracción de flavonoides y poder analizarla, fue necesario un paso de separación y concentración por extracción en fase sólida. Tras el proceso de aislamiento, en la fracción obtenida quedaba algo de picrocrocina pero no interfería en la determinación de los flavonoides. Mediante una hidrólisis ácida pudimos establecer que todos los flavonoides identificados tenían como aglicona el kaempferol. En total, se elucidó tentativamente la estructura de cinco compuestos a través de sus espectros UV-

vis, tiempos de retención, espectros de masas: MS/MS y la comparación con los patrones disponibles.

De los cinco derivados de kaempferol encontrados, dos aparecían en los cromatogramas como mayoritarios, a los tiempos de retención 7,7 y 20,9 minutos. Sus espectros de masas mostraban los iones moleculares deprotonados, $[M - H]^-$, m/z 771 y 609 y además, el ion m/z 285 correspondiente a la aglicona deprotonada. Por tanto, se trataba de derivados del kaempferol con tres y dos hexosas, respectivamente. Además, sus patrones de fragmentación coincidían con los de los patrones del kaempferol-3-*O*-soforósido-7-*O*-glucósido y kaempferol-3-*O*-soforósido procedentes de coliflor (*Brassica oleracea* L. var. *botrytis*) de la colección del grupo de investigación en calidad, seguridad y bioactividad de plantas comestibles del Departamento de Ciencia y Tecnología de los Alimentos del CEBAS-CSIC (Murcia). Por tanto, se identificó como kaempferol-3-*O*-soforósido-7-*O*-glucósido el compuesto de tiempo de retención 7,7 minutos, confirmando la estructura descrita con anterioridad por Straubinger y col. (1997), y como kaempferol-3-*O*-soforósido el compuesto de tiempo de retención 20,9 minutos (Figura 5.3 A, B).

El siguiente compuesto en importancia por su contenido en los cromatogramas aparecía a los 13,8 minutos y presentaba la señal m/z 771 correspondiente a $[M - H]^-$, y en MS2 $[M - H]^-$ el único ion que aparecía era el m/z 609 al perderse una hexosa del ion molecular deprotonado al igual que ocurría en el kaempferol-3-*O*-soforósido-7-*O*-glucósido, indicando la presencia de un monohexósido unido directamente a un grupo hidroxilo del compuesto (Ferreres y col., 2004). Sin embargo, difería del compuesto anteriormente mencionado tanto en su espectro UV-vis como en el resto de la fragmentación de masas. En su espectro UV-vis, el máximo a 333 nm y su baja absorbancia indicaban que el grupo hidroxilo en posición 4' estaba sustituido. Por otra parte, mientras que en el kaempferol-3-*O*-soforósido-7-*O*-glucósido en MS3 $[(M - H)\rightarrow(M - H - 162)]^-$ se observaba la fragmentación típica de un soforósido con el pico base correspondiente a la aglicona deprotonada (m/z 285), en este compuesto el pico

base era el ion m/z 429, resultado de la pérdida de 180 u (162 + 18) y había un 63% de abundancia relativa en el ion m/z 285. Esto demostraba que en éste compuesto los azúcares no estaban en forma de disacárido, sino que estaban unidos a diferentes grupos hidroxilo de la molécula. Con todos estos datos, el compuesto de tiempo de retención 13,8 minutos se identificó tentativamente como kaempferol-3,7,4'-triglucósido (Figura 5.3 C). El orden de elución del kaempferol-3,7,4'-triglucósido respecto al kaempferol-3-*O*-soforósido-7-*O*-glucósido era difícil de prever debido a la sustitución del grupo hidroxilo en la posición 4'.

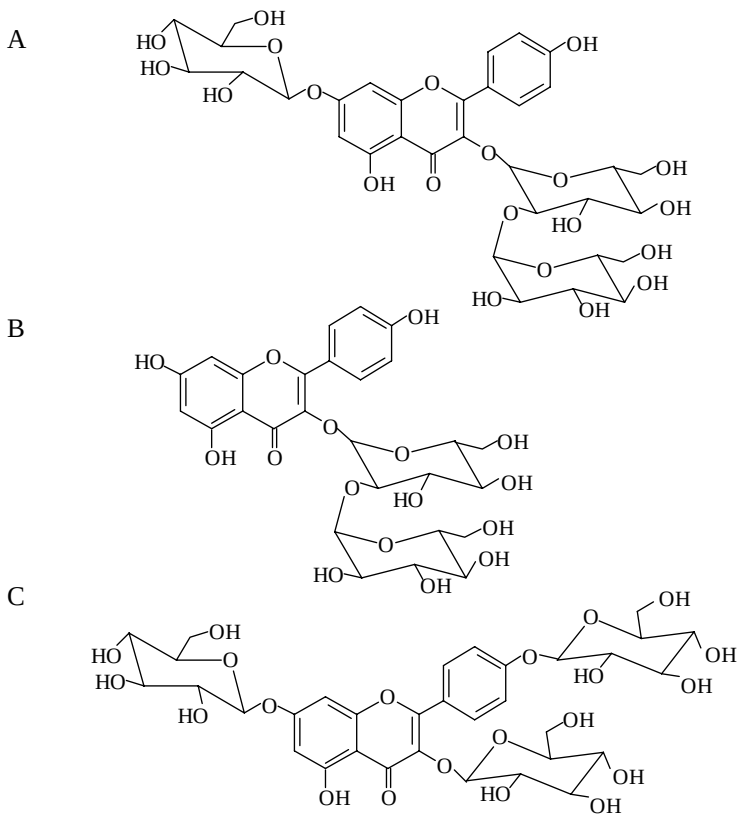

Figura 5.3. Estructura de los principales kaempferoles identificados en el azafrán especia. (A) Kaempferol 3-*O*-soforósido-7-*O*-glucósido; (B) kaempferol 3-*O*-soforósido; (C) kaempferol-3,7,4'-triglucósido.

Se observaron otros dos derivados del kaempferol en menor cantidad. Los dos presentaban un espectro UV-vis característico de derivados sustituidos al menos en la posición 3 (banda de absorción 348-355 nm). El primero de ellos eluyó a los 6,3 minutos y su análisis MS mostraba las señales m/z 933 correspondiente a $[M - H]^-$ y m/z 285 procedente de la aglicona. Sin embargo, su fragmentación de masas no estaba bien definida y no se le pudo asignar una estructura específica, simplemente pudimos asegurar que se trataba de un kaempferol tetrahexósido. El segundo compuesto, cuyo tiempo de elución era 18,2 minutos, parecía tener dos hexosas (m/z 609, $[M - H]^-$) y ser un isómero del kaempferol-3-*O*-soforósido. Su espectro UV-vis era casi igual al de éste indicando en ambos el bloqueo de la posición 3. También había grandes semejanzas en su fragmentación de masas, pues en ambos aparecía el pico base m/z 285 que nos indicaba que los dos residuos glicosilados estaban unidos a un único grupo hidroxilo. En el kaempferol-3-*O*-soforósido se observaba el ion m/z 429 de la pérdida de 162 + 18 u a partir del ion molecular, y en este compuesto de tiempo de retención 18,2 minutos se observaba el ion m/z 447 de la pérdida de 162 u a partir del ion molecular. Con los datos disponibles sólo pudimos afirmar que se trataba de un kaempferol-3-dihexósido.

Una vez tentativamente identificados los compuestos más relevantes de la fracción de los flavonoides del azafrán, procedimos a la cuantificación del kaempferol-3-*O*-soforósido-7-*O*-glucósido, del kaempferol-3,7,4'-triglucósido y del kaempferol-3-*O*-soforósido, mediante una recta de calibrado realizada con un patrón de rutina y expresando los resultados como mg equivalentes de rutina/g de azafrán en base seca. No se tuvieron en cuenta los otros dos derivados del kaempferol por su menor contenido.

En la comparación de los resultados de las muestras de azafrán de España, Grecia, Irán y Marruecos no se apreciaban diferencias cualitativas, es decir, en el tipo de glicósidos de kaempferol presentes, aunque sí se observaron diferencias cuantitativas que podrían deberse a las condiciones edafoclimáticas del cultivo, o bien a diferentes tratamientos poscosecha como el proceso de deshidratación, tal y como se describe en la bibliografía para los ésteres de crocetina (Carmona y col., 2005). Las muestras

españolas presentaban como flavonoide mayoritario el kaempferol-3-*O*-soforósido (47,1%), seguido del kaempferol-3-*O*-soforósido-7-*O*-glucósido (37,4%) y por último en contenido se encontraba el kaempferol-3,7,4'-triglucósido (15,5%). En las muestras griegas el kaempferol-3-*O*-soforósido-7-*O*-glucósido con un 63,2% del contenido de flavonoides fue el mayoritario seguido del kaempferol-3,7,4'-triglucósido (20,4%) y del kaempferol-3-*O*-soforósido (16,4%). En las muestras iraníes y marroquíes el kaempferol-3-*O*-soforósido-7-*O*-glucósido fue el mayoritario con un 50,9% y un 48,7%, respectivamente, seguido del kaempferol-3-*O*-soforósido con un 29,5% y un 28,8%, respectivamente y el kaempferol-3,7,4'-triglucósido fue el de menor contenido con un 19,7% y un 22,4%, respectivamente.

Los resultados indicaban que el contenido en kaempferol-3-*O*-soforósido-7-*O*-glucósido de las muestras españolas era significativamente mayor que el de las iraníes y lo mismo sucedía con su contenido en kaempferol-3,7,4'-triglucósido. Además, el contenido en kaempferol-3-*O*-soforósido de las muestras de azafrán español era significativamente mayor que el del resto de las muestras estudiadas.

En cuanto a los contenidos totales de los tres kaempferoles mayoritarios, se encontraron valores comprendidos entre 3,13 y 6,79 mg equivalentes de rutina/g de azafrán, correspondiendo los valores menores a las muestras iraníes y los mayores a las españolas. En el análisis discriminante las muestras analizadas se separaban claramente por su contenido en kaempferol-3-*O*-soforósido, que era capaz de explicar el 100% de la varianza utilizando como variable de agrupación el origen geográfico.

5.2. Identification of the flavonoid fraction in saffron spice by LC/DAD/MS/MS: Comparative study of samples from different geographical origins

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Abstract

The flavonoid fraction in saffron spice has been analysed, for the first time, by LC-DAD-MS/MS ESI and five kaempferol derivatives have been found. Compounds such as kaempferol-3-sophoroside, kaempferol-3-sophoroside-7-glucoside and kaempferol-3,7,4′-triglucoside were tentatively identified, whereas other compounds, such as kaempferol tetrahexoside and kaempferol-3-dihexoside were detected. Saffron samples from different geographical origins were clearly separated by their kaempferol 3-sophoroside contents that were able to explain 100% of the variance when a discriminant test was carried out.

Keywords: Kaempferol-3-sophoroside-7-glucoside; Kaempferol-3,7,4′-triglucoside; Kaempferol-3-sophoroside; LC-DAD MS/MS; Saffron spice

1. Introduction

Saffron spice is made up of dried stigmas of *Crocus sativus* L., which is cultivated in different countries, such as Greece, India, Iran, Morocco and Spain. Nowadays, it is appreciated by consumers as a colorant for foodstuffs as well as for its aromatic and flavouring properties. Yet, in ancient times, its use as a drug in folk medicine was even more remarkable. Its extracts and tinctures have been used as an antispasmodic, eupeptic, gingival sedative, anticatarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac and emenagogue (Basker & Negbi, 1983; Sampathu, Shivashankar, & Lewis, 1984). In recent decades, biological and medical properties of this spice and its constituents have again focussed scientific attention. It has been proposed that saffron is effective against arteriosclerosis, while reducing cholesterol levels in the blood (Gainer & Jones, 1975; Miller, Willett, Moss, Miller, & Belinka, 1982). Many in vivo tests on tumors in rats, as well as in vitro trials on established cellular lines, have been carried out (Escribano, Alonso, Coca-Prados, & Fernández, 1996; Escribano et al., 2000; Jagedeeswaran, Thirunavukkarasu, Gunasekaran, Ramamurty, & Sakthisekaran, 2000; Konoshima et al., 1998; Morjani, Tarantilis, Polissiu, & Manfait, 1990). In vitro cell toxicity tests proved that saffron stigma extracts inhibited the growth and synthesis of nucleic acids in tumor cells, while normal cells were less susceptible and even completely unsusceptible (Abdullaev & Frenkel, 1992; Nair, Pannikar, & Panikkar, 1991). It was verified that concentrations which induced inhibition of 50% of tumor cell growth were only slightly higher than those for all-*trans* retinoic acid but without its secondary effects (Tarantilis et al., 1992; Tarantilis, Tsoupras, & Polissiou, 1995). In addition, its lower toxicity compared to retinoic acid (Martín, Goh, & Neff,

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2002), plus the absence of cases of sensitization to saffron ingestion (Moneret-Vautrin, Morisset, Lemerdy, Croizier, & Kanny, 2002), open up the possible extension of its pharmacological use. In fact, saffron constituents have been proposed as alternative antitumor agents which, alone or in combination with other chemical substances, could achieve a certain relevance in future treatment of some cancers (Abdullaev, 2002; Winterhalter & Straubinger, 2000).

The compounds considered to be pharmacologically active in saffron are the bitter principles and the pigment derivatives from the carotenoid crocetin (Ríos, Recio, Giner, & Manez, 1996). In addition to picrocrocin, that is to say 4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde, the major compound responsible for saffron bitterness, other compounds with this organoleptic property have been characterized in saffron spice. These are related to picrocrocin and flavonoids (Straubinger, Bau, Eckestein, Fink, & Winterhalter, 1998; Straubinger, Jezussek, Waibel, & Winterhalter, 1997). Flavonoids have many functions in the biochemistry, physiology and ecology of plants, and they are important in both human and animal nutrition (Forkmann & Martens, 2001). The antioxidant activity of flavonoids towards free radicals and reactive oxygen species, plus their potential oestregenic and anticancer activity, draw attention to their health-protecting role in human and animal foods (Harborne & Williams, 2000). Some of these health properties may be due to the flavonoid content, as has been reported for anticonceptive and anti-inflammatory effects (Hosseinzadeh, Karimi, Khaleghpanah, & Niapoor, 2003).

The first identification of a flavonoid in saffron spice (by mass spectrometry) was by Tarantilis et al. (1995), proposing a kaempferol structure with a disaccharide moiety. Straubinger et al. (1997) identified kaempferol 7-*O*-glucopyranosyl-3-*O*-sophoroside and kaempferol 7-*O*-sophoroside by NMR and MS after counter-current preparative chromatography. Taking this determination into account, the same authors considered that the identification of a new flavonoid named kaempferol 3-*O*-gentiobioside carried out by Lozano, Castellar, Simancas, and Iborra (1999) was not correct (Winterhalter & Straubinger, 2000). Moreover, other flavonoids may be found in saffron spice, as they have already been described in other *Crocus* species (Nørbæk & Kondo, 1999).

The purpose of this study was to identify the flavonoid fraction in saffron spice by LC-DAD/MS/MS ESI. Differences in the flavonoid contents of samples from various geographical origins were also studied in order to know whether they could be used as biomarkers for the determination of saffron origin.

2. Materials and methods

2.1. Plant material and standards

A total of 12 saffron spice samples produced in different countries (3 from Iran, 3 from Greece, 3 from Morocco and 3 from Spain) were analysed in triplicate. The reduced number of samples was due to the fact that they were obtained directly from the producers with the guarantee of their origin and lack of adulteration. All samples were of Category I according to ISO/TS 3632 Normative (2003).

For standards, kaempferol-3-*O*-sophoroside-7-*O*-glucoside and kaempferol-3-*O*-sophoroside, from the collection of the Research group on Quality, Safety and Bioactivity of Plant Foods, CEBAS-CSIC (Murcia, Spain), were used. Both standards came from cauliflower (*Brassica oleracea* L. var. *botrytis*) (Llorach, Gil-Izquierdo, Ferreres, & Tomas-Barberán, 2003).

2.2. Extraction and isolation of the flavonoid fraction

A solution of 200 mg of powdered saffron in 200 ml of water was stirred for 1 h at room temperature in the dark. A C_{18} solid-phase cartridge (Waters, Milford, MA, USA), was used for the isolation and concentration step which had been previously conditioned with acetonitrile (2 ml) followed by water (5 ml), acetonitrile (2 ml) and water (5 ml). Four ml of the saffron extract were added to the SPE cartridge and washed with water (15 ml) and further eluted with 10 ml of a solution of acetonitrile 10% in order to elute the flavonoids. The water–acetonitrile extract was taken to dryness and redissolved in 1 ml of water:methanol (1:1 v/v) for chromatographic analysis.

2.3. Acid hydrolysis

This was carried out by adding HCl (1 N) up to pH 0.1 to the flavonoid saffron fraction obtained, as described in Section 2.2, which was heated for 30 min at 80 °C. The extract was then taken to dryness and redissolved in 1 ml of water:methanol (1:1 v/v) for chromatographic analysis.

2.4. LC-DAD/MS/MS ESI

Analysis was achieved with a LichroCART column (250 nm × 4 mm, RP-18, 5 μm particle size, Lichrospher 100 stationary phase, Merck, Darmstadt, Germany), protected with a LichroCART guard column (4 mm × 4 mm, RP-18, 5 μm particle size, Lichrospher 100 stationary phase, Merck, Darmstadt, Germany). The mobile phase consisted of two solvents: A, water–acetic acid (1%) and methanol (B), while a linear gradient, starting with 20% B, was installed to reach 50% B at 35 min and 80% B at 37 min. The flow rate was 1 ml/min, and the injection volume 40 μl. Spectral data from all peaks were accumulated in the range of 240–400 nm and chromatograms were recorded at 355 nm since, in a previous screening with chromatograms at 280 and 355 nm, the spectra of the obtained peaks showed that 355 nm was the suitable wavelength.

The LC/DAD/MS/MS analysis was carried out in an Agilent 1100 chromatograph equipped with a diode array detector and mass detector in series (Agilent Technologies,

Waldbronn, Germany). The HPLC was controlled by Chemstation software (Agilent, v.08.03). The mass detector was an ion trap spectrometer equipped with an electrospray ionization interface and controlled by LCMSD software (Agilent, v.4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebuliser pressure was 65 psi and the nitrogen flow rate was 11 ml/min. The full scan mass covered the range from *m/z* 200 up to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycles from 0.3 up to 2 V. All mass spectrometry data were recorded in the negative ion mode. Total ion chromatograms were recorded as alternating automatic events: full scan mass spectra (MS) and MS/MS of the pseudomolecular ion, and MS^n ($n =$ up to 3) in order to fragment the major ions obtained in every step. Table 1 shows most frequent ions which characterize the fragmentation of these flavonoid *O*-glycosides. Other ions were found but they have not been included owing to their low significance for the MS behaviour ions.

3. Results and discussion

3.1. Identification of flavonoids from saffron spice

The chromatogram profile of the flavonoid saffron extract is shown in Fig. 1. Of flavonoid family, only flavonol compounds were found. Five compounds were tentatively identified by combination of the UV and mass spectra by HPLC-DAD-ESI-MS/MS, while the peak at 12.5 min observed in the chromatogram corresponds to picrocrocin (*m/z* 353 and 185). After acid hydrolysis, all the flavonoids gave kaempferol as an aglycone. The MS spectra of the major compounds (**2** and **5**) showed deprotonated molecular ions at *m/z* 771 and 609 and ions at *m/z* 285 corresponding to a deprotonated aglycone. Therefore, they were kaempferol derivatives with three and two

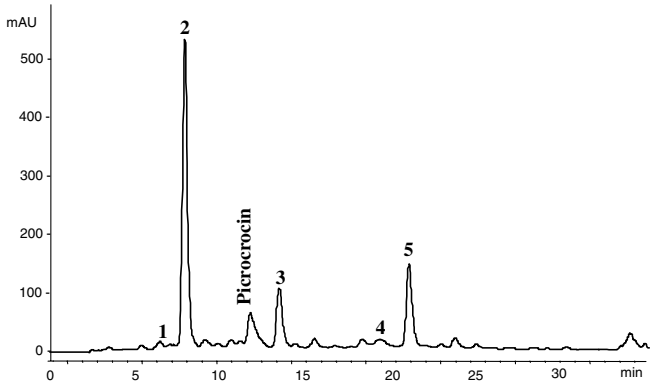

Fig. 1. HPLC profile of the flavonoid fraction from saffron extracts recorded at 355 nm: **1**, kaempferol tetrahexoside; **2**, kaempferol-3-sophoroside-7-glucoside; **3**, kaempferol-3,7,4′-triglucoside; **4**, kaempferol-3-dihexoside; **5**, kaempferol-3-sophoroside.

Table 1
Rt, UV, –MS: $[M-H]^-$, –MS2$[M-H]^-$ and –MS3$[(M-H)\rightarrow(M-H-162)]^-$ data for kaempferol glycoside derivatives in saffron

Compounds[a]	Rt (min)	UV (nm)	$^*[M-H]^-$ (*m/z*)	*-MS2$[M-H]^-$ (*m/z*) (%)		*-MS3$[(M-H)\rightarrow(M-H-162)]^-$ (*m/z*) (%)	
				Kaempferol tetrahexoside			
1	6.3	265, 319sh, 349	933	Fragmentation pattern is not clearly defined			
				Kaempferol trihexosides			
				−162		−(162 + 18)	−(162 × 2)
2	7.7	265,321sh, 348	771	609(100)		429(20)	285(100)
3	13.8	267,295sh,333	771	609(100)		429(100)	285(63)
				Kaempferol dihexosides			
				−162	−(162 + 18)	−(162 × 2)	
4	18.2	265, 319sh, 349	609	447(37)		285(100)	
5	20.9	265, 320sh, 348	609		429(53)	285(100)	

* The fragmentation ions shown are the most significant ones.

[a] **1**, kaempferol tetrahexoside; **2**, kaempferol-3-sophoroside-7-glucoside; **3**, kaempferol-3,7,4′-triglucoside; **4**, kaempferol-3-dihexoside; **5**, kaempferol-3-sophoroside.

hexoses, respectively (Table 1). Their fragmentation patterns coincided with two standards, the -3-sophoroside-7-glucoside and the -3-sophoroside of kaempferol, respectively (Ferreres, Llorach, & Gil-Izquierdo, 2004). All these data confirm the structures reported previously by Straubinger et al. (1997) for the main saffron flavonoids.

Another flavonoid observed with a relevant relative abundance was compound **3**. Its deprotonated molecular ion at *m/z* 771 indicated that it was an isomer of compound **2**, but its MS fragmentation pattern, as well as its UV spectrum (Table 1), differed from those of compound **2**. In the MS2$[M-H]^-$ fragmentation, it was observed that, in both compounds, the only ion that appeared was at *m/z* 609, produced by a hexose loss from the deprotonated molecular ions and indicating the occurrence of a monohexoside linked directly to a phenolic hydroxyl (Ferreres et al., 2004). However, the fragmentation MS3$[(M-H)\rightarrow(M-H-162)]^-$ was different (Table 1, Fig. 2), and while in compound **2** the characteristic fragmentation of a sophoroside was observed, with a base peak corresponding to the deprotonated aglycone (*m/z* 285), in compound **3**, the base peak is the ion at *m/z* 429, produced by the loss of 180 m.u. (162 + 18), with a relative abundance for the ion at *m/z* 285 of 60%. This showed that, in **3**, both sugars were not in the form of a disaccharide, but they were linked to different phenolic hydroxyls and, consequently compound **3** should be tentatively identified as kaempferol-3,7,4′-triglucoside. Such structure was in accordance with the UV spectrum observed (Fig. 3), since the maximum at 333 nm and its low absorbance supported the idea that the hydroxyl at 4′ was substituted. The chromatographic behaviours of compound **2** and **3** were difficult to explain due to the fact that the hydroxyl substitution in position 4′ might modify its chromatographic mobility, as was experimentally observed.

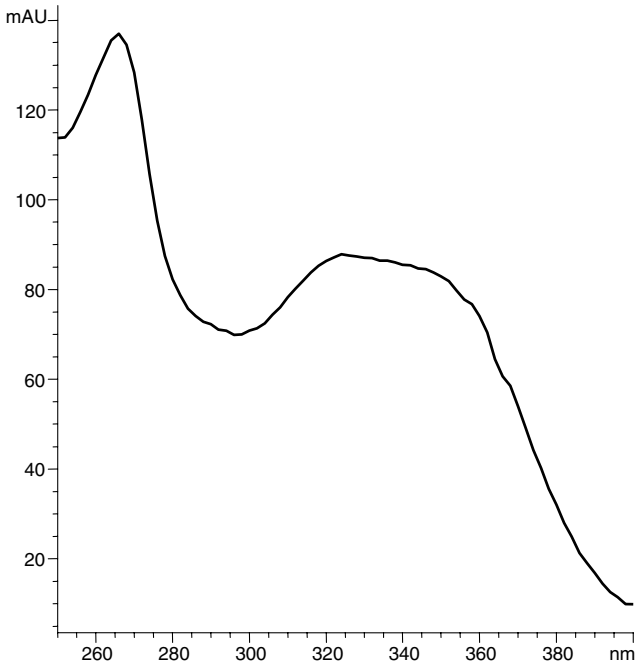

Fig. 3. UV spectrum for compound **3**: kaempferol-3,7,4′-triglucoside.

In addition to these three flavonoids, two other minor ones were observed in trace amounts (**1** and **4**). Both showed a characteristic UV spectrum of kaempferol derivatives substituted, at least, at position 3 (Table 1). In the MS analysis of compound **1,** a deprotonated molecular ion at *m/z* 933 and an ion at *m/z* 285, corresponding to the aglycone, were observed. Even so, its fragmentation pattern was not well defined and a specific structure was not assigned, although the mass coincided with that of a kaempferol tetrahexoside. Compound **4** (Table 1) was an isomer of **5** with a very similar MS. The fragmentation of

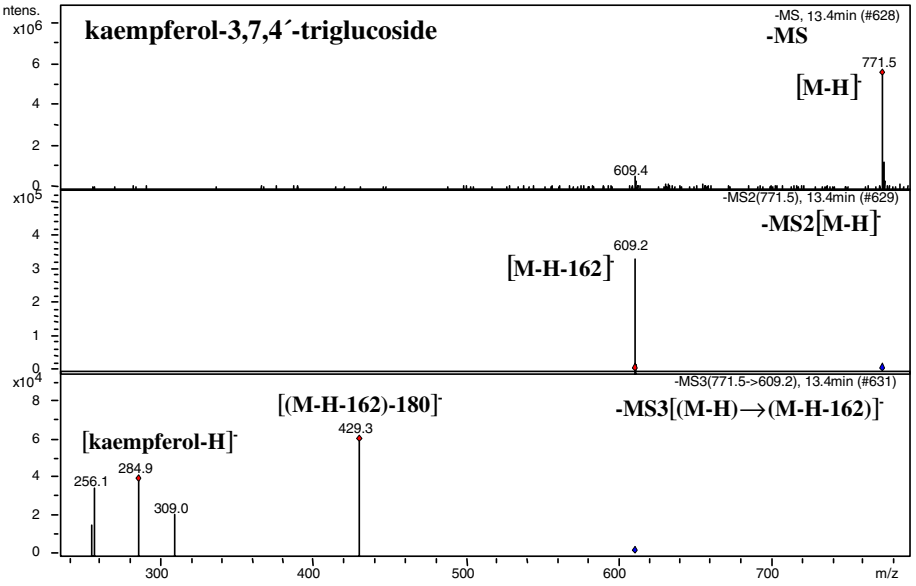

Fig. 2. $-MS^n$ analysis of kaempferol-3,7,4′-triglucoside (**3**).

these compounds revealed a base peak at m/z 285, indicating that both sugar moieties corresponded to a disaccharide, and therefore they were linked to a single phenolic hydroxyl group. UV–Vis spectra showed that the hydroxyl group at the 3 position was blocked in both compounds. Besides, in compound **5**, an ion at m/z 429 was observed, with loss of 162 + 18 m.u. from the molecular ion, and in compound **4** the ion m/z 447 (loss of 162 m.u. from the molecular ion) was similar to the mentioned signal m/z of 429, confirming the isomer's tentative identification, as kaempferol-3-dihexoside.

3.2. Saffron origin discrimination

No qualitative differences in relation to the flavonoid fraction were observed when samples from different geographical origins were analysed. This could be due to the poor genetic variability between cultivars. It is proposed that, saffron, being a sterile plant, all available vegetal material contains the same genetic information (Chichiriccó, 1987, 1989; Grilli & Chichiricco, 1991). Besides, heterogeneous edaphoclimatic conditions and postharvesting treatments, necessary to convert *C. sativus* stigmas into saffron spice (Carmona et al., 2005), generate a different flavonoid profile. While the content of compounds **1** and **4** did not offer valuable information because the presence of both of them was in trace amounts, the rest of the compounds quantified led us to establish that Spanish saffron was the one with the highest flavonoid content (Table 2).

Saffron samples were clearly separated by their kaempferol 3-sophoroside contents (compound **5**), which was able to explain 100% of the variance when a discriminant test was carried out with the geographical origin functioning as the differentiating variable. Although the number of samples is reduced, the flavonoid fraction was shown as a reliable tool for origin discrimination. Some other efforts have been carried out with other saffron constituents (Carmona et al., 2005; Semiond et al., 1996), although no analytical tool is available for quality control laboratories, at an international level, to certify saffron origin. Saffron price depends greatly on origin, with fraudulent interchanges in the international market frequently being produced: the cheapest saffron, coming from Iran, is sold as if it had come from other traditional areas, such as Spain, Greece or Italy, where saffron quality is considerably better.

This study confirms the idea expressed by Tarantilis et al. (1995) that more flavonoids should be found in *C. sativus* L. spice than the ones already characterized. The fact that exactly the same flavonoids have been found in samples coming from various countries suggested that its different content is the result of different edaphoclimatic conditions and postharvesting treatments. The main difficulty to overcome, in any future approach, is obtaining certified samples from different origins with detailed dehydration conditions, as has occurred in this study. Finally, it remains to be seen whether some of the traditionally recognized pharmacological properties of saffron could be attributed to the flavonoids identified at the concentration detected.

Table 2
Flavonoid contents for saffron samples of differing origin

Saffron	Compound **2**	Compound **3**	Compound **5**
	Content[a]	Content[a]	Content[a]
Spain	2.58 (37.4)[b]	1.09 (15.5)[b]	3.12 (47.1)[b]
Greece	2.24 (63.2)[a,b]	0.73 (20.4)[a,b]	0.61 (16.4)[a]
Iran	1.47 (50.9)[a]	0.59 (19.7)[a]	1.07 (29.5)[a]
Morocco	1.91 (48.7)[a,b]	0.88 (22.4)[a,b]	1.24 (28.8)[a]

Compound **2**, kaempferol-3-sophoroside-7-glucoside; Compound **3**, kaempferol-3,7,4′-triglucoside; Compound **5**, kaempferol-3-sophoroside.

[a] Flavonoid content expressed as equivalent mg of rutin/g of saffron (relative content expressed as % of the total fraction); Different superscript letters between columns indicate significant differences ($p < 0.05$).

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5.3. Cinética de degradación de cada uno de los ésteres de crocetina en extractos acuosos de azafrán (*Crocus sativus* L.) cuando se someten a tratamiento térmico en oscuridad


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Kinetics of Individual Crocetin Ester Degradation in Aqueous Extracts of Saffron (*Crocus sativus* L.) upon Thermal Treatment in the Dark

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En este artículo estudiamos los cambios que experimentaron los ésteres de crocetina cuando estaban disueltos en agua y se sometieron a tratamiento térmico en la oscuridad. Para ello, se estudiaron sus cinéticas en dos tipos distintos de extractos de azafrán: el primero se designó como "NFS" y fue preparado siguiendo la especificación técnica ISO/TS 3632 (2003) pero sin realizar la última dilución, es decir, dejando una concentración de 500 mg L^{-1} y sin retirar la materia vegetal procedente del azafrán del extracto; el segundo se denominó "FS" se preparó como el anterior pero se filtró para retirar la materia vegetal. Además, se siguió la evolución en agua de una fracción enriquecida en ésteres de crocetina, a la que se llamó "CE" y que había sido obtenida por un proceso previo de purificación mediante cromatografía en columna.

También se estudió la cinética de la pérdida de poder colorante que experimentaron los extractos, para poder determinar la relación existente entre el deterioro global de los ésteres de crocetina y los cambios que se producen en $E_{1cm}^{1\%}$ 440 nm.

Cambios en el espectro UV-vis

Se observó que, a todas las temperaturas estudiadas, excepto a 5 ºC, los extractos de azafrán que no se habían filtrado conservaban mejor el poder colorante que los extractos filtrados. Esto podría deberse a un aporte de ésteres de crocetina procedente de la materia vegetal, que se veía favorecido por la temperatura. En las mismas condiciones, la fracción enriquecida en ésteres de crocetina sufrió una pérdida de poder colorante más pronunciada que cualquiera de los extractos de azafrán. Este resultado indicaba que, o bien hay otros componentes polares del azafrán como la picrocrocina o ciertos flavonoides que contribuyen a la estabilidad de los ésteres de crocetina, o bien que el proceso de purificación tiene un efecto perjudicial en la estabilidad de los mismos.

Los cambios más destacables en el espectro UV-vis de las disoluciones estudiadas consistieron en:

a) Un descenso continuo y pronunciado del máximo situado en torno a los 440 nm, acompañado por un ligero desplazamiento hacia longitudes de onda menores.
b) Un aumento de la absorbancia en la región comprendida entre 275 y 315 nm, seguido de un descenso de la misma conforme pasa el tiempo.
c) Un aumento, también temporal para los extractos de azafrán, de la absorbancia en la región comprendida entre 315 y 380 nm a todas las temperaturas estudiadas, menos a 5 ºC.
d) Un pequeño descenso en el máximo situado en torno a 256 nm y simultáneamente un desplazamiento hacia longitudes de onda menores en los extractos de azafrán y en sentido contrario para la disolución de ésteres de crocetina.

Los resultados indicaron que la temperatura aceleraba esta evolución haciendo que los cambios se produjeran en un periodo de tiempo más corto. Así mismo, se observó que en los extractos de azafrán las distintas zonas del espectro se veían afectadas de distinta manera según la temperatura, por ejemplo, a temperaturas más altas

la banda del espectro situada en torno a 400-500 nm era más ancha; pero se modificaban por igual en la fracción purificada de los ésteres de crocetina.

No todos estos cambios se debían a los ésteres de crocetina, sino también a la evolución de otros componentes del azafrán. Sin embargo en este trabajo nos limitamos a estudiar en profundidad los ésteres de crocetina.

Cambios en el perfil cromatográfico y su relación con los cambios en el espectro UV-vis

Observando por HPLC los cambios producidos en cada componente se concluyó que el descenso de absorbancia en la región comprendida entre 400 y 500 nm se correspondía con el descenso del área total debida a los ésteres de crocetina. Con el método cromatográfico utilizado, durante la degradación de los ésteres de crocetina no se observó la aparición de ningún compuesto nuevo a 440 nm, por lo que, o bien sus productos de degradación no absorbían a esta longitud de onda, o bien no podían ser detectados con el método utilizado. El efecto hipsocrómico del máximo alrededor de 440 nm estaba más relacionado con el cambio que se produjo en la proporción de los ésteres de crocetina con máximos a longitudes de onda menores de 440 nm, incluyendo los isómeros cis, que a la isomerización de las formas trans en cis. La diferente anchura de la banda situada entre 400 y 500 nm descrita para la evolución de un mismo extracto a mayor o menor temperatura pareció estar causada por el diferente contenido relativo de cada éster de crocetina.

No se observaron aumentos ni del porcentaje ni del contenido absoluto de ninguno de los ésteres de crocetina en las disoluciones CE. Por el contrario, en NFS después de 9 horas se observaron aumentos del contenido absoluto de *cis*-3-Gg a 5 y 30 ºC y de *trans*-2-gg a 70 ºC respecto al contenido inicial. Esta misma tendencia se observó en FS, durante las primeras 6 horas de los experimentos para *trans*-5-nG a 5, 30 y 70 ºC; *trans*-3-Gg a 5 ºC; *trans*-2-gg a 5, 30 y 50 ºC; *cis*-4-GG, *cis*-3-Gg y *trans*-1-g a 5, 30, 50 y 70 ºC; y *trans*-2-G a 50 y 70 ºC. En relación a los contenidos relativos, se

produjeron aumentos respecto al momento inicial en todos los ésteres de crocetina menos en el *trans*-4-GG, llegando el *trans*-3-Gg a sobrepasarlo. Precisamente, en la comparación de estos dos ésteres de crocetina en medidas consecutivas en el tiempo de los extractos de azafrán se observó que cuando el porcentaje de *trans*-4-GG aumentaba el de *trans*-3-Gg disminuía y viceversa, para todas las temperaturas menos para 30 ºC en NFS donde el porcentaje de *trans*-3-Gg fluctuaba mientras el de *trans*-4-GG decrecía, y a 70 ºC en FS, donde ambos disminuían. En las disoluciones CE, estos porcentajes se mantenían estables o disminuían al mismo tiempo. A pesar de las distintas velocidades de degradación, estos resultados apoyarían la generación del resto de los ésteres de crocetina a partir del *trans*-4-GG, tal y como se ha descrito en la bibliografía para soluciones metanólicas (Vickackaite y col., 2004).

Los principales cambios observados en los cromatogramas a 250 nm fueron el pequeño descenso del pico correspondiente a la picrocrocina, la desaparición de las señales de los ésteres de crocetina y la aparición tras 23 horas a 70 ºC de un pico al tiempo de retención de 8,7 minutos, cuyo espectro UV-vis presentaba un máximo a 286 nm. Además, el pico cuyo tiempo de retención era 11,2 minutos y cuyo espectro UV-vis era similar al de la picrocrocina aumentó ligeramente a 70 y a 50 ºC y creció de forma más pronunciada con el paso del tiempo a las temperaturas más bajas, 30 y 5 ºC. En base a su espectro UV-vis, tiempo de retención y en comparación con los datos de la bibliografía (Himeno y Sano, 1987; Iborra y col., 1992) este pico podría corresponder al HTCC.

Tras examinar los cromatogramas, pudimos concluir que los cambios que se produjeron en el espectro en torno a los 330 nm eran atribuibles tanto a una aumento en el contenido de isómeros cis, como a la aparición de algunos productos de degradación con absorbancia en esta parte del espectro. Además, se observó que el momento y duración de estos cambios dependía de la temperatura.

Parámetros cinéticos

En general, fue posible aplicar una cinética de primer orden a la degradación de la mayoría de los ésteres de crocetina y a la pérdida de poder colorante. Para todos ellos, se calcularon la constante de velocidad de la reacción (*k*) y el tiempo de vida media ($t_{1/2}$) para las temperaturas de 5, 30, 50 y 70 ºC en los distintos extractos. Además, en NFS se calcularon dichos parámetros para temperatura ambiente (20 ± 2 ºC), 35, 40 y 60 ºC pero, al observar las similitudes entre las temperaturas más próximas y la posibilidad de interpolar, se decidió llevar a cabo los estudios para FS y CE sólo a las temperaturas de 5, 30, 50 y 70 ºC.

En el poder colorante, las diferencias encontradas en *k* dependiendo del tipo de extracto indicaron que podían considerarse como distintos medios de reacción en los que el resto de los componentes modulan la degradación tal y como se ha descrito para los carotenoides del pimentón (Jarén-Galán y Mínguez-Mosquera, 1997; Pérez-Gálvez y col., 2000). Los resultados también indicaron el efecto de la temperatura sobre el poder colorante, aumentando, en general, de forma importante el valor absoluto de *k* al aumentar la misma. Comparando los tipos de extractos, los valores de *k* en CE fueron siempre mayores que en los extractos de azafrán, demostrando una vez más su menor estabilidad. Sin embargo, sólo a 50 y 70 ºC se encontraron valores significativamente mayores en FS que en NFS. Los periodos de vida media de la pérdida de $E_{1cm}^{1\%}$ 440 nm variaron desde 151 horas para FS a 5 ºC hasta 3 horas para CE a 50 y 70 ºC.

Del mismo modo, el valor de *k* para cada éster de crocetina era claramente dependiente de la temperatura, aumentando con la misma. Tan sólo en el caso del *cis*-4-GG no se encontraron diferencias entre los valores de *k* a 50 y a 70 ºC.

En los extractos NFS, los ésteres de crocetina que se degradaron más lentamente fueron el *trans*-3-Gg a 5 ºC ($t_{1/2}$ = 198 horas), el *trans*-2-gg a 30 y a 50 ºC ($t_{1/2}$ = 301 horas y 59 horas, respectivamente) y el *cis*-4-GG a 70 ºC ($t_{1/2}$ = 14 horas). Por el

contrario, los ésteres de crocetina que presentaron las mayores constantes de degradación fueron el *trans*-5-tG a 5 ºC ($t_{1/2}$ = 99 horas) y el *trans*-4-GG para el resto de temperaturas ($t_{1/2}$ = 53 horas a 30 ºC, 8 horas a 50 ºC, 6 horas a 70 ºC).

En los extractos FS, los ésteres de crocetina más estables fueron: el *trans*-2-G ($t_{1/2}$ = 165 horas) y el *trans*-5-tG ($t_{1/2}$ = 136 horas) a 5 ºC; a 30 y a 50 ºC, el *trans*-5-nG ($t_{1/2}$ = 32 horas y 9 horas, respectivamente); y a 70 ºC, el *cis*-4-GG ($t_{1/2}$ = 7 horas). Por otra parte, los ésteres de crocetina con mayores constantes de degradación fueron: el *trans*-4-GG ($t_{1/2}$ = 104 horas), el *trans*-3-Gg ($t_{1/2}$ = 106 horas) y el *cis*-4-GG ($t_{1/2}$ = 123 horas) a 5 ºC; el *trans*-4-GG a 30 ºC ($t_{1/2}$ = 12 horas); el *trans*-2-G a 50 ºC ($t_{1/2}$ = 4 horas); y a 70 ºC el *trans*-2-gg y el *trans*-4-GG ($t_{1/2}$ = 3 horas).

En las disoluciones CE, el *trans*-5-nG fue el éster de crocetina más estable ($t_{1/2}$ = 60 horas), mientras que el *trans*-2-G y el *cis*-3-Gg fueron los más lábiles para todas las temperaturas estudiadas ($t_{1/2}$ = 23 horas a 5 ºC, 3 horas a 30 ºC, 2 horas a 50 y 70 ºC), aunque a 70 ºC sus diferencias con el *trans*-4-GG no eran significativas.

Los tiempos de vida media de los ésteres de crocetina variaron entre 198 horas a 5 ºC y 2 horas a 70 ºC.

Comparando con los valores de *k* obtenidos para el poder colorante, se comprobó que estos últimos se situaban entre los valores máximos y mínimos encontrados para los ésteres de crocetina de forma individualizada. En general, el poder colorante mostró menores valores de *k* que los dos ésteres de crocetina mayoritarios, el *trans*-4-GG y el *trans*-3-Gg.

No se encontró ninguna relación entre el número o el tipo de radical glicosilado de cada éster de crocetina y su estabilidad.

Los resultados también indicaban que los incrementos de temperatura afectaban de diferente forma a cada éster de crocetina, por lo que fue necesario estudiar ciertos parámetros termodinámicos como la energía de activación (E_a).

Parámetros termodinámicos

Los resultados mostraron diferencias significativas en la E_a de los distintos ésteres de crocetina. Así, por ejemplo, en FS, el *trans*-2-G tenía el mayor valor de E_a seguido del *trans*-5-tG, del *trans*-3-Gg y del *trans*-4-GG. Sin embargo, el *cis*-4-GG presentó los valores más pequeños. Para NFS el orden de los ésteres de crocetina según su E_a fue: *trans*-2-gg > *trans*-4-GG > *trans*-3-Gg > *trans*-5-nG > *cis*-4-GG; mientras que para CEs ese orden fue el siguiente: *trans*-2-gg > *trans*-5-nG > *trans*-5-tG > *trans*-4-GG > *cis*-4-GG > *trans*-3-Gg > *trans*-2-G. También se observó que el valor de la E_a para la pérdida del poder colorante, 46,5 ± 0,4 kJ mol^{-1} (11,1 ± 0,1 kcal mol^{-1}), se encontraba dentro del rango de valores de los ésteres de crocetina, al igual que ocurría con los valores de *k*.

En cuanto a los distintos tipos de extractos, los valores de E_a fueron mayores en FS, seguidos por los obtenidos en NFS y CE, con la excepción del *cis*-4-GG donde la E_a en CE era mayor que en NFS.

Según la teoría isocinética, una misma reacción puede tener diferentes parámetros cinéticos y termodinámicos, dependiendo de las condiciones en las que transcurra la reacción (Mínguez-Mosquera y Jarén-Galán, 1995; Jarén-Galán y col., 1997; Rhim y col., 1989, 1990; Canjura y col., 1991). Para determinar si la reacción de degradación de los ésteres de crocetina era la misma en los distintos tipos de extractos (FS, NFS y CE) se interpretaron los resultados para un sistema cinéticamente compensado. En un sistema cinéticamente compensado los diferentes parámetros termodinámicos definen la siguiente línea isocinética:

$$\Delta H^* = T_{isok}\Delta S^* + \Delta G_{isok}$$

Para la mayoría de los ésteres de crocetina, en la representación del incremento de entropía de activación (ΔS*) frente al incremento de entalpía de activación (ΔH*) se obtuvo una línea recta (R^2 > 0,9, salvo para el *trans*-5-nG y el *trans*-3-Gg donde R^2 > 0,8). El incremento de energía libre de Gibbs (ΔG_{isok}) estaba comprendido entre 97,1 kJ mol^{-1} para el *trans*-5-nG y 121,6 kJ mol^{-1} para el *trans*-2-gg. Las temperaturas isocinéticas (T_{isok}) variaban entre 273 y 383 K, incluyendo por tanto, las temperaturas estudiadas (278-343 K) y también los valores descritos en la bibliografía para la co-oxidación del β-caroteno y la capsantina por la lipoxigenasa (Jarén-Galán y Mínguez-Mosquera, 1997) y para otros pigmentos carotenoides de las oleorresinas del pimentón (Pérez-Gálvez y col., 2000).

Se pudo concluir que la reacción de degradación de los ésteres de crocetina era la misma tanto si se encontraban en los distintos extractos de azafrán (FS, NFS), como si habían sido purificados (CE). Teóricamente esa reacción consistiría en la decoloración de cada éster de crocetina, debida a la pérdida del sistema de dobles enlaces conjugados de la molécula. Factores externos, como el resto de grupos funcionales de la molécula, el medio en el que se produjo la reacción y las condiciones ambientales que rodearon a la misma fueron los responsables de las modificaciones de los parámetros cinéticos y termodinámicos encontradas.

5.3. Kinetics of individual crocetin ester degradation in aqueous extracts of saffron (*Crocus sativus* L.) upon thermal treatment in the dark

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Kinetics of individual crocetin ester degradation in aqueous extracts of saffron upon thermal treatment in the dark has been studied. Special attention has been paid to the comparison between saffron extracts and aqueous solutions of a crocetin ester rich fraction, with a lower stability of the latter observed. The degradation reaction was the same for all crocetin esters whether they were in saffron extracts or whether they were purified, although it was affected by external factors that modified their kinetic and thermodynamic parameters, making some of them less stable than others.

KEYWORDS: Crocetin esters; saffron (*Crocus sativus* L.); thermal degradation; kinetics; thermodynamics

INTRODUCTION

Color is the most important quality characteristic of saffron spice (dried stigmas of *Crocus sativus* L.), and coloring strength values ($E_{1\,cm}^{1\%}$440 nm) of its aqueous extracts are critical for the commercial value of the spice. The yellowish red hues of the latter are due to the presence of a variety of water-soluble crocetin esters ($C_{44}H_{64}O_{24}$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid) with several glucose, gentiobiose, and neapolitanose moieties (*1*) that are also known as crocins. With appropriate flower harvest, stripping, and, above all, an adequate dehydration process, a high initial quality of spice can be achieved. However, its quality will deteriorate progressively during storage and packaging or even upon application. As evidenced by several kinetic studies in the past, optimum storage conditions of saffron should involve a dark, inert atmosphere, low water activity level (<0.43), and less than ambient temperatures (<25 °C) (*2–7*).

Due to the high water solubility of saffron pigments, most of the applications of the spice, for example, in cookery, the food and pharmaceutical industries, or even dyeing, are based on the use of aqueous extracts. The few previous studies of saffron aqueous extracts have shown that color degradation follows first-order kinetics; it is sensitive upon exposure to light, thermal treatment, and acidic environment as well as to the presence of additives (*8–10*). Vickackaite et al. studied photochemical and thermal processes that degrade saffron in methanolic solutions (*11*). Still, it should be noted that up to now most findings about crocetin ester degradation and, consequently, about color loss have been based on changes in $E_{1\,cm}^{1\%}$440 nm values. These values reflect the total result of the degradation of all the crocetin esters and are not valid for studying the changes in each one.

However, and differently from other carotenoids, such as paprika ones or carotene (*12–20*), the individual changes that each crocetin ester undergoes are not yet known. As with other spices (*21*), qualitative composition regarding trans and cis isomers is important in saffron because geometrical isomers differ in physiochemical properties (*22*), which may have an impact on quality characteristics and nutritional value (*23*) and also could discriminate saffron origins and dehydration processes (*24, 25*). Further knowledge about the kinetics of individual crocetin ester degradation in aqueous extracts of saffron would provide important information for the prevailing mechanism as well as for application in the food and pharmaceutical industries.

The aim of this work was to study overall and individual changes that crocetin esters undergo in aqueous extracts when subjected to mild thermal treatment in the dark. Special attention has been paid to kinetics in aqueous extracts prepared directly from the spice or from a crocetin ester rich fraction. The methods employed were HPLC coupled with diode array detection system and LC-MS as well as UV–vis spectrophotometry.

MATERIALS AND METHODS

Samples and Chemicals. Spanish saffron spice (*C. sativus* L.) of the Protected Designation of Origin "Azafrán de La Mancha" was collected from the 2004 harvest. HPLC-grade acetonitrile, cyclohexane, and phosphoric acid were from Scharlau (Barcelona, Spain). Gallic acid was purchased from Sigma-Aldrich (Madrid, Spain). Ultrahigh-purity water was produced using a Milli-Q system (Millipore, Bedford, MA).

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PTFE filters (11 mm, 0.45 μm) were also purchased from Millipore and C_{18} packing material (125×10^{-8} cm pore size, 55–105 μm particle size) from Waters (Milford, MA).

Saffron Aqueous Extract Preparation. Three different types of aqueous extracts were used throughout the study (500 mg L^{-1}). Two of them were prepared according to the ISO 3632 Technical Specification, 2003 (*26*), trade standard without and after removal of vegetal matter by filtration through filter paper. The extracts were designated nonfiltered saffron extract (NFS) and filtered saffron extract (FS), respectively. The third extract was prepared using as starting material a crocetin ester rich fraction (CE) that was purified by column chromatography according to a procedure described in the next paragraph.

Crocetin Ester Rich Fraction Preparation. Removal of nonpolar compounds was achieved with 30 mL of cyclohexane added to 5 g of powdered saffron for 24 h at room temperature in the dark with sporadic agitation. The organic solvent was discarded, and the residue was dried under vacuum before 60 mL of water was added and the mixture was bubbled with nitrogen. The resulting solution was magnetically stirred for 1 h at room temperature in the dark. Then, the extract was centrifuged at 4000 rpm for 10 min, and the supernatant was collected and transferred to a plastic LC column (8 cm high $\times$ 2.7 cm i.d.) filled with C_{18} packing material. Crocetin esters were eluted with 50 mL of acetonitrile/water 80% v/v after removal of flavonoids and picrocrocin with 20 mL of acetonitrile/water 2% v/v and 90 mL of acetonitrile/water 20% v/v. The solvent was evaporated under vacuum, and the crocetin ester rich fraction was kept at −20 °C until analysis.

Thermal Treatment. *NFS.* Eight aliquots of a NFS (200 mL each) were transferred to 250 mL borosilicate glass bottles, hermetically sealed, and kept in the dark at different temperatures: 5 °C (refrigerator), room temperature (20 ± 2 °C), and 30, 35, 40, 50, 60, and 70 °C (different thermostated ovens) for 91 days until total degradation of crocetin esters was attained.

FS. Ten aliquots of approximately 20 mL were put in 50 mL Falcon tubes sealed and kept in the dark at 5, 30, 50, and 70 °C for 28 days. Samples were withdrawn periodically, at intervals of 1, 2, 6, and 9 h during the first stages of the study and then after 12 or 24 h at the final stages of the experimental procedure. Each sample was used once and then discarded. All of the samples were filtered through a PTFE filter of 0.45 μm before analysis.

CE. The procedure carried out was similar to that described for FSs, and degradation was monitored for 17 days. All experiments were carried out in duplicate extracts.

Spectrophotometric Analysis. Changes in specific spectral characteristics of NFSs, FSs, and CEs at 440, 330, and 257 nm were monitored periodically by scanning from 190 to 700 nm using a Perkin-Elmer Lambda 25 spectrophotometer (Norwalk, CT). $E_{1\,cm}^{1\%}$ at 440 nm, $E_{1\,cm}^{1\%}$ at 257 nm, and $E_{1\,cm}^{1\%}$ at 330 nm values were calculated according to the method of ref *26* on a dry sample basis. Triplicate measurements for every sample at each time point were taken.

RP-HPLC of Crocetin Esters. Simultaneously to spectrophotometric analysis, gallic acid was added to the sample up to a concentration of 10 mg L^{-1}, and 20 μL of the mixture was injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm $\times$ 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column thermostated at 30 °C. The addition of gallic acid just before the injection was used to check the correct working of the HPLC system. Crocetin esters were eluted using a gradient system consisting of a mixture of water +0.1% phosphoric acid (A) and acetonitrile (B) (20% B, 0–5 min; 20–80% B, 5–15 min and 80% B, 15–20 min). The flow rate was 0.8 mL min^{-1}. For each condition studied, duplicate extracts were prepared, and each was chromatographed twice. Apart from retention time (t_R), the relative retention (r) was calculated as the ratio of the adjusted retention time (the total elution time minus the hold-up time) of each crocetin ester relative to that of gallic acid, obtained under identical conditions (*27*).

Identification and Quantification of Crocetin Esters. Identification of crocetin esters by LC-DAD-MS was carried out as previously described (*1*). Respective maxima in the UV–vis region and retention times were used as additional means of identification. Due to the lack of pure standards of each crocetin ester, quantification was based on the equation

$$\%\ \text{of crocetin ester } i \text{ on dry basis} = \frac{\text{Mw}_i(E_{1\text{cm}}^{1\%}440\text{nm})A_i}{10\epsilon_{\text{t,c}}} \quad (1)$$

where Mw_i stands for the molecular weight of the crocetin ester i, $E_{1\,cm}^{1\%}440$ nm is the coloring strength, A_i is the percentage peak area of the crocetin ester i at 440 nm, and $\epsilon_{t,c}$ is the molecular coefficient absorbance value (89000 for *trans*-crocetin esters and 63350 for *cis*-crocetin esters (*22*)).

Kinetic Studies. The kinetic parameters of each reaction—reaction order, rate constants (k), and half-life periods ($t_{1/2}$)—were obtained using the integral method (*28*). This method uses a trial-and-error procedure to find reaction order. If the order assumed is correct, the appropriate plot of the concentration−time data [concentration against time (zero-order), ln concentration against time (first-order), and concentration^{-1} against time (second-order)] should be linear. The result showing the best correlation coefficient (R^2) was selected. Absolute temperature (T) dependence of the degradation rate constant was determined by the Arrhenius equation

$$\ln k = \ln A - \frac{E_a}{RT} \quad (2)$$

where R is the gas constant, E_a is the activation energy, and A is the pre-exponential factor. Therefore, E_a was estimated on the basis of linear regression analysis of ln k versus T^{-1}.

Thermodynamic Studies. According to the activated complex theory, the enthalpy and entropy of activation (ΔH^* and ΔS^*) were determined by the equation

$$\ln k = \ln \frac{k_B T}{h} - \frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \quad (3)$$

where k is the degradation constant at temperature T, k_b is the Boltzmann constant, and h is the Planck constant. The pairs of ΔH^* and ΔS^* obtained were linearly correlated according to the equation

$$\Delta H^* = T_{\text{isok}}\Delta S^* + \Delta G_{\text{isok}} \quad (4)$$

from which the isokinetic temperature of reaction (T_{isok}) and its corresponding Gibbs free energy of the reaction (ΔG_{isok}) were calculated.

Nomenclature for Crocetin Esters. Abbreviations in nomenclature were adopted from Carmona et al. (*1*): *trans*-5-tG, *trans*-crocetin (β-D-triglucosyl)-(β-D-gentibiosyl) ester; *trans*-5-nG, *trans*-crocetin (β-D-neapolitanosyl)-(β-D-gentibiosyl) ester; *trans*-4-GG, *trans*-crocetin di-(β-D-gentibiosyl) ester; *trans*-3-Gg, *trans*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester; *trans*-2-G, *trans*-crocetin (β-D-gentibiosyl) ester; *trans*-2-gg, *trans*-crocetin di-(β-D-glucosyl) ester; *trans*-1-g, *trans*-crocetin (β-D-glucosyl) ester; *cis*-4-GG, *cis*-crocetin di-(β-D-gentibiosyl) ester; and *cis*-3-Gg, *cis*-crocetin (β-D-glucosyl)-(β-D-gentibiosyl) ester.

Statistics. Evaluation of the statistical significance of differences was performed using analysis of variance (ANOVA) with the aid of the SPSS 14.0 for Windows (SPSS Inc.) statistical program.

RESULTS AND DISCUSSION

Saffron Quality Characteristics and Initial Content in Crocetin Esters. Quality characteristics were evaluated according to ISO 3632 (2003) specifications (*26*). Results indicated that the sample used belonged to commercial category I: moisture and volatile matter content, 5.5%; coloring strength ($E_{1\,cm}^{1\%}440$ nm), 261; $E_{1\,cm}^{1\%}257$ nm, 100; $E_{1\,cm}^{1\%}330$ nm, 29. **Table 1** shows the individual crocetin ester composition as percentage on a dry basis. Each compound shown in this table was identified by LC-DAD-MS, and the results (data not shown) were totally in agreement with those previously reported (*1*). The five major

Table 1. Individual Crocetin Ester Composition, Spectral Characteristics in the UV–vis Region, Retention Times (t_R) and Relative Retention (r)

	compd								
	trans-5-tG	*trans*-5-nG	*trans*-4-GG	*trans*-3-Gg	*trans*-2-gg	*trans*-2-G	*cis*-4-GG	*cis*-3-Gg	*trans*-1-g
mean content[a] (g/100 g)	0.36	0.38	15.72	7.64	0.60	0.98	0.48	0.24	0.09
SD[b]	0.01	0.01	0.03	0.04	0.02	0.02	0.03	0.01	0.01
UV−vis max (nm)	263, 443, 467	263, 422sh, 440, 467sh	262, 442, 465	262, 441, 465	261, 439, 464	259, 434, 459	262, 327, 435, 458	262, 325, 434, 458	257, 434, 459
t_R (min)	9.6	9.9	10.2	10.8	11.4	12.4	12.0	12.6	13.4
r (min)	5.6	5.8	6.0	6.4	6.8	7.5	7.2	7.6	8.1

[a] Values are the means of two extracts conducted in duplicate ($2 \times 2n$) (g of compd/100 g of saffron, dry basis). [b] Standard deviation.

crocetin esters were, in a decreasing order *trans*-4-GG > *trans*-3-Gg > *trans*-2-G > *trans*-2-gg > *cis*-4-GG. It is noteworthy that the first one alone was found to represent 59% of the total crocetin ester content of the aqueous saffron extract, whereas the first three mentioned accounted for >90% of the total esters recorded. Respective maxima in the UV–vis region, t_R, and r are also shown in **Table 1**.

Changes in Coloring Strength ($E_{1\,cm}^{1\%}$440 nm) Values and Other Spectral Characteristics of Aqueous Extracts during Storage. The results of experiment with NFSs showed certain similarities between the closest temperatures. Thus, in light of these similarities, it was decided to rule out 20, 40, and 60 °C in the thermal treatment for FSs and CEs. The percentage of $E_{1\,cm}^{1\%}$440 nm values retained throughout the thermal treatment of NFSs, FSs, and CEs are displayed in **Figure 1**. Upon thermal treatment of NFSs and FSs, a greater retention of $E_{1\,cm}^{1\%}$440 nm values for the former was evidenced at all temperatures studied, except for 5 °C. A continuous extraction of crocetin esters from the remaining vegetal material in NFSs that was enhanced at higher temperatures could justify this observation. Kinetic curves at each temperature indicate a greater loss in coloring strength values of CEs, with regard to those of NFSs and FSs, when exposed to the same storage conditions. Such a finding might imply that either coexisting polar saffron constituents such as picrocrocin and flavonoids contribute to the stability of color in nonpurified aqueous extracts or that the purification process has a detrimental effect on individual crocetin esters. These results will be broached from a kinetic and thermodynamic point of view later on, in the two sections devoted to them.

The most outstanding changes observed in UV–vis spectra upon storage of aqueous extracts at different temperatures (**Figure 2**) consisted of (a) a continuous and dramatic decrease in the maximum at about 440 nm and, as a result, a progressive discoloration that was accompanied by a slight shift (**Table 2**) toward lower wavelengths; (b) an increase in absorbance at 275–315 nm followed by a decrease when time passed; (c) an increase in the absorbance around 315–380 nm at all temperatures studied but 5 °C that also disappeared over time in FSs and NFSs; and (d) a slight decrease in the maximum at about 256 nm accompanied by a slight shift of this peak toward lower wavelengths in saffron extracts but toward higher ones in CEs (**Table 2**). Obviously, not all of these changes came only from crocetin esters but also might come from other saffron components such as picrocrocin and flavonoids. Nonetheless, this study will focus mainly on crocetin esters with only brief descriptions of other components, because these components are currently under study in our laboratory.

The mentioned changes were more marked as temperature increased. In FSs, the absorption band between 400 and 500 nm regions disappeared after the extracts were kept for 23 h at 70 and 50 °C, whereas it was not until 56 h at 30 °C and 672 h at 5 °C when this region was insignificant. The same happened in NFSs at 21, 45, 2184, and 816 h at 70, 50, 30, and 5 °C, respectively, whereas in CEs this happened at 6 h at the three first temperatures and at 50 h at 5 °C.

Even so, experimentation showed some differences in the spectra of NFSs and FSs recorded for the highest temperature with regard to those obtained with the same extracts kept at lower ones for a longer time (**Figure 3**). When we compared the spectra of two samples from the same saffron extract kept at different temperatures, with similar absorbance at about 440 nm, a broader visible band with a slight shift toward lower wavelengths was observed at the highest temperature. The increment of absorbance in 275–380 nm was also higher, whereas the decrement at 256 nm was lower. The lack of

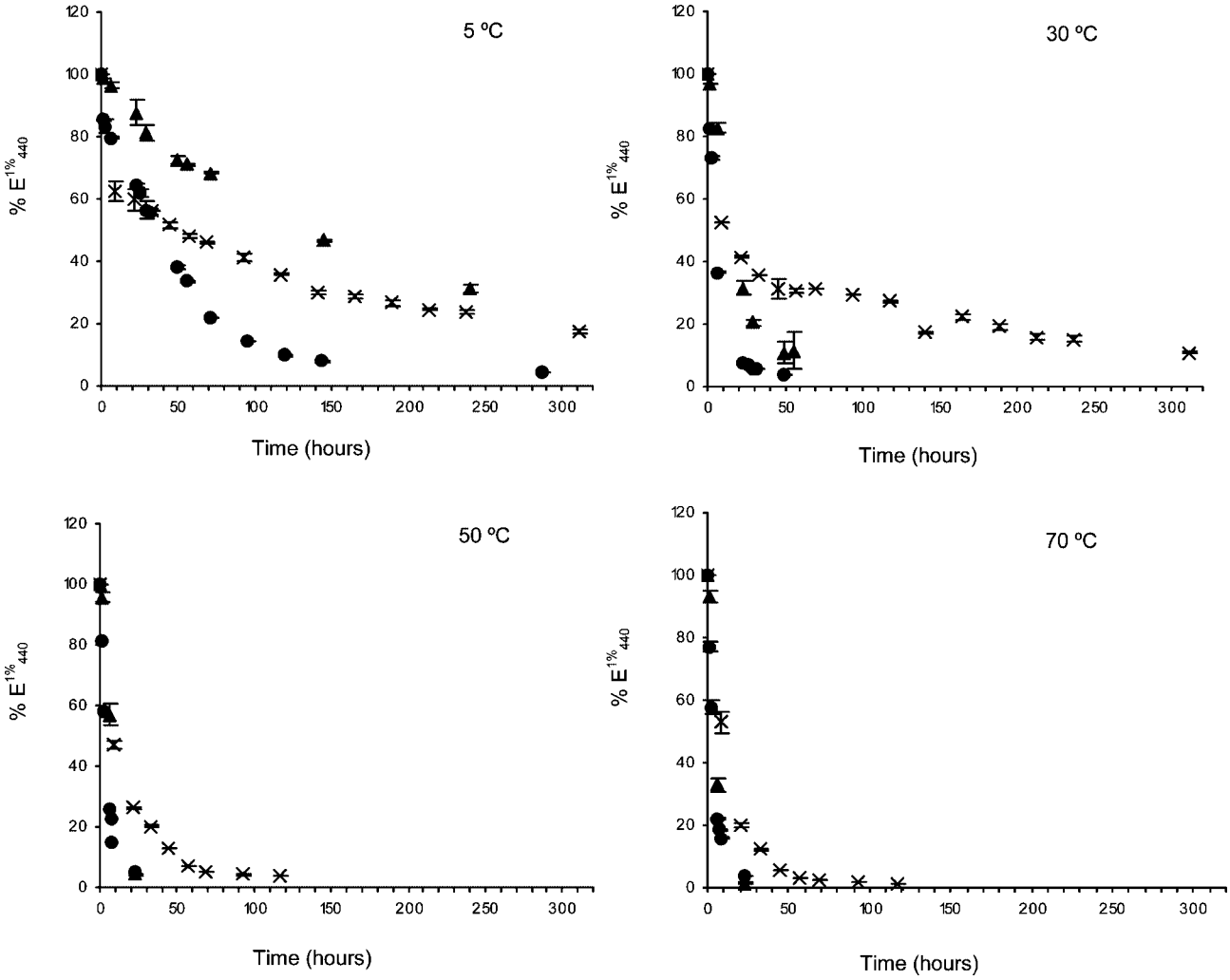

Figure 1. Percentage [mean value of two extracts conducted in triplicate ($3 \times 2n$) $\pm$ standard deviation] of $E^{1\%}_{1\,cm}440$ nm retained throughout the thermal treatment of crocetin ester rich fraction solutions: CE (●), nonfiltered saffron aqueous extracts, NFS (×), and filtered saffron aqueous extracts, FS (▲).

parallelism between this behavior and that of CEs, in which case both spectra were overlapped, was noteworthy. Unlike the main maximum in the visible region, the maximum at 254, 256, and 263 nm of NFS, FS, and CE, respectively, was more stable and showed a slight decrease in absorbance. This decrease was more noticeable at lower temperatures after a longer time elapsed. Therefore, this maximum in time became the only peak in the spectra of saffron extracts.

Changes in HPLC Profile during Storage. To match the previous information with changes shown individually by crocetin esters, their chromatographic profiles were carefully examined. The results from HPLC analyses showed that the decrease in absorbance of the band between 400 and 500 nm corresponded to the decrease in the area of total crocetin esters, until reaching almost total disappearance for all temperatures studied. Also, as has been noted for UV–vis changes, higher temperatures accelerated degradation.

No significant appearance of new compounds was detected at 440 nm, indicating that degradation products had no absorbance at this wavelength or that they could not be detected by our chromatographic method. Moreover, small differences (<10% of their remaining values at each time) between the evolution of $E^{1\%}_{1\,cm}440$ nm and the percentage of the total crocetin ester content were found.

To justify the hypsochromic effect at approximately 440 nm along with the varying width of the visible band, the relative content of each crocetin ester as well as that of trans and cis isomers was examined. Taking into account that the total crocetin ester content was influenced by both time and temperature, the results were normalized by expressing each crocetin ester content as a percentage of the whole. Also, the change observed in the proportion of *trans*- and *cis*-crocetin esters could explain the hypsochromic shift shown. However, moments with maximum shifts were not in line with moments with maximum proportions of *cis*-crocetin esters. Thus, our research focused on the sum of crocetin esters with the lowest maximum wavelength (**Table 1**). The results were in better agreement with the only exceptions of NFS at 5 and 50 °C, where maximum proportions of these crocetin esters were reached earlier than maximum hypsochromic shifts (**Figure 4**). On the basis of the above findings, the hypsochromic effect was generated by a change in the proportion of crocetin esters with maximum wavelength lower than 440 nm, which included cis isomers, rather than by an isomerization of *trans*-crocetin esters to *cis*-crocetin esters. The different width of the band between 400 and 500 nm seemed to be caused by the different relative content of each crocetin ester.

It has been reported (*24*) that the proportion of *trans*-4-GG in saffron spice (dry stigmas), as well as its absolute amount, were higher after 2 h at 50 °C and after 4 h at 70 °C when compared with a nonaged sample. It was concluded that saffron could increase its *trans*-4-GG content when it was resubmitted

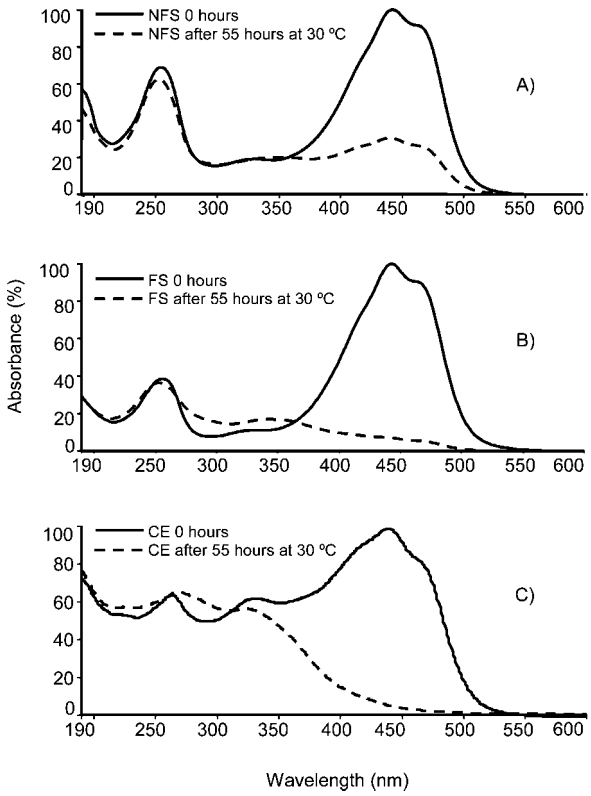

Figure 2. UV−vis spectra of (**A**) nonfiltered saffron aqueous extracts (NFS), (**B**) filtered saffron aqueous extracts (FS), and (**C**) crocetin ester-rich fraction solutions (CE) at the initial moment (—) and after 55 h (- - -) at 30 °C.

to a heating treatment. However, this appears not to happen in saffron crocetin ester rich fraction solutions, because increments in neither the percentage nor the absolute content of the above compound were observed. The same happened for all crocetin esters quantified in CEs. On the contrary, higher absolute contents than the initial ones in NFS for *cis*-3-Gg at 5 and 30 °C after 9 h and for *trans*-2-gg at 70 °C after 9 h have been observed. In FS, within the first 6 h of the experiments, the same trend was found for *trans*-5-nG at 5, 30, and 70 °C; for *trans*-3-Gg at 5 °C; for *trans*-2-gg at 5, 30, and 50 °C; for *cis*-4-GG, *cis*-3-Gg, and *trans*-1-g at 5, 30, 50, and 70 °C; and for *trans*-2-G at 50 and 70 °C. Concerning their relative proportion, increases were shown in all crocetin esters except *trans*-4-GG, which gave rise to situations in which *trans*-3-Gg surpassed *trans*-4-GG. By comparison of these two major carotenoids, *trans*-4-GG and *trans*-3-Gg, it was shown that when the latter increased, the former decreased and vice versa in saffron extracts, at all temperatures but 30 °C in NFS, at which the percentage of *trans*-3-Gg fluctuated while the percentage of *trans*-4-GG decreased, and at 70 °C in FS, at which both decreased. In CE both remained stable or decreased at the same time.

Despite the different rates of degradation, these findings would support the generation of the other crocetin esters from *trans*-4-GG by the detachment or attachment of glucose moieties, as previously reported (*11*) in methanolic solutions along with the cis−trans isomerization promoted by light.

The main changes shown by the HPLC results at 250 nm were a slight decrease in the picrocrocin peak area, the disappearance of signals corresponding to crocetin esters, and the appearance of a peak with a $t_R = 8.7$ min ($r = 5.0$ min) and $\lambda_{max} = 286$ nm after 23 h at 70 °C. On the contrary, a peak with $t_R = 11.2$ min ($r = 6.7$ min) and with a spectrum similar

Table 2. Maxima Shifts of UV–vis Spectra Observed in Saffron Aqueous Extracts and Crocetin Ester Rich Fraction Solutions upon Thermal Treatment

T (°C)	starting material[a]	λ peak at initial time (nm)	max shift[b] (nm)	time of max shift (h)
70	NFS	443	−3.0 ± 0.3	9
		254	−3.0 ± 0.4	2184
	FS	443	−3.0 ± 0.3	6
		256	−4.0 ± 0.5	672
	CE	439	−4.0 ± 0.2	2
		263	+3.0 ± 0.4	6
50	NFS	443	−6.0 ± 0.3	33
		254	−2.0 ± 0.1	93
	FS	443	−1.0 ± 0.4	6
		256	−3.0 ± 0.5	672
	CE	439	−4.0 ± 0.3	2
		263	+4.0 ± 0.2	387
30	NFS	443	−14.0 ± 0.4	984
		254	−9.0 ± 0.3	2184
	FS	443	−3.0 ± 0.6	30
		256	−3.0 ± 0.5	50
	CE	439	−2.0 ± 0.2	2
		263	+6.0 ± 0.4	385
5	NFS	443	−13.0 ± 0.3	575
		254	−2.0 ± 0.2	2184
	FS	443	−1.0 ± 0.3	240
		256	−3.0 ± 0.5	672
	CE	439	−4.0 ± 0.4	32
		263	+8.0 ± 0.3	385

[a] Nonfiltered saffron aqueous extracts (NFS), filtered saffron aqueous extracts (FS), and crocetin ester rich fraction solutions (CE). [b] Values are the means of two extracts conducted in triplicate ($3 \times 2n$). Shifts toward lower wavelengths are indicated by (−), whereas shifts toward higher wavelengths are indicated by (+).

to that of picrocrocin slightly increased at this temperature and at 50 °C, but showed a more prominent increase at 30 and 5 °C at longer times. On the basis of its UV–vis spectrum and retention time in comparison to the bibliography data (*29, 30*), this peak could possibly be 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (HTCC).

To justify the increase in absorbance observed between 275 and 380 nm, special attention was paid to specific moments. For example, in FSs at 70 °C, this change, unnoticed after 1 h, was observed after 6 h (**Figure 5**). Thus, the increase in absorbance at 330 nm occurred between 1 and 6 h. Comparison of the chromatograms at 330 nm for those times showed that several peaks with t_R between 3 and 4 min (r between 1.2 and 1.9 min) and around 7 min ($r = 3.9$ min), for which λ_{max} was between 325 and 385, appeared and could have contributed to the mentioned increase. Among them, the most noticeable peak eluted at $t_R = 4.2$ min ($r = 2.0$ min), and its spectrum had two maxima at 241 and 335 nm, the latter being higher. A baseline increase in the region of the chromatogram where crocetin esters were eluted was also observed. However, neither the *cis*-4-GG nor the *cis*-3-Gg area showed a higher level after 6 h, even though the previous measurement observed increases of 31 and 6% in each. The effect of these increases in the UV–vis spectrum might become apparent in delay with regard to the HPLC signal. Consequently, the increase in absorbance around 330 nm could be attributed to the isomerization of crocetin esters, together with the appearance of some degradation products. After 23 h at 70 °C, the spectrum showed slightly higher absorbance between 275 and 315 nm, but it decreased at higher wavelengths. This decrease was in consonance with the decrease in the peaks with retention times around 4 min and with the decreases in

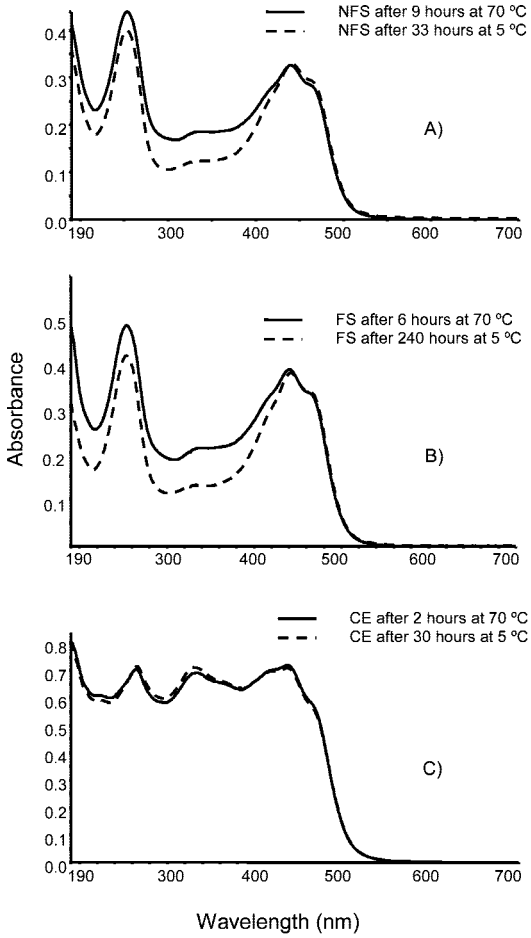

Figure 3. Comparison of UV−vis spectra of (**A**) a nonfiltered saffron extract (NFS), (**B**) a filtered saffron extract (FS), and (**C**) a crocetin ester rich fraction solution (CE) when their coloring strengths were the same but subjected to different temperatures.

picrocrocin and in crocetin esters shown in the chromatograms of the extracts after 23 h.

Therefore, the changes in the UV–vis spectrum from 315 to 380 nm could be due to such compounds present in the first minutes of chromatograms.

The same changes in the spectra and chromatograms were observed in FS at 50 °C, the only difference being that after 23 h, the absorbance between 275 and 360 nm was higher than at 6 h and also the area of the peaks with t_R around 4 min was higher. At 30 °C, these changes happened later in time (between 6 and 23 h) and remained longer (the increase in the absorbance was still noticeable after 240 h).

Kinetic Parameters. Table 3 shows rate constants (k) and half-life periods ($t_{1/2}$) of coloring strength loss ($E_{1\,cm}^{1\%}$440 nm), according to a first-order kinetic model, in saffron aqueous extracts and crocetin ester rich fraction solutions at 5, 30, 50, and 70 °C. Prior to discussing these results, it is necessary to point out that all rate constants were negative (as was degradation), with increases or decreases being expressed in absolute value.

The differences found in the k, depending on the initial material used, except for 5 and 30 °C when NFSs and FSs were compared, indicated that they could be considered as different reaction media in which the rest of the components modulate the degradation as stated for paprika carotenoids (*16, 31*). These k values showed a prominent increase with temperature, especially in FSs, for which k at 5 °C was approximately 10, 30, and 40 times lower than those at 30, 50, and 70 °C,

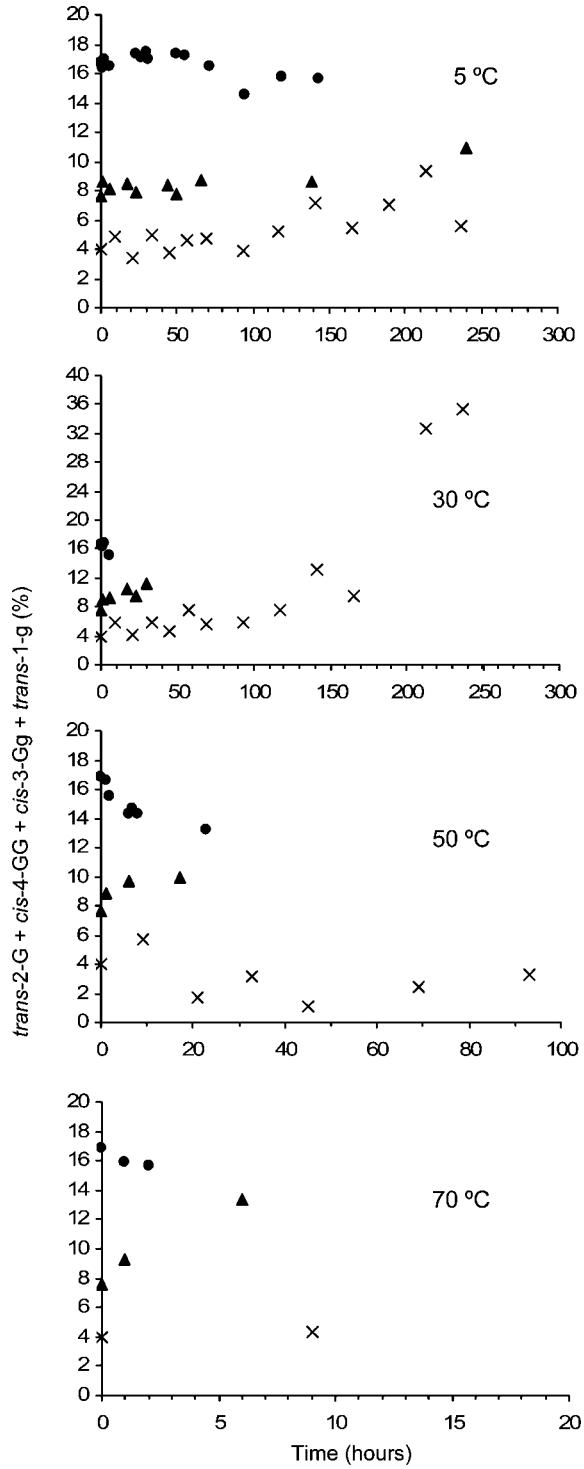

Figure 4. Sum of *trans*-2-G, *cis*-4-GG, *cis*-3-Gg, and *trans*-1-g, as percent of the total crocetin esters content, in crocetin ester rich fraction solutions (CE, ●), nonfiltered saffron aqueous extracts (NFS, ×), and filtered saffron aqueous extracts (FS, ▲) upon thermal treatment.

respectively. The only exceptions were CEs at 50 and 70 °C, which had very close k. These results illustrate the great effect of temperature on coloring strength stability and also confirmed the results previously reported (*9*). Degradation rate constants for CE were always higher than those for saffron extracts, giving evidence, once more, of their higher lability. However, only at 50 and 70 °C were k values of FSs significantly higher than those of NFSs. Half-life periods of the $E_{1\,cm}^{1\%}$440 nm degradation ranged from 151 h for FSs at the lowest temperature (5 °C) to 3 h for CEs at 50 and 70 °C. With regard to each crocetin ester,

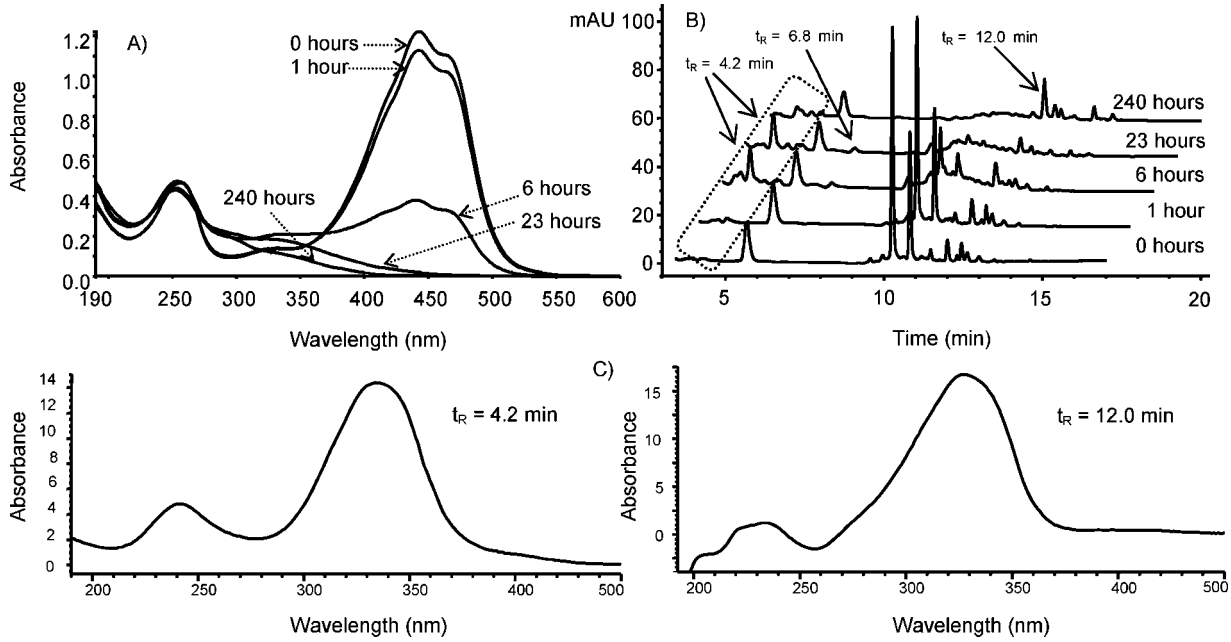

Figure 5. Evolution of filtered saffron aqueous extracts (FS) kept at 70 °C: (**A**) UV−vis spectra; (**B**) HPLC chromatograms at 330 nm; (**C**) UV−vis spectra of peaks with t_R values of 4.2 and 12.0 min.

Table 3. Rate Constants (k), Determination Coefficients (R^2), and Half-Life Periods ($t_{1/2}$) of Coloring Strength Loss ($E^{1\%}_{1cm}$440 nm) in Saffron Aqueous Extracts and Crocetin Ester Rich Fraction Solutions upon Thermal Treatment

T (°C)	starting material[a]	($k \pm$ SD)[b] $\times 10^3$ (h^{-1})	R^2(m)[c]	$t_{1/2}$ (h)
5	NFS	4.7 a ± 0.4	0.920 (45)	147
	FS	4.6 a ± 0.5	0.998 (16)	151
	CE	19.1 b ± 1.0	0.991 (26)	36
30	NFS	40.6 a ± 0.9	0.883 (9)	17
	FS	43.7 a ± 5.5	0.955 (11)	16
	CE	103.4 b ± 5.2	0.987 (12)	7
50	NFS	62.9 a ± 2.4	0.976 (9)	11
	FS	140.0 b ± 4.2	0.994 (9)	5
	CE	224.7 c ± 11.2	0.992 (12)	3
70	NFS	76.5 a ± 0.4	0.996 (9)	9
	FS	184.2 b ± 8.8	0.999 (9)	4
	CE	234.0 c ± 11.4	0.996 (12)	3

[a] Nonfiltered saffron aqueous extracts (NFS), filtered saffron aqueous extracts (FS), crocetin ester rich fraction solutions (CE). [b] Values are the means of two extracts conducted in triplicate (3 × 2n), SD = standard deviation. [c] Minimum number of experimental data points. At each temperature, different letters between rows indicate significant differences at the 0.05% level.

in most cases studied their degradation adjusted to a first-order kinetics model (**Table 4**), but it was not always possible to apply such a model to the degradation because either their areas were stable or they increased slightly in the first hours and then decreased. The k of each crocetin ester was also clearly dependent on temperature and increased along with temperature. Only *cis*-4-GG had similar k values for the higher temperatures (50 and 70 °C) in the three starting solutions. Besides, low differences were found between k of CE at 50 and 70 °C.

In the first thermal treatment for NFSs, the crocetin esters that had the lowest k and therefore degraded more slowly were the *trans*-3-Gg at 5 °C ($t_{1/2}$ = 198 h), whereas at 30 and 50 °C they were the *trans*-2-gg ($t_{1/2}$ = 301 and 59 h, respectively) and at 70 °C it was the *cis*-4-GG ($t_{1/2}$ = 14 h). On the contrary, crocetin esters with the highest degradation rates were *trans*-5-tG at 5 °C ($t_{1/2}$ = 99 h) and *trans*-4-GG at other temperatures ($t_{1/2}$ = 53 h at 30 °C, 8 h at 50 °C, 6 h at 70 °C). In the second thermal treatment for FSs, the most stable crocetin esters were *trans*-2-G ($t_{1/2}$ = 165 h) and *trans*-5-tG ($t_{1/2}$ = 136 h) at 5 °C, whereas at 30 and 50 °C they were the *trans*-5-nG ($t_{1/2}$ = 32 and 9 h, respectively) and at 70 °C it was the *cis*-4-GG ($t_{1/2}$ = 7 h). On the other hand, crocetin esters with the highest degradation rates were *trans*-4-GG ($t_{1/2}$ = 104 h), *trans*-3-Gg ($t_{1/2}$ = 106 h), and *cis*-4-GG ($t_{1/2}$ = 123 h) at 5 °C; *trans*-4-GG at 30 °C ($t_{1/2}$ = 12 h); *trans*-2-G at 50 °C ($t_{1/2}$ = 4 h); and *trans*-2-gg and *trans*-4-GG at 70 °C ($t_{1/2}$ = 3 h). Finally, in the third thermal treatment for CEs, the *trans*-5-nG was the most stable crocetin ester ($t_{1/2}$ = 60 h), whereas *trans*-2-G and *cis*-3-Gg were the most labile ones at all temperatures ($t_{1/2}$ = 23 h at 5 °C, 3 h at 30 °C, 2 h at 50 and 70 °C), although at 70 °C there were no significant differences with the *trans*-4-GG.

No evidence of any relationship between the number or type of glycoside moiety and lability of crocetin esters was found. Furthermore, at almost all temperatures, the k values of the two mayor crocetin esters (*trans*-4-GG and *trans*-3-Gg) did not present significant differences. However, *trans*-3-Gg at 5, 50, and 70 °C in NFSs and at 30 °C in FSs degraded at a lower rate, whereas it was less stable than *trans*-4-GG at 5 °C in CEs. Analogously, *cis*-4-GG and *cis*-3-Gg had equal k values at 30 °C but *cis*-4-GG was slightly more stable than *cis*-3-Gg at 50 °C in FSs. The crocetin ester *cis*-4-GG showed equal or lower k values compared to the major *trans*-crocetin esters (*trans*-4-GG, *trans*-3-Gg, and *trans*-2-G) except for NFS at 50 °C, at which *trans*-3-Gg had a higher stability, and for FS at 5 °C, at which the same happened with *trans*-2-G. The crocetin ester *cis*-4-GG was less stable than *trans*-5-nG in all cases but in NFS and FS at 70 °C. Half-life periods of crocetin esters ranged from 198 h at 5 °C to 2 h at 70 °C. The results also indicated that each crocetin ester was affected in a different way by the temperature increase. For example, in FSs, an increment of temperature from 5 to 30 °C resulted in k values of crocetin ester degradation being multiplied by a factor ranging from 7 to 11 approximately. From 30 to 50 °C, this factor varied between 3 and 4, whereas an increase in temperature from 50 to 70 °C multiplied k by a factor of 1−2. Especially important

Table 4. Degradation Rate Constant (k), Determination Coefficient (R^2), and Half-Life Period ($t_{1/2}$) of Each Crocetin Ester in Saffron Aqueous Extracts and Crocetin Ester Rich Fraction Solutions upon Thermal Treatment

		5 °C			30 °C			50 °C			70 °C		
compd	starting material[a]	($k \pm$ SD)[b] $\times 10^3$(h^{-1})	R^{2c} (m)	$t_{1/2}$ (h)	($k \pm$ SD) $\times 10^3$ (h^{-1})	R^2 (m)	$t_{1/2}$ (h)	($k \pm$ SD) $\times 10^3$ (h^{-1})	R^2 (m)	$t_{1/2}$ (h)	($k \pm$ SD) $\times 10^3$ (h^{-1})	R^2(m)	$t_{1/2}$ (h)
trans-5-tG	NFS	7.0 e ± 0.3	0.968 (11)	99	*			*			*		
	FS	5.1 bc ± 0.4	0.987 (13)	136	45.5 d ± 5.1	0.997 (12)	15	132.8 gh ± 5.9	0.995 (5)	5	196.0 f ± 13.1	0.994 (5)	4
	CE	16.8 g ± 0.8	0.982 (27)	41	100.8 f ± 5.0	0.988 (16)	7	194.2 l ± 9.7	0.992 (12)	4	222.4 gh ± 11.1	0.991 (12)	3
trans-5-nG	NFS	5.6 cd ± 1.0	0.944 (13)	124	9.7 ab ± 1.7	0.955 (11)	71	32.6 c ± 2.2	0.980 (7)	21	74.1 b ± 12.0	0.979 (4)	9
	FS	*			21.9 c ± 3.4	0.985 (12)	32	75.0 e ± 3.4	0.988 (9)	9	136.1 d ± 17.4	0.952 (5)	5
	CE	11.6 f ± 0.6	0.996 (19)	60	65.8 e ± 3.3	0.982 (18)	11	148.4 i ± 7.4	0.982 (12)	5	156.0 e ± 7.8	0.998 (14)	4
trans-4-GG	NFS	5.6 cd ± 0.5	0.982 (20)	124	13.0 b ± 5.1	0.981 (16)	53	92.4 f ± 4.6	0.989 (7)	8	112.2 c ± 5.6	0.992 (6)	6
	FS	6.7 de ± 0.5	0.985 (13)	104	58.3 e ± 6.6	0.997 (12)	12	165.4 jk ± 8.1	0.994 (7)	4	229.3 ghi ± 13.3	0.994 (5)	3
	CE	25.5 j ± 1.3	0.990 (29)	27	153.9 i ± 7.7	0.991 (14)	5	281.5 o ± 14.1	0.995 (12)	2	300.4 kl ± 15.0	0.996 (12)	2
trans-3-Gg	NFS	3.5 a ± 0.2	0.972 (18)	198	5.4 ab ± 0.3	0.930 (17)	128	15.8 b ± 0.8	0.896 (15)	44	78.1 b ± 0.8	0.991 (7)	9
	FS	6.6 de ± 1.7	0.961 (13)	106	43.8 d ± 5.0	0.992 (12)	16	169.3 k ± 8.1	0.995 (7)	4	222.9 gh ± 2.1	0.999 (5)	3
	CE	27.4 k ± 1.4	0.983 (27)	25	159.7 i ± 8.0	0.987 (14)	4	278.1 o ± 13.9	0.996 (12)	2	292.8 k ± 2.9	0.996 (12)	2
trans-2-gg	NFS	*			2.3 a ± 0.2	0.911 (19)	301	11.7 ab ± 0.6	0.965 (15)	59	67.8 b ± 3.4	0.982 (5)	10
	FS	*			*			152.7 i ± 5.2	0.991 (7)	5	243.7 ij ± 7.8	0.993 (5)	3
	CE	16.1 g ± 0.8	0.979 (27)	43	116.9 g ± 5.8	0.986 (10)	6	240.9 n ± 12.0	0.997 (12)	3	256.3 j ± 10.3	0.994 (12)	3
trans-2-G[d]	FS	4.2 ab ± 0.1	0.979 (13)	165	45.2 d ± 10.7	0.989 (12)	15	182.6 l ± 10.9	0.980 (7)	4	215.6 g ± 7.1	0.997 (5)	3
	CE	29.6 l ± 1.5	0.976 (27)	23	211.3 j ± 10.6	0.994 (12)	3	331.5 p ± 16.6	0.995 (12)	2	315.4 l ± 15.8	0.993 (12)	2
cis-4-GG	NFS	*			*			53.8 d ± 2.7	0.972 (6)	13	51.3 a ± 2.6	0.961 (5)	14
	FS	5.7 cd ± 0.2	0.979 (13)	123	37.5 d ± 7.6	0.983 (12)	19	103.5 f ± 6.0	0.987 (7)	7	106.6 c ± 18.3	0.963 (5)	7
	CE	22.3 i ± 1.1	0.983 (27)	31	133.5 h ± 6.7	0.990 (10)	5	241.7 n ± 12.1	0.995 (12)	3	235.5 hi ± 11.8	0.996 (12)	3
cis-3-Gg[e]	FS	*			37.9 d ± 8.4	0.983 (12)	18	121.1 g ± 8.9	0.973 (5)	6	*		

[a] Nonfiltered saffron aqueous extracts (NFS), filtered saffron aqueous extracts (FS), crocetin ester rich fraction solutions (CE). [b] Values are the means of two extracts conducted in duplicate ($2 \times 2n$), SD = standard deviation. [c] Minimum number of experimental data points. [d] Separation of the peak was not possible in NFS, and it was coeluted with *cis*-3-Gg in CE. [e] It was coeluted with *trans*-2-G in CE, and it did not follow a first-order kinetics in NFS. *, first-order kinetics was not followed. Different letters between rows indicate significant differences at the 0.05% level.

Table 5. Arrhenius Equation Parameters, Activation Energy (E_a), and Pre-exponential Factor of Each Crocetin Ester Degradation and Coloring Strength Loss ($E_1^{1\%}{}_{cm}$440 nm) in Saffron Aqueous Extracts and Crocetin Ester Rich Fraction Solutions

	starting material[a]	$E_a \pm$ SD[b] (kJ mol^{-1})	ln $A \pm$ SD (A, h^{-1})	R^2
trans-5-tG	NFS	*	*	*
	FS	45.6 jk ± 1.2	14.7	0.958
	CE	32.3 cd ± 0.7	10.1	0.912
trans-5-nG	NFS	32.4 i ± 0.7	8.7	0.871
	FS	39.8 gh ± 0.8	12.0	0.998
	CE	33.0 de ± 0.8	10.1	0.913
trans-4-GG	NFS	41.2 hi ± 0.8	12.6	0.946
	FS	44.3 j ± 0.9	14.4	0.951
	CE	30.9 bc ± 1.1	10.0	0.890
trans-3-Gg	NFS	39.2 g ± 1.1	10.7	0.846
	FS	45.4 jk ± 2.7	14.7	0.962
	CE	29.6 b ± 1.2	9.5	0.882
trans-2-gg	NFS	55.8 m ± 1.2	16.6	0.982
	FS	*	*	*
	CE	34.8 e ± 1.3	11.2	0.895
trans-2-G[c]	NFS	*	*	*
	FS	50.3 l ± 0.9	16.5	0.950
	CE	29.5 b ± 0.8	9.6	0.826
cis-4-GG	NFS	21.9 a ± 1.3	5.2	0.981
	FS	37.4 f ± 1.3	11.3	0.926
	CE	29.7 b ± 1.3	9.4	0.867
$E_1^{1\%}{}_{cm}$ 440 nm	NFS	34.1 e ± 0.3	9.8	0.873
	FS	46.5 k ± 0.4	15.0	0.961
	CE	31.8 cd ± 0.4	10.0	0.911

[a] Nonfiltered saffron aqueous extracts (NFS), filtered saffron aqueous extracts (FS), crocetin ester rich fraction solutions (CE). [b] Values are the means of two extracts conducted in duplicate ($2 \times 2n$) ± standard deviation (SD) for crocetin esters and the means of two extracts conducted in triplicate ($3 \times 2n$) ± SD for $E_1^{1\%}{}_{cm}$440 nm. [c] Separation of the peak was not possible in NFS and was coeluted with *cis*-3-Gg in CE. *, there were not enough data to calculate the activation energy. Different letters between rows indicate significant differences at the 0.05% level.

was the change in the degradation rate of crocetin esters when temperature was increased from lower temperatures, justifying the necessity to study certain thermodynamic parameters such as the activation energy (E_a).

Now that all degradation rate constants have been discussed, we point out that k values of coloring strength were situated among the k values corresponding to crocetin esters, as well as its E_a (described below). In general, the coloring strength (**Tables 3** and **4**) was more stable (lower k) than the two major crocetin esters with the following exceptions: no significant differences were found with *trans*-3-Gg at 30 °C in FS and at 5 and 70 °C in NFS; at 50 °C in NFS, *trans*-3-Gg had lower k than $E_1^{1\%}{}_{cm}$440 nm.

Thermodynamic Parameters. Thermodynamic results are presented in **Tables 5** and **6**. Significant differences were found among crocetin ester E_a (**Table 5**), with the highest E_a in line with crocetin esters that showed the most important differences when k values for 5 °C were compared to those for 70 °C. For instance, in FSs, *trans*-2-G showed the highest E_a (the mentioned increase was >50 times) followed by *trans*-5-tG, *trans*-3-Gg, and *trans*-4-GG (their k increased by >34 times). On the other hand, *cis*-4-GG showed the lowest E_a (k increment of 20 times approximately). In addition, the order in E_a for NFSs was *trans*-2-gg > *trans*-4-GG > *trans*-3-Gg > *trans*-5-nG > *cis*-4-GG, whereas that for CEs was *trans*-2-gg > *trans*-5-nG > *trans*-5-tG > *trans*-4-GG > *cis*-4-GG > *trans*-3-Gg > *trans*-2-G. It was also found that the changes in k with temperature for the loss of coloring strength, and as a consequence the E_a, were always in the range of the corresponding values for crocetin esters.

As has been observed for degradation constant rates, there were no important differences between the E_a values of *trans*-4-GG and *trans*-3-Gg. The activation energy for the loss of coloring strength, 46.5 ± 0.4 kJ/mol (11.1 ± 0.1 kcal/mol), was higher when compared with that obtained by Tsimidou and Tsatsaroni (*9*) for temperatures ranging from 4 to 62 °C and pH 7 (7.2 ± 0.1 kcal/mol) but lower than that reported by Alonso et al. (*8*) for temperatures ranging from 0 to 35 °C (124.11 kJ/mol). The E_a was always higher for FSs, followed by NFSs and CEs, except for *cis*-4-GG, for which the E_a values of CEs were higher than those of the NFSs. Mathematically, the interpretation is that changes in temperature modify the reaction rate constant more when the extract is filtered than when they remain unfiltered or when crocetin esters are purified from the rest of saffron components. With these values, according to the activated complex theory, the energy requirement for each crocetin ester in FSs to become an activated complex is well above that of crocetin esters in NFSs and CEs. However, once energy is supplied to the reaction system (in this case an aqueous medium), it is the other parameter of the Arrhenius equation, A (**Table 5**), that determines the degree to which the reaction proceeds. The result was that for the majority of crocetin esters, the number of molecules able to form the activated complex was higher in FSs than it was in NFSs and CEs. Thus, for these reaction conditions, there was a degradative effect on the FSs.

Table 6. Increase of Activation Entalpy (ΔH^*), Entropy (ΔS^*), Gibbs Free Energy (ΔG_{isok}), and Isokinetic Temperature (T_{isok}) of Each Crocetin Ester Degradation and Coloring Strength Loss ($E_1^{1\%}{}_{cm}$440 nm) in Saffron Aqueous Extracts and Crocetin Ester Rich Fraction Solutions

	NFS[a]			FS[a]			CE[a]					
	ΔH^* (kJ/mol)	ΔS^* (J/mol K)	R^2	ΔH^* (kJ/mol)	ΔS^* (J/mol K)	R^2	ΔH^* (kJ/mol)	ΔS^* (J/mol K)	R^2	ΔG_{isok} (kJ/mol)	T_{isok} (K)	R^2
trans-5-tG	36.7	221.5	1.000	43.0	199.6	0.952	29.7	237.3	0.896	112.9	348	0.984
trans-5-nG	31.4	245.1	0.926	37.0	222.1	0.970	30.4	237.9	0.899	97.1	273	0.817
trans-4-GG	37.7	221.1	0.910	41.7	201.8	0.944	28.3	238.6	0.870	115.7	361	0.938
trans-3-Gg	34.2	239.5	0.859	42.4	200.3	0.958	27.1	242.5	0.860	102.2	297	0.834
trans-2-gg	59.3	164.6	0.984	18.8	271.2	1.000	32.2	228.2	0.878	121.6	383	0.994
cis-4-GG	15.0	292.8	0.745	35.0	227.4	0.907	27.2	243.8	0.843	100.3	292	0.976
trans-2-G[b]	–	–	–	47.6	184.1	0.933	27.0	241.6	0.797	–	–	–
cis-3-Gg	–	–	–	44.7	192.8	1.000	–	–	–	–	–	–
trans-1-g	–	–	–	–	–	–	33.0	221.9	0.769	–	–	–
$E_1^{1\%}{}_{cm}$ 440 nm	31.2	241.8	0.860	43.9	197.0	0.940	29.2	238.1	0.895	105.4	313	0.960

[a] Nonfiltered saffron aqueous extracts (NFS), filtered saffron aqueous extracts (FS), crocetin ester rich fraction solutions (CE). [b] Separation of the peak was not possible in NFS and was coeluted with *cis*-3-Gg in CE. –, no available data.

To elucidate whether there were formal, kinetic, and thermodynamic differences among the situations studied (NFS, FS, and CE), the results obtained were interpreted as a kinetically compensated system. According to the isokinetic theory, a single reaction can have different kinetic and thermodynamic parameters depending on the reaction conditions, although in all cases the reaction is the same (*14, 16, 32–34*). A kinetically compensated system requires that the different thermodynamic parameters obtained for the same reaction in different environments define an isokinetic line. This theoretical line includes all of the different kinetic and thermodynamic coordinates of a single reaction, having as slope the isokinetic temperature (at which the rate constant of the reaction is unique and independent of the medium) and as ordinate at the origin the increase in Gibbs free energy of all the reactions at the isokinetic temperature (eq 4). **Table 6** shows the variation of activation enthalpy, entropy, and Gibbs free energy for the degradation of each crocetin ester and loss of coloring strength. These values have been expressed as absolute values because, as rate constants must have positive values for the thermodynamic study, the sign of the calculated parameters has only a mathematical meaning. It was found that in most of crocetin esters studied, an isokinetic line was obtained ($R^2 > 0.9$). However, *trans*-5-nG and *trans*-3-Gg presented a lower determination coefficient (0.817 and 0.834). The variation of Gibbs free energy varied between 97.1 kJ/mol for *trans*-5-nG and 121.6 kJ/mol for *trans*-2-gg. The isokinetic temperatures ranged from 273 to 383 K, including the range of temperatures under study (278–343 K) and also the values previously reported for β-carotene and capsanthin co-oxidation by lipoxygenase (*16*) and carotenoid pigments in paprika oleoresins (*31*). The fact that all situations defined the same isokinetic line showed that the reaction was the same and that the environment which surrounded the reaction was responsible for the displacement of the thermodynamic parameters along the same isokinetic line. In this case, the theoretical single reaction is represented by each colored crocetin ester that becomes colorless, possibly due to the loss of conjugation in its molecule. The other functional groups of the crocetin ester (not included in the chromophore) can be considered as external factors that can modify the amount of energy required for loss of conjugation. The medium in which the reaction occurs or the environmental conditions are also external factors that do not modify the pattern of the reaction, but change its speed and temperature dependence. Also, the nature of the compounds that promote the loss of conjugation is an external factor affecting the reaction quantitatively but not qualitatively.

In conclusion, the degradation reaction was the same for all crocetin esters whether they were in saffron extracts or whether they were purified, but it was affected by external factors that modified their kinetic and thermodynamic parameters, hence making some of them more labile than others. In general, an overall loss, more marked with increasing temperature, of all crocetin esters was found and a lower stability of the purified crocetin esters was observed. Throughout degradation, the proportion of the different crocetin esters showed changes that contributed to hypsochromic effects in the UV–vis spectra of aqueous extracts. The parameters for the loss of coloring strength were always between the maximum and minimum values for individual crocetin esters, so it could be considered as a global result of their degradation. Some punctual increases in percentage of cis isomers were observed that could be responsible for changes at about 330 nm in the UV–vis spectra, together with the appearance of some degradation products.

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Supporting Information Available: Degradation rate constant (k), determination coefficient (R^2), and half-life period ($t_{1/2}$) of each crocetin ester in nonfiltered saffron aqueous extracts at room temperature, 35, 40, and 60 °C. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting information table. Degradation rate constant (*k*), determination coefficient (R^2) and half-life period ($t_{1/2}$) of each crocetin ester in non filtered saffron aqueous extracts (FS) at room temperature, 35, 40 and 60 ºC.

Compound	$(k \pm SD)^a \times 10^3$ $(hours)^{-1}$	$R^2(m)^b$	$t_{1/2}$ (hours)	$(k \pm SD)^a \times 10^3$ $(hours)^{-1}$	$r^2(m)^b$	$t_{1/2}$ (hours)	$(k \pm SD)^a \times 10^3$ $(hours)^{-1}$	$r^2(m)^b$	$t_{1/2}$ (hours)	$(k \pm SD)^a \times 10^3$ $(hours)^{-1}$	$r^2(m)^b$	$t_{1/2}$ (hours)
	Room Temperature			35 ºC			40 ºC			60 ºC		
Trans-5-tG		*			*		46.6 d ± 5.0	0.994 (4)	15		*	
Trans-5-nG	7.0 c ± 0.4	0.935 (12)	99		*		26.6 b ± 0.2	0.982 (7)	26		*	
Trans-4-GG	7.8 d ± 0.4	0.973 (18)	89	12.1 b ± 0.6	0.962 (15)	57	52.3 e ± 2.6	0.995 (7)	13	117.1 e ± 5.9	0.965 (6)	6
Trans-3-Gg	4.4 b ± 0.2	0.951 (18)	158	5.7 a ± 0.3	0.935 (18)	122	9.2 a ± 0.5	0.877 (17)	75	48.3 b ± 2.4	0.982 (9)	14
Trans-2-gg	1.6 a ± 0.1	0.913 (20)	433				8.2 a ± 0.4	0.868 (16)	85	34.5 a ± 1.7	0.943 (9)	20
Trans-2-G[b]												
Cis-4-GG	20.3 e ± 1.0	0.940 (6)	34	34.4 c ± 1.7	0.959 (4)	20	43.3 c ± 2.2	0.991 (5)	16	67.2 c ± 3.4	0.936 (5)	10
Cis-3-Gg		*			*			*			*	

[a]Values are the means of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. [b]Minimum number of experimental data points. [d]The separation of the peak was not possible. *First-order kinetics was not followed. Different letters between rows indicate significant differences at the 0.05% level.

5.4. Picrocrocina: cinética de su degradación en extractos acuosos de azafrán especia (*Crocus sativus* L.) sometidos a tratamiento térmico y umbral de percepción gustativa

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Picrocrocin: Kinetics of its Degradation in Aqueous Extracts of Saffron (*Crocus sativus* L.) Spice upon Thermal Treatment and Taste Detection Threshold

ANA M. SÁNCHEZ, MANUEL CARMONA, JOSÉ E. PARDO, AND GONZALO L. ALONSO*

El objetivo de este trabajo fue el estudio de la cinética de degradación de la picrocrocina en extractos acuosos de azafrán y de forma aislada cuando se somete a tratamiento térmico. Además, se llevaron a cabo análisis sensoriales para establecer el umbral de percepción gustativa de la picrocrocina.

Al igual que en los ésteres de crocetina, se estudiaron dos tipos de extractos de azafrán: el primero, designado como "NFS" se preparó siguiendo la especificación técnica ISO/TS 3632 (2003) pero dejando una concentración de 500 mg/L y sin retirar la materia vegetal procedente del azafrán del extracto; el segundo se denominó "FS" se preparó como el anterior pero se filtró para retirar la materia vegetal. En NFS los análisis se llevaron a cabo a 5, 20, 30, 35, 40, 50, 60 ºC y 70 ºC, mientras que en FS se decidió estudiar sólo 5, 30, 50 y 70 ºC debido a las semejanzas observadas a las temperaturas más próximas. Además, se siguió la evolución en agua de una fracción purificada de picrocrocina, a la que denominamos "PI", sometida un calentamiento a reflujo a aproximadamente 100 ºC. Se eligió este tratamiento térmico no sólo por ser extremo, sino también, para simular condiciones térmicas de cocinado.

Cambios en el contenido de picrocrocina

En general, se observó un descenso lento del contenido de picrocrocina en todos los tratamientos térmicos. Sin embargo, a 70 ºC en los extractos NFS el contenido de picrocrocina permaneció estable hasta algo más de 300 h aunque después sí se observó un descenso. Además, a esta temperatura se vieron aumentos puntuales del contenido de picrocrocina a 237 y 311 h. En los dos tipos de extractos de azafrán, el tratamiento térmico a 30 ºC fue el que produjo mayores descensos de picrocrocina, observándose la pérdida casi completa de picrocrocina a 311 h en NFS y a 461 h en FS. Los extractos FS mostraron mayores valores de picrocrocina retenida a todas las temperaturas estudiadas, excepto a 70 ºC, pareciendo indicar que el filtrado del extracto contribuía a la estabilidad de la picrocrocina a esas temperaturas.

El tratamiento térmico de PI a 100 ºC también demostró una alta estabilidad de la picrocrocina pues el descenso del contenido de picrocrocina no fue mayor del 34% de su valor inicial tras 6 h y un descenso de aproximadamente el 10% fue observado tras 1 h de tratamiento. Considerando la picrocrocina como responsable del sabor amargo del azafrán, estos resultados sugerían que un tratamiento térmico de ebullición en el cocinado podría influir levemente en dicho sabor. Simultáneamente se observó un aumento continuado del safranal en las primeras 5 h de calentamiento seguido de un leve descenso.

Cambios en los valores de $E_{1cm}^{1\%}$ 257 nm y de ΔEpic

Para poder comparar con los resultados que aparecen en la bibliografía y con los obtenidos para el contenido de picrocrocina, decidimos seguir también la evolución $E_{1\mathrm{cm}}^{1\%}$ 257 nm y ΔEpic en los extractos de azafrán. La tendencia general de estos parámetros consistió en un lento descenso, salvo en el caso de $E_{1\mathrm{cm}}^{1\%}$ 257 nm a 50 y 70 ºC para el extracto NFS, donde se observó un aumento en las primeras 50 horas y una posterior estabilización en torno al 110% de su valor inicial. Comparando los dos tipos

de extractos, los descensos fueron menores en NFS que en FS para todas las temperaturas estudiadas menos para 30 ºC en NFS, donde no se apreciaron diferencias entre ambos extractos. Al contrario que para el contenido de picrocrocina, el proceso de filtrado redujo la estabilidad de los valores de $E_{1cm}^{1\%}$ 257 nm y ΔEpic. A pesar de las semejanzas encontradas en algunos casos, comprobamos que la evolución de estos parámetros no reflejaba la del contenido de picrocrocina. Esto podría justificarse por los cambios que se producen en otros componentes de los extractos como los ésteres de crocetina o por la aparición de productos de degradación.

Parámetros cinéticos y termodinámicos

Los mejores ajustes de los resultados experimentales se encontraron, en general, para un modelo cinético de segundo orden. Sin embargo, la cinética de la picrocrocina se ajustaba mejor al orden cero desde 5 hasta 35 ºC en NFS y a 30 ºC en FS. Además, a algunas temperaturas, las diferencias con los ajustes de primer y de orden cero no fueron importantes. Las constantes de velocidad de la reacción (k) y los tiempos de vida media ($t_{1/2}$) de la picrocrocina reflejaron los cambios ya descritos para su contenido en los distintos tratamientos térmicos. Así, por ejemplo, los valores de k fueron menores en FS que en NFS indicando una mayor estabilidad después de haber retirado la materia vegetal del extracto. También se encontraron mayores valores de energía de activación, Ea, y de variación de entalpía de activación, ΔH^*, para FS (38,3 kJ mol^{-1} y 35,5 kJ mol^{-1} respectivamente) que para NFS (12,5 kJ mol^{-1} y 15,1 kJ mol^{-1}, respectivamente). Por el contrario, la variación de entropía, ΔS^*, fue mayor para NFS (408,3 kJ mol^{-1} K^{-1}) que para FS (243,0 kJ mol^{-1} K^{-1}).

Análisis sensorial: umbral de percepción gustativa de la picrocrocina

Se estableció el umbral de percepción gustativa de la picrocrocina, según una prueba triangular, en 10 mg/L a un nivel de significación del 1% (α = 0,01).

5.4. Picrocrocin: kinetics of its degradation in aqueous extracts of saffron (*Crocus sativus* L.) spice upon thermal treatment and taste detection threshold

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Kinetics of picrocrocin degradation in aqueous extracts of saffron upon thermal treatment from 5 to 70 ºC has been studied, together with the degradation of purified picrocrocin at 100 ºC. In general, the best fits were found for a second-order kinetic model. However, good fits were also found for zero and first-order models. Picrocrocin showed a great stability with half-life periods ($t_{1/2}$) ranging from more than 4000 h at 5 ºC in saffron extracts to 9 h in the experiments with purified picrocrocin at 100 ºC. The results also showed that a removal of vegetal matter in saffron extracts contributed to picrocrocin stability at all temperatures studied, except 70 ºC. Although similarities were occasionally found, the parameters $E_{1cm}^{1\%}$ 257 nm and ΔEpic did not reflect the evolution of picrocrocin. The evolution of safranal, was also studied in the solution of picrocrocin at 100 ºC, and showed increments up to 5 h of thermal treatment. Finally, on the basis of the results of a triangle test, the picrocrocin detection threshold was set at 10 mg L^{-1} ($\alpha = 0.01$).

KEYWORDS: Picrocrocin, saffron (*Crocus sativus* L.), kinetics, safranal, sensory analysis, taste detection threshold.

INTRODUCTION

Saffron spice, the dried stigmas of *Crocus sativus* L., is highly valued in cookery and in the food industry for the coloring properties provided by a group of glycosyl esters of crocetin ($C_{20}H_{24}O_4$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid). However, its alluring aroma and pleasant bitter taste are what mainly differentiate saffron from other natural or synthetic colorants such as safflower, curcumin, gardenia, and tartrazine. Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound in the volatile fraction of saffron spice, whereas picrocrocin (4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) is thought to be the foremost contributor to its bitter taste. Color compounds as well as volatiles have been thoroughly investigated in the last decades (*1-4*), but only a few studies have focused on the taste of saffron (*5-8*) and, in particular, on picrocrocin and bitter taste (*9*). In other products such as red wine, cocoa, coffee or tea, recent papers have shown a growing concern for corresponding analytical data to sensory data (*10-13*). This correspondence is also important in saffron in order to take advantage of its taste potential and optimize its usage in food, especially when considering its high price.

It is known that picrocrocin is converted to safranal either by a two-step enzymatic/dehydration process involving the intermediate 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde (HTCC) or directly by thermal degradation. There is also evidence of this conversion at extreme pH (*14-17*).

Many applications of saffron spice imply a thermal cooking process at high temperature. Carmona et al. reported that thermal treatment changes aroma profile of saffron (*18-19*), but the remaining question is the effect of thermal treatment on picrocrocin and its impact on saffron taste. Previous kinetic studies have focused on establishing the best saffron storage conditions; giving evidence that a dark, inert atmosphere, low water activity level (< 0.43) and less than ambient temperatures (< 25 ºC) should be maintained (*20-25*). They have also dealt with aqueous extracts of saffron, showing that color and crocetin ester degradation follow first-order kinetics; are sensitive upon exposure to light, thermal treatment and acidic environment as well as to the presence of additives

(*26-28*). In comparison, only a few approximate studies deal with picrocrocin degradation. Among these studies, kinetic results based on measurements of $E_{1cm}^{1\%}$ at 257 nm, the maximum of picrocrocin absorbance in water, and the parameter defined by Corradi and Micheli (*29*) as $\Delta Epic = E_{257}^{1\%} - E_{297}^{1\%}$ have been reported, showing good fits to either first or second-order reaction models for the loss of $E_{1cm}^{1\%}$ 257 nm (*23*) and a second-order kinetics for Δ*E*pic (*25*). Castellar (*30*) in the only study done with purified picrocrocin, to our knowledge, gave evidence of its high stability at room temperature, 4 and -20 ºC at pH 7.

The purpose of this research was to study the changes that picrocrocin undergoes in aqueous extracts when subjected to thermal treatment through its kinetic and thermodynamic parameters. Moreover, a first insight into the impact of this treatment on saffron taste was obtained by determination of the taste detection threshold of picrocrocin.

MATERIALS AND METHODS

Standards and Chemicals. Gallic acid and safranal were purchased from Sigma-Aldrich (Madrid, Spain). HPLC-grade acetonitrile, ciclohexane and phosphoric acid were from Scharlau (Barcelona, Spain). Ultra-high-purity water was produced using a Milli-Q System from Millipore (Bedford, MA). PTFE filters (11 mm, 0.45 μm) were also purchased from Millipore while C_{18} packing material (125×10^{-8} cm pore size, 55-105 μm particle size) was supplied by Waters, (Milford, MA).

Samples. Saffron spice (*Crocus sativus* L.) was used from the 2004 harvest of the Protected Designation of Origin *Azafrán de La Mancha*. Three different types of aqueous extracts were used. Two of them were prepared according to the ISO 3632 Technical Specification, 2003 (*31*) but at a 500 mg L^{-1} concentration, without and after removal of vegetal matter by filtration through filter paper. The extracts were designated NFS (Non Filtered Saffron extract) and FS (Filtered Saffron extract), respectively. The third extract was prepared using as starting material a picrocrocin fraction (PI) that was purified by column chromatography according to the procedure reported by Sánchez et al. (*32*).

Thermal Treatment. NFS. Eight aliquots of an NFS (200 mL each) were transferred to 250 mL borosilicate glass bottles, hermetically sealed, and kept in the dark at different temperatures: 5 ºC (refrigerator), room temperature (20 ± 2 ºC), and 30, 35, 40, 50, 60, and 70 ºC (different thermostated ovens).

FS. Ten aliquots of approximately 20 mL were put in 50 mL Falcon tubes sealed and kept in the dark at 5, 30, 50, and 70 ºC. Samples were withdrawn periodically, at intervals of 1, 2, 6, and 9 h during the first stages of the study and then after 12 or 24 h at the final stages of the experimental procedure. Each sample was used once and then discarded. All of the samples were filtered through a PTFE filter of 0.45 μm before analysis.

PI. A reflux heating at about 100 ºC was applied to PI in order to simulate thermal treatment when cooking. The picrocrocin and safranal contents were monitored every hour for 6 h.

All experiments were conducted in duplicate extracts.

Chemical Analyses

Spectrophotometric analysis. Changes in specific spectral characteristics of NFS, FS and PI were monitored periodically by scanning from 190 to 700 nm using a spectrophotometer Perkin-Elmer Lambda 25 (Norwalk, CT). $E_{1cm}^{1\%}$ 440 nm, $E_{1cm}^{1\%}$ at 257 nm and $E_{1cm}^{1\%}$ at 330 nm values were calculated according to (*31*) on a dry weight basis. Triplicate measurements for every sample at each time point were taken.

RP-HPLC analysis. Simultaneously to spectrophotometric analysis, gallic acid was added to the sample up to a concentration of 10 mg L^{-1} and 20 μL of the mixture were injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm

× 4.6 mm i.d., 5 µm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column thermostated at 30 °C. The addition of gallic acid just before the injection was used to check the correct functioning of the HPLC system. Crocetin esters were eluted using a gradient system consisting of a mixture of water + 0.1% phosphoric acid (A) and acetonitrile (B) [20% B, 0-5 min; 20-80% B, 5-15 min and 80% B, 15-20 min]. The flow rate was 0.8 mL min^{-1}. For each condition studied, duplicate extracts were prepared and each was chromatographed twice.

Stir bar sorptive extraction (SBSE). The picrocrocin solution was poured into a 10 mL volumetric flask. Compounds were extracted by introducing a polydimethylsiloxane coated stir bar (0.5 mm film thickness, 10 mm length, Twister, Gerstel GmbH, Mülheim and der Ruhr, Germany) into the sample by stirring (700 rpm) at room temperature for 60 min. The stir bar was then removed from the sample, rinsed with distilled water, dried with a cellulose tissue, and later transferred into a thermal desorption tube for TD-GC-MS analysis.

TD-GC-MS of safranal. A joined system made up of thermal desorption Perkin-Elmer ATD-400 equipment (Norwalk, CT, USA), a gas chromatograph HP-6890 and a mass spectrometer HP-5973 provided with a NIST library (Hewlett-Packard, Palo Alto, CA, USA) were used. The carrier gas was helium of chromatographic purity (220 kPa). In the thermal desorption tube, the volatile compounds were desorbed from the stir bar at the following conditions: oven temperature at 290 ºC; desorption time, 1 min; cold trap temperature, −30 ºC; helium inlet flow, 45 mL min^{-1}. The compounds were transferred into the Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard LC 3D mass detector (Palo Alto, USA) with a fused silica capillary column (BP21 stationary phase 50 m length, 0.22 mm i.d., and 0.25 µm film thickness) (SGE, Ringwood, Australia). The chromatographic program was set at 50 ºC (held for 2 min), raised to 230 ºC at 12 ºC min^{-1} and held for 20 min. For mass spectrometry analysis, electron impact mode (EI) at 70 eV was used. The mass range varied from 35 to 500 units, and the detector temperature was 150 ºC.

Identification and quantification of picrocrocin and safranal. Identification of picrocrocin by LC-DAD-MS was carried out as previously described (*33*). Three series of safranal standard solutions in 75% ethanol/water (v/v) with 0.01, 0.1, 0.25, 0.5 and 1 mg L^{-1} concentration were prepared and analyzed by TD-GC-MS after the stir bar sorptive extraction described above. A calibration curve was established for the series of safranal standards as a function of safranal's peak area.

Kinetic Parameters. The kinetic parameters, i.e. reaction order, rate constants (k), and half-life periods ($t_{1/2}$) were obtained using the integral method (*34*). Absolute temperature (T) dependence of the degradation rate constant was determined by the Arrhenius equation:

$$\ln k = \ln \mathrm{A} - \frac{\mathrm{Ea}}{RT} \qquad (1)$$

where R is the gas constant, Ea is the activation energy and A is the preexponential factor. Therefore, Ea was estimated on the basis of linear regression analysis of ln k versus T^{1}.

Thermodynamic parameters. According to the activated complex theory, the enthalpy and entropy of activation (ΔH^* and ΔS^*) were determined by the equation:

$$\ln k = \ln \frac{k_B T}{h} - \frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R} \qquad (2)$$

where k is the degradation constant at temperature T, k_B is the Boltzmann constant, and h is the Planck constant.

Sensory Analysis. Picrocrocin detection threshold, the minimum concentration of picrocrocin needed to give rise to a taste sensation, was determined by means of the triangle test according to ISO 4120 (*35*). Aqueous solutions of purified picrocrocin in bottled water (Bezoya; very low mineralization, 26 mg L^{-1}) were presented in order of increasing concentrations: 2.5, 5, 10 and 15 mg L^{-1} to a semi-trained panel of 24 subjects (16 women and eight men, ages 25-50 years, with no history of known taste disorders) using the sip-and-spit method. The semi-training sessions included familiarizing the

Table 1. Saffron Composition in Picrocrocin and Crocetin Esters, Spectral Characteristics Retention Times (t_R) and Relative Retention (r)

Compound	% on a dry basis[a]	UV-vis λmax (nm)	t_R (min)	r (min)
Picrocrocin	19.69 ± 2.35	250	5.8	3.0
trans-5-tG	0.36 ± 0.01	263, 443, 467	9.6	5.6
trans-5-nG	0.38 ± 0.01	263, 422sh, 440, 467sh	10.0	5.8
trans-4-GG	15.72 ± 0.03	262, 442, 465	10.3	6.0
trans-3-Gg	7.64 ± 0.04	262, 441, 465	10.9	6.4
trans-2-gg	0.60 ± 0.02	261, 439, 464	11.4	6.8
trans-2-G	0.98 ± 0.02	259, 434, 459	12.4	7.5
trans-1-g	0.09 ± 0.01	257, 434, 459	13.4	8.1
cis-4-GG	0.48 ± 0.03	262, 327, 435, 458	12.0	7.2
cis-3-Gg	1.32 ± 0.06	262, 325, 434, 458	12.6	7.6
total crocetin glycosides	27.57 ± 0.28			

[a]Values are expressed as Mean ± Standard Deviation of two extracts conducted in duplicate (2 × 2n).

panelists with the assessment procedure and objective. Four equal series of six sets each were prepared and distributed at random among the sensory panelists. In each set, one or two out of the three samples randomly contained picrocrocin and the rest of the samples contained bottled water. The volume of each sample was 15 mL and the panelists were asked to identify the sample which was different from the others by tasting. Sensory analyses were performed in a sensory panel room at 22 ºC-25 ºC in two different sessions. Criteria for significant detection of picrocrocin were based on binomial distribution tables.

Statistics. Significant differences in obtained data were determined by analysis of variance (ANOVA) using the SPSS 15.0 for Windows (SPSS Inc.) statistical program.

Nomenclature for Crocetin Esters. Abbreviations were adopted from Carmona et al. (*33*): *trans*-5-tG, *trans*-crocetin (β-D-triglucosyl)-(β-D-gentiobiosyl) ester; *trans*-5-nG, *trans*-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester; *trans*-4-GG, *trans*-crocetin di-(β-D-gentiobiosyl) ester; *trans*-3-Gg, *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; *trans*-2-G, *trans*-crocetin (β-D-gentiobiosyl) ester; *trans*-2-gg, *trans* crocetin di-(β-D-glucosyl) ester; *trans*-1-g, *trans* crocetin (β-D-glucosyl) ester; *cis*-4-GG, *cis*-crocetin di-(β-D-gentiobiosyl) ester and *cis*-3-Gg, *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester.

RESULTS AND DISCUSSION

Saffron Quality Characteristics and Composition. Results of moisture and volatile matter content = 5.5%; coloring strength ($E_{1cm}^{1\%}$ 440 nm) = 261; $E_{1cm}^{1\%}$ 257 nm = 100 and $E_{1cm}^{1\%}$ at 330 nm = 29, indicated that the saffron belonged to the highest commercial category, category I, according to (*31*). **Table 1** displays the composition of the saffron in picrocrocin and crocetin esters. Respective maxima in the UV-vis region, t_R and r are also shown in this Table. Each compound was identified by LC-DAD-MS and the results (data not shown) were totally in agreement with those previously reported (*33*).

Changes in Picrocrocin Content. The results of the experiment with NFS showed certain similarities between the closest temperatures. In the light of these similarities, we decided to rule out 20, 35, 40 and 60 ºC in the thermal treatment of FS. The picrocrocin retained throughout the thermal treatment of NFS and FS is shown in **Figure 1**. For purposes of comparison, only the results for 5, 30, 50 and 70 ºC are displayed in this Figure. In NFS (**Figure 1A**), the picrocrocin went down slowly at 5, 30 and 50 ºC, whereas at 70 ºC the values fluctuated very close to the initial level and only sporadic increases lower than 10% were observed at 237 and 311 h. Nevertheless, after this time, a continuous decrease of the retained picrocrocin was also observed at 70 ºC. In FS (**Figure 1B**), all thermal treatments caused a drop in

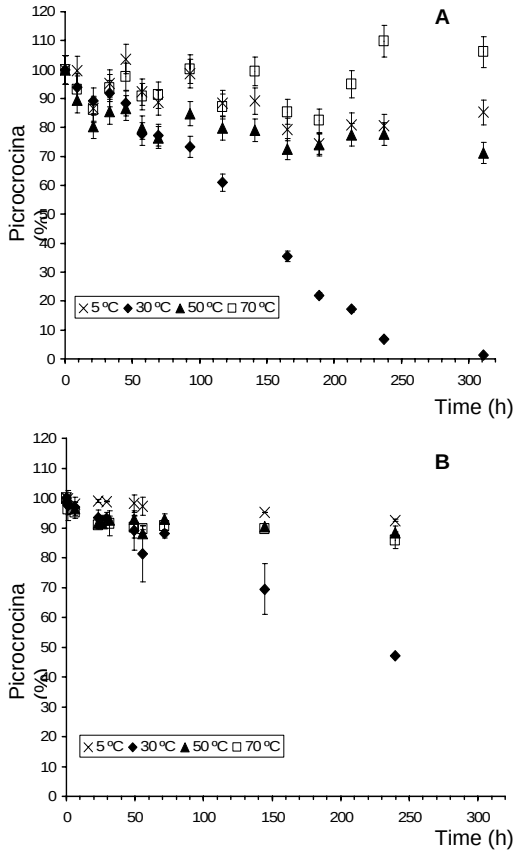

Figure 1. Percentage (mean value ± standard deviation) of picrocrocin retained throughout the thermal treatment of non filtered saffron aqueous extracts (A) and filtered saffron aqueous extracts (B).

picrocrocin, with the retained picrocrocin slightly greater than in NFS at all temperatures except 70 ºC.

In both kinds of extracts, NFS and FS, a more prominent decrease in picrocrocin was found at 30 ºC when compared to the other temperatures. Moreover, a complete loss of picrocrocin at about 311 h was observed in NFS and at about 461h in FS. From these results it may follow that the filtration process contributed to picrocrocin stability at all temperatures studied, except 70 ºC. These changes observed in picrocrocin content were in total agreement with our previously reported results on the evolution of the HPLC profile at 250 nm of aqueous extracts of saffron (*2*).

The next step was to study the effect of thermal treatment on picrocrocin after purification (PI). **Figure 2** shows the evolution of the percentage of picrocrocin retained throughout the thermal treatment at 100 ºC, and that of the absorbance at 250 nm. A progressive fall to just 66% at 6 h was found in the retained picrocrocin, providing evidence of the high stability of picrocrocin not only at low temperatures, as reported by Castellar et al. (*30*), but also at extreme temperatures. Taking into account that picrocrocin is responsible for the bitter taste of saffron, this finding means only a slight loss of this taste when the spice is submitted to a thermal cooking process. As regards the absorbance at 250 nm that was retained, significant differences with the picrocrocin retained were not found after 3 h of thermal treatment. The formation of degradation products could explain such later differences.

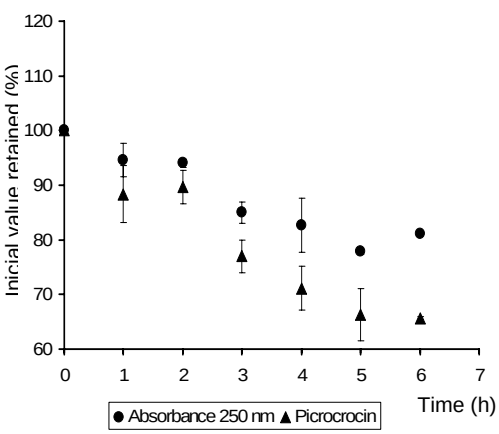

Figure 2. Percentage (mean value ± standard deviation) of picrocrocin and absorbance at 250 nm retained throughout the thermal treatment at 100 ºC of PI.

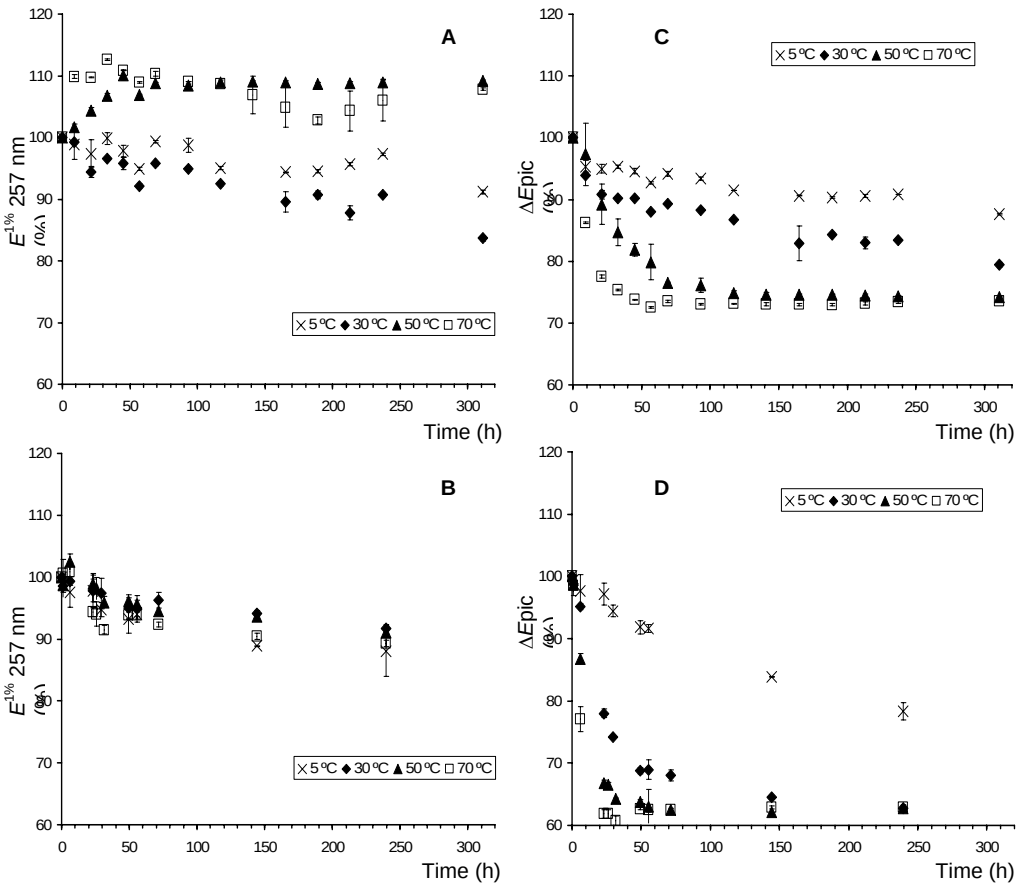

Figure 3. Percentage (mean value ± standard deviation) of $E^{1\%}_{1cm}$ 257 nm and Δ*E*pic retained throughout the thermal treatment of non filtered saffron aqueous extracts (A, C) and filtered saffron aqueous extracts (B, D).

Changes in the UV-vis data concerning picrocrocin: $E^{1\%}_{1cm}$ 257 nm and Δ*E*pic values. Despite the low selectivity of the parameters $E^{1\%}_{1cm}$ 257 nm and Δ*E*pic, it was interesting to follow their evolution in order to connect the results obtained with previous studies. **Figure 3** shows the evolution of $E^{1\%}_{1cm}$ 257 nm and Δ*E*pic values retained throughout different thermal treatments of NFS and FS as a percentage of their respective initial levels. With regard to $E^{1\%}_{1cm}$ 257 nm values, high stability was generally observed with decrements lower than 20% at about 300 h at all temperatures studied. This observation was in agreement with the previously reported data for saffron aqueous extracts, and contrasted to the pronounced decrease in $E^{1\%}_{1cm}$ 440 nm values due to the degradation of crocetin esters (*2, 23, 30*). In NFS (**Figure 3A)**, results for 5 and 30 ºC presented a slight decrease over time that was less prominent for the first temperature, whereas the results for 50 and 70 ºC showed an increase in $E^{1\%}_{1cm}$ 257 nm values in the first hours, which was faster at 70 ºC, and then remained constant with a value of about 110%. On the other hand, the

evolution of $E_{1cm}^{1\%}$ 257 nm values in FS (**Figure 3B**) was the same at all temperatures studied, with only slight differences among thermal treatments being found in some moments. Similar to the behavior at the two lowest temperatures in NFS, a progressive decrease in the $E_{1cm}^{1\%}$ 257 nm retained was observed in FS. Comparing the results for both, NFS and FS, a greater retention of $E_{1cm}^{1\%}$ 257 nm values for the former was evidenced at all temperatures studied, except for 30 ºC, in which case significant differences were not observed. It seemed that a filtration process reduced stability to a certain extent in the $E_{1cm}^{1\%}$ 257 nm value. This finding was in consonance with the reported effect of filtration on the loss of coloring strength (*2*), which was justified by a possible extraction of crocetin esters from the remaining vegetal material in NFS. Although similarities were found, especially in FS, the results demonstrated that $E_{1cm}^{1\%}$ 257 nm values do not reflect the evolution of picrocrocin.

The same occurred with the parameter Δ*E*pic, which evolved differently from picrocrocin and its changes were more marked as temperature increased (**Figures 3C** and **3D**). These results seemed to corroborate the effect of other components apart from picrocrocin in $E_{1cm}^{1\%}$ 257 nm and Δ*E*pic. The simultaneous changes happening in crocetin esters throughout the thermal treatment, and the possible presence of degradation products with absorbance at 257 nm could explain these discrepancies.

Kinetic and Thermodynamic Parameters. Prior to initiating the discussion of the results, it is necessary to point out that the majority of rate constants corresponded to degradation and have been expressed in **Tables 2-4** in absolute value, except when increases of the magnitude measured were observed. In such cases, the *k* values have been expressed with a minus sign.

Table 2 shows rate constants (*k*) and half-life periods ($t_{1/2}$) of picrocrocin, according to a zero, first and second-order kinetic models, in NFS. Up to 35 ºC the best fits were found for a zero-order model while at higher temperatures, data fitted better in a second-order model. However, R^2 values also showed good fits at 35 ºC in a first and second-order model and they were lower than 0.9 upon thermal treatments at 50-70 ºC. The *k* values increased as temperature increased from 5 to 35 ºC, but decreased from 40 to 60 ºC and the *k* value of picrocrocin at 70 ºC was higher than that of 60 ºC. Thus, the lowest $t_{1/2}$ values were obtained at about 35 ºC. These $t_{1/2}$ values ranged from 2776 h (60 ºC) to 128 h (35 ºC).

The *k* values and $t_{1/2}$ of $E_{1cm}^{1\%}$ 257 nm and Δ*E*pic loss are also shown in **Table 2**. When these *k* values were compared to the picrocrocin ones, it was found that the former were lower than the latter at temperatures below 50 ºC. Due to the fact that $E_{1cm}^{1\%}$ 257 nm and Δ*E*pic showed two stages in their kinetics at 50-70 ºC, the comparison of their *k* values and the picrocrocin one was not possible. In the case of $E_{1cm}^{1\%}$ 257 nm, the first of these two stages showed increases in its values, whereas Δ*E*pic presented a faster degradation in the first hours (first stage) followed by a slower one. We designated as "turning point" the time when regression lines of both stages intersected with each other (**Tables 2** and **3**). This point was lower at higher temperatures and was in agreement with the moment of almost total disappearance of the crocetin esters in the saffron extract.

The same parameters assessed in **Table 2** for NFS are presented in **Table 3** for FS. For picrocrocin degradation, all the R^2 values were ≥ 0.9, and there were low differences among reaction orders. The best fits of the data corresponded to a second-reaction model at 5 ºC, to a zero one at 30 ºC and none of the orders was dominant at 50 and 70ºC. With regard to $E_{1cm}^{1\%}$ 257 nm, worse fits were found to the reaction orders studied. However, Δ*E*pic also showed R^2 values > 0.9 in most cases.

Table 2. Rate Constants (k), Determination Coefficients (R^2) and Half-Life Periods ($t_{1/2}$) of $E_{1cm}^{1\%}$ at 257 nm, ΔEpic and Picrocrocin Degradation According to a Zero, First and Second-Order Kinetics, in Non Filtered Saffron Aqueous Axtracts (NFS) upon Thermal Treatment

Reaction order		*Picrocrocin*			$E_{1cm}^{1\%}$ at 257 nm				ΔEpic			
	T (ºC)	($k \pm$ SD)[a] × 10^3	R^2 (m)[b]	$t_{1/2}$ (h)	Turning point (h)[c]	($k \pm$ SD)[a] × 10^3	R^2 (m)[b]	$t_{1/2}$ (h)	Turning point (h)[c]	($k \pm$ SD)[a] × 10^3	R^2 (m)[b]	$t_{1/2}$ (h)
zero	5	60.9 ± 1.8	0.923 (20)	591		8.7 ± 0.4	0.875 (21)	4908		7.4 ± 0.4	0.896 (21)	4439
	20	162.2 ± 2.8	0.948 (18)	285		22.7 ± 0.9	0.975 (21)	1881		17.3 ± 1.0	0.967 (21)	1899
	30	259.5 ± 7.8	0.967 (15)	139		25.7 ± 2.2	0.981 (21)	1662		19.8 ± 1.2	0.972 (21)	1659
	35	280.7 ± 6.8	0.958 (13)	128		42.3 ± 1.7	0.978 (20)	1010		22.5 ± 1.4	0.903 (21)	1460
	40	148.6 ± 1.2	0.842 (14)	242		31.4 ± 1.6	0.823 (21)	1360		23.8 ± 1.4	0.821 (21)	1380
	50	39.0 ± 1.2	0.635 (16)	924	45	-188.6 ± 9.4	0.996 (5)		57	240.9 ± 14.5	0.908 (6)	136
						4.8 ± 0.2	0.919 (17)	8896		1.6	0.644 (16)	20530
	60	13.4 ± 0.6	0.644 (21)	2688	33	-266.7 ± 13.3	0.948 (4)		33	467.7 ± 28.1	0.897 (4)	70
						11.5 ± 0.6	0.969 (18)	3713		5.0	0.891 (18)	6570
	70	16.7 ± 0.5	0.712 (21)	2157	21	12.8 ± 0.6	0.874 (21)	3336	21	692.0 ± 41.6	0.956 (3)	47
										7.9	0.976 (19)	4158
first	5	1.64 ± 0.05	0.898 (21)	433		0.12 ± 0.01	0.887 (21)	5776		0.13 ± 0.01	0.924 (21)	5332
	20	5.00 ± 0.05	0.902 (19)	139		0.40 ± 0.02	0.967 (21)	1733		0.44 ± 0.03	0.941 (21)	1575
	30	4.19 ± 0.13	0.956 (10)	165		0.48 ± 0.02	0.987 (21)	1444		0.53 ± 0.03	0.979 (21)	1308
	35	6.91 ± 0.13	0.953 (10)	100		0.54 ± 0.03	0.959 (21)	1284		0.62 ± 0.04	0.976 (21)	1118
	40	3.53 ± 0.02	0.872 (14)	196		0.54 ± 0.04	0.878 (21)	1284		0.72 ± 0.02	0.931 (21)	963
	50	0.68 ± 0.02	0.676 (16)	990	45	-2.11 ± 0.11	0.997 (5)		57	4.21 ± 0.25	0.927 (6)	165
						0.05 ± 0.01	0.921 (17)	13863		0.03	0.654 (16)	23105
	60	0.25 ± 0.01	0.724 (21)	2773	33	-2.96 ± 0.15	0.942 (4)		33	8.20 ± 0.49	0.914 (4)	85
						0.14 ± 0.01	0.977 (18)	4951		0.12	0.889 (18)	5776
	70	0.33 ± 0.01	0.802 (21)	2310		0.16 ± 0.01	0.900 (21)	4332	21	11.97 ± 0.72	0.969 (3)	58
										0.19	0.977 (19)	3648
second	5	0.015 ± 0.001	0.787 (18)	694		0.002 ± 0.001	0.894 (21)	5854		0.002 ± 0.001	0.944 (21)	7611
	20	0.040 ± 0.002	0.811 (15)	347		0.008 ± 0.002	0.920 (21)	1464		0.012 ± 0.004	0.935 (21)	1268
	30	0.079 ± 0.003	0.932 (10)	174		0.006 ± 0.001	0.968 (19)	1951		0.016 ± 0.003	0.919 (21)	951
	35	0.172 ± 0.006	0.951 (10)	81		0.010 ± 0.002	0.976 (21)	1171		0.019 ± 0.005	0.986 (21)	801
	40	0.067 ± 0.002	0.910 (13)	207		0.010 ± 0.001	0.921 (21)	1171		0.030 ± 0.008	0.981 (21)	507
	50	0.012 ± 0.001	0.711 (16)	1388	45	-0.024 ± 0.003	0.998 (5)		57	0.074 ± 0.021	0.943 (6)	206
						0.001 ± 0.001	0.923 (17)	16727		0.001	0.664 (16)	21745
	60	0.005 ± 0.001	0.785 (21)	2776	33	-0.033 ± 0.002	0.935 (4)		33	0.145 ± 0.009	0.929 (4)	105
						0.002 ± 0.001	0.981 (18)	5854		0.003	0.883 (19)	5074
	70	0.007 ± 0.001	0.846 (21)	1983	21	-0.047 ± 0.004	0.665 (3)		21	0.208 ± 0.014	0.980 (3)	73
						0.002 ± 0.001	0.976 (19)	5854		0.005	0.969 (19)	3044

[a]Values are the means of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. Units of k corresponding to concentration in mg L^{-1} and time in h. A minus sign before k value means no degradation but increments of the measurements. [b]Minimum number of experimental data points. [c]Turning point: time when regression lines of two phases intersected with each other.

Table 3. Rate Constants (k), Determination Coefficients (R^2) and Half-Life Periods ($t_{1/2}$) of $E_{1cm}^{1\%}$ at 257 nm, ΔEpic and Picrocrocin Degradation According to a Zero, First and Second-Order Kinetics, in Filtered Saffron Aqueous Extracts (FS) upon Thermal Treatment

Reaction order	Picrocrocin					$E_{1cm}^{1\%}$ at 257 nm				ΔEpic			
	T (ºC)	Turning point (h)[a]	($k \pm$ SD)[b] × 10^3	R^2 (m)[c]	$t_{1/2}$ (h)	Turning point (h)[a]	($k \pm$ SD)[b] × 10^3	R^2 (m)[c]	$t_{1/2}$ (h)	Turning point (h)[a]	($k \pm$ SD)[b] × 10^3	R^2 (m)[c]	$t_{1/2}$ (h)
zero	5		20.1 ± 0.7	0.936 (20)	2413		117.4 ± 5.9	0.838 (15)	423		48.9 ± 1.9	0.948 (15)	812
	30		207.4 ± 5.0	0.996 (11)	233		78.3 ± 13.9	0.988 (16)	635	50	538.5 ± 20.6	0.923 (12)	74
											31.8 ± 9.1	0.997 (6)	1250
	50	23	306.5 ± 28.7	0.996 (7)	158		30.8 ± 1.5	0.801 (20)	1614		937.0 ± 17.7	0.958 (12)	42
			21.0 ± 1.1	0.925 (13)	2304								
	70	21	407.2 ± 8.3	0.991 (6)	119	32	274.8 ± 13.7	0.789 (12)	181		996.0 ± 13.0	0.893 (12)	40
			26.2 ± 1.3	0.899 (10)	1908		22.4 ± 1.1	0.821 (10)	2219				
first	5		0.22 a ± 0.01	0.945 (20)	3466		1.20 ± 0.06	0.838 (15)	578		0.69 ± 0.01	0.964 (15)	1005
	30		3.11 b ± 0.07	0.966 (12)	223		0.29 ± 0.01	0.839 (14)	2390	56	7.05 ± 0.08	0.953 (12)	98
											0.47 ± 0.02	0.928 (6)	1475
	50	23	3.66 ± 0.81	0.997 (7)	190		0.84 ± 0.04	0.720 (18)	825		14.51 ± 0.11	0.972 (11)	48
			0.25 ± 0.01	0.933 (14)	2773								
	70	21	4.40 ± 0.01	0.990 (6)	158		0.39 ± 0.02	0.469 (22)	1777		15.98 ± 0.04	0.918 (12)	43
			0.32 ± 0.02	0.912 (10)	2166								
second	5		0.003 ± 0.001	0.953 (20)	4133		0.006 ± 0.002	0.924 (15)	1829		0.010 ± 0.001	0.976 (15)	1326
	30		0.035 ± 0.005	0.965 (10)	295		0.007 ± 0.005	0.991 (16)	1547	56	0.107 ± 0.004	0.965 (12)	118
											0.008 ± 0.001	0.914 (7)	1575
	50	23	0.039 ± 0.008	0.997 (7)	265		0.003 ± 0.001	0.817 (20)	3353		0.228 ± 0.001	0.981 (12)	55
			0.003 ± 0.002	0.941 (15)	3444								
	70	21	0.048 ± 0.001	0.990 (6)	218	32	0.030 ± 0.002	0.771 (12)	335		0.261 ± 0.004	0.939 (12)	48
			0.004 ± 0.001	0.924 (10)	2501		0.003 ± 0.001	0.849 (11)	3353				

[a]Turning point: time when regression lines of two phases intersected with each other. [b]Values are the means of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. Units of k corresponding to concentration in mg L^{-1} and time in h. [c]Minimum number of experimental data points.

Table 4. Rate Constants (*k*), Determination Coefficients (R^2) and Half-Life Periods ($t_{1/2}$) of Picrocrocin Degradation According to a Zero, First and Second-Order Kinetics, in Water (PI) at 100 ºC

Reaction order	$(k \pm SD)^a \times 10^3$	R^2 (m)^b	$t_{1/2}$ (h)
zero	2794.0 ± 332.1	0.933 (6)	9
first	73.85 ± 2.79	0.932 (6)	9
second	1.99 ± 0.09	0.930 (6)	11

[a]Values are the means of two extracts conducted in duplicate (2 × 2n), SD = standard deviation. Units of *k* corresponding to concentration in mg L^{-1} and time in h. [b]Minimum number of experimental data points.

As with NFS for $E_{1cm}^{1\%}$ 257 nm and Δ*E*pic, a two-staged degradation was observed at 50 and 70 ºC. Comparing the *k* values at all studied temperatures and focusing the attention on the first stage at 50 and 70 ºC, an increment was noticed with temperature. In general, lower *k* values of picrocrocin were found in FS than in NFS (**Tables 2** and **3**). On the contrary, higher *k* values of $E_{1cm}^{1\%}$ 257 nm and Δ*E*pic were found after filtering the saffron extract, as previously reported for crocetin ester degradation at 50 and 70 ºC (*2*). The only study on aqueous extracts of saffron from the literature with k and $t_{1/2}$ values of a parameter related to picrocrocin (*25*), reported $t_{1/2}$ values of Δ*E*pic for a second-order kinetic model slightly lower at 20 ºC (391-480 h) than that reported in this study (1280 h), and of the same magnitude at 40 ºC (223-535 h versus 507 h).

Table 4 displays the kinetic parameters of PI at about 100 ºC according to a zero, first and second-order kinetics. Similar R^2 and $t_{1/2}$ values were found for the three reaction orders, the latter values ranging from 9 to 11 h and demonstrating a high resistance of picrocrocin to thermal treatment.

Due to the observed variation of *k* values with temperature we decided to study the activation energy (*E*a) in NFS and FS for a second-order model. The Arrhenius equation parameters were the following: a) for NFS, *E*a = 12.5 kJ mol^{-1}; Ln A = 16.2 (with A in L mg^{-1} h^{-1}); R^2 = 0.993 and b) for FS, *E*a = 38.3 kJ mol^{-1}; Ln A = 3.7 (with A in L mg^{-1} h^{-1}); R^2 = 0.992. As

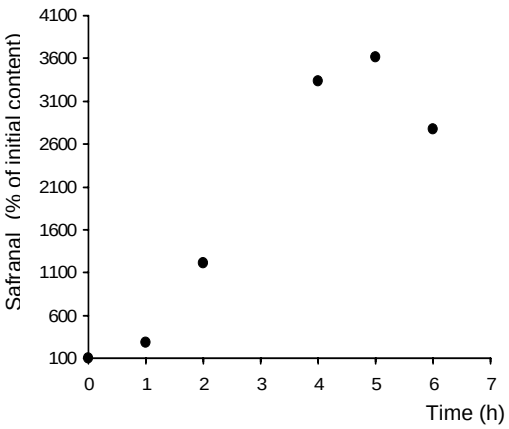

Figure 4. Evolution of safranal throughout the thermal treatment of PI at 100 ºC, expressed as percentage of its initial concentration.

previously described in crocetin esters (*2*), these results showed higher *E*a values in FS than in NFS. The interpretation is that changes in temperature modify the reaction rate constant more when the extract is filtered than when it remain unfiltered.

The variation of activation enthalpy and entropy (Δ*H** and Δ*S**) in NFS were 15.1 kJ mol^{-1} and 408.3 kJ mol^{-1} K^{-1}, respectively. Moreover, in FS these results were Δ*H** = 35.5 kJ mol^{-1} and Δ*S** = 243.0 kJ mol^{-1} K^{-1}.

Degradation Products: Safranal.

According to past studies on picrocrocin (*14-17*), its lost could generate HTCC and safranal. In fact, slight increases of a peak with t_R = 11.2 min (*r* = 6.7 min), which could be HTCC based on its UV-vis spectrum and retention time in comparison to the bibliography data (*17*, *30*), were observed in the chromatograms at all temperatures studied. However, our main research focused on safranal, the major compound in the volatile fraction of saffron. Picrocrocin (PI) was submitted to a strict thermal treatment at about 100 ºC and stir bar sorptive extraction (SBSE) and TD-GC-MS were used to determine safranal. The SBSE offers the advantages of low sample manipulation and great capacity of capturing volatiles at trace levels, and was assayed for

the first time in saffron matrixes by Carmona et al. (*4*), who determined safranal, among other volatile compounds. **Figure 4** shows the results of safranal evolution throughout the thermal treatment, confirming the generation of this volatile compound in the first 5 h. After this time, safranal decreased.

Picrocrocin Detection threshold. A triangle test with 24 assessors was conducted to evaluate whether a perceptible taste difference exists between water and picrocrocin solutions at different concentrations. With a picrocrocin solution of 15 mg L^{-1}, a total of 18 assessors, i.e. 75%, correctly identified the odd sample. According to (*35*), 18 correct responses indicated strong evidence (α = 0.001) that a perceptible difference existed. Evidently, the number of correct responses decreased along with picrocrocin concentration, which was 62.5%, 50% and 37.5% for 10, 5, and 2.5 mg L^{-1}, respectively. The proportion of correct responses with a picrocrocin solution of 10 mg L^{-1} indicated moderate evidence (α = 0.01) for the difference. Moreover, with 5 mg L^{-1}, slight evidence of the difference was found ($\alpha = 0.1$). On the contrary, significant differences were not found between the taste of water and that of a 2.5 mg L^{-1} solution of picrocrocin. The 90% confidence intervals (lower - upper confidence limit) assessed following the indications of (*35*) were 0.455 - 0.795 for samples containing 15 mg L^{-1} of picrocrocin; 0.248 - 0.627 for a 10 mg L^{-1} picrocrocin solution, and 0.54 - 0.446 for a picrocrocin concentration of 5 mg L^{-1}. In order to avoid the false conclusion that a difference exists, we proposed α= 0.01, and on the basis of these results the picrocrocin detection threshold was set at 10 mg L^{-1}.

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5.5. Determinación rápida de los ésteres de crocetina y de la picrocrocina del azafrán especia (*Crocus sativus* L.) mediante espectrofotometría UV–visible para el control de calidad

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Rapid Determination of Crocetin Esters and Picrocrocin from Saffron Spice (*Crocus sativus* L.) Using UV–Visible Spectrophotometry for Quality Control

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El objetivo de este trabajo fue desarrollar modelos multivariantes de aplicación al control de calidad del azafrán especia que predijesen el contenido de los principales ésteres de crocetina y de la picrocrocina a partir del espectro UV-vis de los extractos acuosos.

Este objetivo estaba motivado por las limitaciones de las determinaciones espectrofotométricas recogidas en ISO/TS 3632 (2003), que no permiten distinguir el contenido de cada uno de los ésteres de crocetina, ni evitan interferencias causadas por otros componentes del extracto con absorbancia a las longitudes de onda establecidas. Aunque existen numerosos métodos de análisis de los principales componentes del azafrán que utilizan HPLC y que han demostrado su capacidad de resolución, el problema es que, a diferencia de lo que ocurre con los espectrofotómetros, pocas empresas disponen de un cromatógrafo de líquidos.

Por su potencial en el control de calidad de gran número de muestras de forma rápida y sencilla, sin necesidad de nuevos instrumentos, el desarrollo de modelos

multivariantes suponía la creación de herramientas de gran interés para el control de calidad del azafrán en las empresas del sector. Los resultados obtenidos demostraron la validez de tales modelos para la determinación de los principales componentes de la especia.

En total se obtuvieron siete modelos PLSR1 para la predicción de los contenidos de *trans*-4-GG, *trans*-3-Gg, *cis*-4-GG, *cis*-3-Gg, *trans*-2-G, la suma de todos esos ésteres de crocetina y la picrocrocina. También se generó con anterioridad a ellos un modelo PLSR2 para todos esos componentes, pero los resultados no fueron satisfactorios y se decidió entonces desarrollar un modelo PLSR1 para cada uno de ellos. Tales modelos se generaron a partir de 61 muestras representativas del azafrán disponible en el mercado español durante una determinada cosecha, la mayoría de las cuales pertenecían a la máxima categoría, categoría I, establecida por ISO/TS 3632 (2003).

La matriz de variables predictoras, X, estaba formada por los espectros UV-vis entre 190 y 700 nm de los extractos acuosos de las muestras, mientras que la matriz de variables respuesta, Y, estaba constituida por los contenidos en base seca de cada uno de los compuestos estudiados y de la suma de los ésteres de crocetina, previamente determinados por HPLC.

Se encontraron contenidos de *trans*-4-GG, *trans*-3-Gg y de picrocrocina mayores que los descritos por Alonso y col. (2001a), del orden de tres veces mayores, pero los contenidos de *cis*-4-GG y *cis*-3-Gg fueron muy similares. Los tres ésteres mayoritarios fueron el *trans*-4-GG, el *trans*-3-Gg y el *cis*-4-GG, representando entre los tres más del 95% del contenido total en ésteres de crocetina.

Antes de realizar la calibración multivariante, se estudiaron las correlaciones lineales entre los contenidos en los principales ésteres de crocetina, la suma de los mismos, el contenido en picrocrocina, el poder colorante ($E_{1cm}^{1\%}$ 440 nm), $E_{1cm}^{1\%}$ 257 nm

y $E_{1cm}^{1\%}$ 330 nm. Los mayores coeficientes de correlación de Pearson, *R*, se encontraron entre el poder colorante y los siguientes contenidos: contenido total de ésteres de crocetina (*R* = 0,994), % de *trans*-3-Gg (*R* = 0,929) y $E_{1cm}^{1\%}$ 257 nm (*R* = 0,925).

En problemas de calibración multivariante que implican matrices complejas puede ser difícil reproducir la variabilidad de la composición de las muestras reales a través del diseño óptimo de experimentos. En tales casos, se debe extraer del conjunto de muestras reales un conjunto de calibración que sea representativo. Además, para asegurar la calidad del modelo se han de seleccionar las muestras de validación. El muestreo aleatorio es una técnica muy utilizada por su sencillez y porque un grupo de datos extraídos al azar de un conjunto mayor, sigue la distribución estadística de todo el conjunto. Sin embargo, el muestreo aleatorio no garantiza la representatividad del grupo, ni tampoco impide los problemas al extrapolar. La alternativa al muestreo aleatorio por la que optamos para este artículo fue el algoritmo de Kennard-Stone (Kennard y Stone, 1969; Kanduc y col., 2003). Dicho algoritmo abarca el espacio multidimensional de un modo uniforme, maximizando las distancias euclídeas entre los vectores de las respuestas instrumentales de las muestras seleccionadas. Utilizamos el algoritmo de Kennard-Stone con algunas modificaciones (Galvao y col., 2005) para separar las muestras en los conjuntos de calibración y de validación.

En los modelos que se construyeron con 11 muestras en la predicción y con conjuntos de calibración y de validación formados por 40 y 10 muestras respectivamente, los valores del RMSEP, error estándar de la predicción de muestras desconocidas, fueron suficientemente pequeños para la aplicación de los modelos en la práctica. En todos los modelos, excepto en el de la picrocrocina y en el del *cis*-3-Gg, los valores del RMSEP fueron menores o similares a los del error estándar de calibración, RMSEC, mostrando la robustez y capacidad de predicción.

En el modelo PLSR definitivo para el *trans*-4-GG (con 48 muestras en el conjunto de calibración y 13 en el de validación), la dimensión óptima determinada con

el mínimo error estándar en la validación, RMSEV, fue dos. Esas dos componentes principales explicaban el 97,8% de la variabilidad de X (información del espectro UV-vis) y el 87,9% de la variabilidad de Y (contenido en *trans*-4-GG). Las longitudes de onda que presentaron mayores coeficientes de regresión en valor absoluto fueron 475, 328 y 255 nm y no coincidían con los máximos en el espectro UV-vis del *trans*-4-GG. Destacaba la importancia que dio el modelo a la región en torno a 330 nm, a pesar de que el espectro UV-vis del *trans*-4-GG no tiene un máximo ahí. En esta zona, a diferencia del resto, los coeficientes de regresión eran negativos, indicando que cuanto menor es la absorbancia en el espectro en torno a 330 nm y mayor es en el resto del espectro, mayor es el contenido en *trans*-4-GG del extracto. La bondad del ajuste de este modelo, junto con la del resto de modelos construidos para los ésteres de crocetina, se presentó en la Figura 4 del artículo. Por último respecto a este modelo, decir que se obtuvieron buenos resultados para el RMSE, siendo 0,824 en calibración (RMSEC) y 0,470 en validación (RMSEV).

El modelo PLSR generado para el *trans*-3-Gg presentaba sólo una variable latente con capacidad de explicar el 96,4% de la variabilidad del conjunto de datos espectrales y el 86,2% de la variabilidad del contenido en *trans*-3-Gg. En cuanto a las longitudes de onda con mayores coeficientes de regresión, éstas estaban muy próximas a los máximos del espectro UV-vis del *trans*-3-Gg y fueron 444, 460 y 255 nm. A diferencia del modelo anteriormente descrito para el *trans*-4-GG, en este modelo la región en torno a 330 nm no fue apenas tenida en consideración. El RMSEC fue de 0,568 y el RMSEV fue de 0,452.

El número óptimo de componentes principales del modelo construido para el *cis*-4-GG fue cinco, las cuales explicaban el 99,8% de la variabilidad de X y el 74,6% de la de Y. Este resultado fue razonable ya que el contenido en *cis*-4-GG era menor de diez veces el de *trans*-4-GG y menor de cinco veces el de *trans*-3-Gg. Su mayor coeficiente de variación pudo ser el responsable del mismo. De entre las longitudes de onda del espectro que mostraban mayores coeficientes de regresión (326, 363, 485 y 197

nm) sólo la de 326 nm coincidió con uno de los máximos del espectro UV-vis del *cis*-4-GG. Además, a diferencia de lo que ocurría en los dos modelos descritos con anterioridad, los coeficientes de regresión en torno a 330 nm fueron positivos. El RMSEC fue de 0,423 y el RMSEV fue de 0,254.

Respecto al modelo correspondiente al *cis*-3-Gg, sus nueve componentes principales eran capaces de explicar el 99,9% de X y el 90,0% de Y. Los mayores coeficientes de regresión positivos se encontraron a 327, 193 y 478 nm, mientras que los coeficientes negativos con mayores valores absolutos correspondieron a 198, 293 y 462 nm. Como en el modelo anteriormente descrito, los coeficientes en la zona en torno a 330 nm fueron positivos. El RMSEC fue de 0,100 y el RMSEV fue de 0,083.

No fue posible obtener un modelo tan bueno para el *trans*-2-G, con cinco componentes principales el modelo generado explicaba el 99,8% de la variabilidad de X pero sólo el 42,1% de la variabilidad de Y.

Para la suma de todos los ésteres de crocetina una componente principal fue capaz de explicar el 96,4% de la variabilidad de X y el 98,0 de la de Y. Las longitudes de onda con mayores coeficientes de regresión coincidieron con las obtenidas para el *trans*-3-Gg. Para este modelo, la región comprendida entre 280 y 360 nm no fue significativa. El RMSEC fue de 0,469 y el RMSEV fue de 0,339.

El número óptimo de componentes principales en el modelo de la picrocrocina fue cuatro y explicaban el 99,8% de la variabilidad de X y el 61,1% de la variabilidad de Y, siendo después del porcentaje de Y explicado para el *trans*-2-G el más bajo de todos los modelos generados. El máximo coeficiente de regresión se encontró a 253 nm, coincidiendo prácticamente con el máximo de absorción de la picrocrocina y la región comprendida entre 330 y 380 nm mostró las menores cargas con coeficientes de regresión negativos. El RMSEC fue de 3,405 y el RMSEV fue de 1,892. Los resultados para este modelo podrían estar relacionados con las interferencias debidas a la

absorbancia de otros componentes, como los ésteres de crocetina o los flavonoides, en la zona del espectro UV-vis próxima a 250 nm.

Si estos modelos, tras su apropiada calibración, se asociaran o incluyeran en el software del espectrofotómetro, permitirían a las pequeñas y medianas empresas envasadoras y comercializadoras del azafrán determinar de forma rápida el contenido de los principales compuestos, mejorando así el control de calidad de sus materias primas y productos sin tener que invertir en nuevos equipos de medida.

5.5. Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV-Visible spectrophotometry for quality control

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The aim of this work was the development of multivariate models able to determine the content of the main crocetin esters and picrocrocin from spectrophotometric data that could be used for routine quality control of saffron. These compounds were determined with HPLC in Spanish saffron, and their absorbance spectra from 190 to 700 nm were simultaneously monitored. Partial least-squares regression (PLSR) models have been obtained and applied to the determination of individual crocetin esters, to the sum of crocetin esters, and to picrocrocin. A modification of the Kennard−Stone algorithm was used to divide the pool of samples into calibration and validation subsets. The best predictions were obtained with the sum of crocetin esters model, followed by the model for *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester, *trans*-crocetin di-(β-D-gentiobiosyl) ester, and *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester, whereas the worst predictions were found with the picrocrocin and *trans*-crocetin (β-D-gentiobiosyl) ester models. These models may enhance quality control in saffron enterprises.

KEYWORDS: Saffron; partial least-squares regression (PLSR); crocetin esters; picrocrocin; UV–vis spectrophotometry

INTRODUCTION

Due to consumer demands and strict regulatory requirements for food quality and safety, quality control is of utmost importance in the entire food sector. Because spice commerce in general, and saffron (the dried stigmas of *Crocus sativus* L.) commerce in particular, are no exceptions to these controls, the development of simple and quick methods of quality control and the application of chemometric tools to treat spectroscopic information is essential for medium- and small-size companies that make up this sector. Moreover, their potential use in a large number of samples without the necessity of additional instruments is of special interest.

Current international research on saffron quality is basically centered on the characterization of its attributes and their subsequent deterioration (*1–4*), the detection and quantification of food and nonfood colorings (*5*), and the revision of the standards used to certify saffron in the international saffron trade (*6*). In recent years, there has also been a growing concern for guaranteeing and defending the quality of saffron historically produced in certain regions (*7–9*). Saffron quality in the food industry is and has been mainly determined by specifications recommended by the ISO 3632, the latest revision of which has given rise to the Technical Specification ISO 3632/TS (*6*). This classifies saffron into three categories with regard to a large number of physical and chemical parameters that define saffron quality: microscopic characteristics, presence of flower waste, moisture and volatile matter content, ash content, $E^{1\%}_{1\,cm}$ 257 nm, $E^{1\%}_{1\,cm}$ 330 nm, $E^{1\%}_{1\,cm}$ 440 nm (coloring strength), etc. These last three categories are historically related to the content of picrocrocin, safranal, and crocins, respectively, the compounds associated with saffron organoleptic characteristics. Picrocrocin [4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde] is considered to be a contributor to saffron's bitter taste, although other compounds such as kaempferols and picrocrocin-related ones with this organoleptic property have been characterized in saffron spice (*10, 11*). In the saffron volatile fraction, safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound, whereas the crocins make up a group of water-soluble carotenoids identified as glycosyl esters of crocetin (8,8′-diapo-Ψ,Ψ′-carotenedioic acid) with glucose, gentiobiose, neapolitanose, or triglucose sugar

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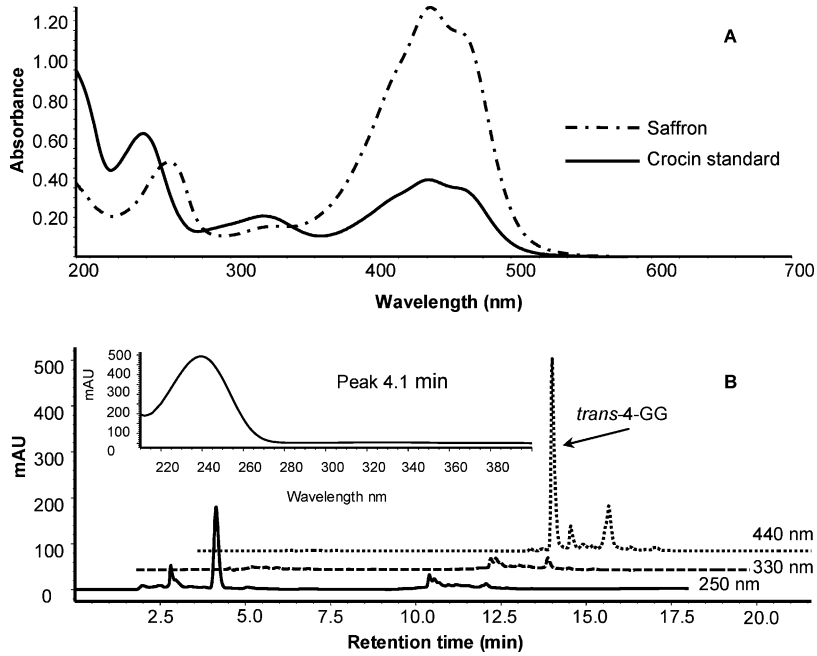


Figure 1. (**A**) UV–vis (— · —) spectrum of saffron spice from the DO "Azafrán de La Mancha", 50 mg/L in water compared to the corresponding one (—) of the commercial crocin standard, 50 mg/L in water (**A**). (**B**) Chromatograms of the commercial crocin standard and absorption spectrum (inset) of peak 4.1 min.

moieties (*1, 12*). The determination of these main characteristics of saffron by UV–vis spectrophotometry, according to ISO standards, leads to the existence of a spectrophotometer in almost all saffron companies. However, in the case of crocetin esters, this procedure by itself does not allow us to distinguish their detailed composition, that is, each *trans*-crocetin ester and each *cis*-crocetin ester. Up to now, other techniques such as thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) have been used to study these carotenoids as well as picrocrocin, with this last technique being considered to be the most effective one (*1, 2, 13, 14*). However, all of these methods are rather time-consuming and require equipment that is seldom found in small- and medium-size companies that process and package saffron spice. These methods are therefore limited to analytical or research laboratories and can hardly be used in a company to monitor raw materials, processes, or final products.

Quality differences found in saffron are due mainly to its drying process, although edaphoclimatic crop conditions, harvesting, stigma separation, handling, storing, and packaging also have an influence (*15–17*). Apart from the loss of humidity necessary to preserve the spice, drying brings about the physical, biochemical, and chemical changes needed to achieve the desired attributes and can therefore be considered the principal step in its manufacturing process. In this sense, Carmona et al. (*16, 18*) claim that temperature and rate during the drying process are factors that determine saffron's final characteristics. Pardo et al. (*19*) have studied the influence of the dehydration process on the sensory properties of saffron as well.

Despite their low selectivity, spectrophotometric measures have already been developed in other spices such as paprika and its oleoresins in order to establish simple quality criteria for the control of both raw material and finished product (*20*). In addition, multivariate chemometric methods currently play a very important role in the multicomponent analysis of mixtures with UV–vis spectrophotometry under computer-controlled instrumentation. Among the various chemometric approaches applied to multicomponent analysis, principal component regression (PCR) and partial least-squares regression (PLSR) have been successfully adopted in many fields of study, for example, quantitative assays of pharmaceutical formulations (*21*), simultaneous determination of dyes in mixtures (*22*), enology (*23*), and the olive oil (*24*) and alcohol industries (*25*). Numerous studies have also dealt with the possible presence of outliers, their treatment and alternatives for checking the validity of the calibration over time, or other perturbations (*25, 26*). Nevertheless, the studies devoted to the application of multivariate calibration to saffron spice are rather limited (*27, 28*).

The aim of this study was the development of multivariate models able to determine the content of the main crocetin esters and picrocrocin from spectrophotometric data that could then be used in saffron companies in routine quality control. A new method proposed by Galvao et al. (*29*), which is a modification of the Kennard−Stone algorithm (*30*), was used to divide the pool of samples into calibration and validation subsets.

MATERIALS AND METHODS

Samples, Chemicals, and Reagents. A total of $N = 61$ samples of Spanish saffron in filaments ($N_1 = 55$ samples) and powder ($N_2 = 6$ samples) were used in this study. Forty-five samples of the total belonged to the Protected Designation of Origin "Azafrán de La Mancha" and were obtained by means of its Regulatory Council. The remaining samples were directly obtained from the producers with the subsequent guarantee of their origin. The crocin [crocetin di-(β-D-gentiobiosyl) ester] was purchased from Fluka (Buchs, Switzerland). HPLC-grade acetonitrile and cyclohexane were from Merck (Steinhem, Germany). Ultrahigh-purity water was produced using a Millipore Milli-Q System (Bedford, MA); PTFE filters (11 mm, 0.45 μm) were also purchased from Millipore, and C_{18} packing material (125×10^{-8} cm pore size, 55–105 μm particle size) was from Waters (Milford, MA).

Experimental Measures. *Moisture and Volatile Matter Content.* These were determined by successive weighing of 1 g of powdered sample introduced into an oven set at 103 ± 2 °C for 16 h. They were calculated with the following ratio:

$$100 \times \frac{\text{initial mass} - \text{constant mass}}{\text{initial mass}} \quad (1)$$

UV–Vis Determinations. A Perkin-Elmer Lambda 25 (Norwalk, CT) spectrophotometer, accompanied by UV Winlab 2.85.04 software (Perkin-Elmer), was used for spectra recording and treatment at the following conditions: start wavelength, 700 nm; end wavelength, 190 nm; data interval, 1 nm; scan speed, 480 nm/min; 1.0 cm pathway quartz cells from Hellma (Jena, Germany). $E_{1\,\mathrm{cm}}^{1\%}$ 440 nm, $E_{1\,\mathrm{cm}}^{1\%}$ 330 nm, and $E_{1\,\mathrm{cm}}^{1\%}$ 257 nm were calculated according to ISO 3632/TS (*6*).

Identification and Analysis of Crocetin Esters and Picrocrocin by LC-DAD-MS. The identification of the main saffron components was carried out according to the method given in ref *12*, and the same procedure was followed for their analysis, the only difference being the sample injection volume (10 μL). The samples were injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) equipped with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column thermostaed at 30 °C. The solvents were water (acidified with 0.25% formic acid for identification) (A) and acetonitrile (B), and the gradient system was the following: 20% B, 0–5 min; 20–80% B, 5–15 min; and 80% B, 15–20 min. The flow rate was 0.8 mL/min.

Quantification of Crocetin Esters and Picrocrocin. The only crocetin ester from saffron available on the market is the crocetin di-(β-D-gentiobiosyl) ester, although it is not suitable for quantitative use in HPLC and UV–vis spectrophotometry because it contains considerable amounts of impurities or byproduct, as could be seen by its low $E_{1\,\mathrm{cm}}^{1\%}$ 440 nm, 79, versus 257 for saffron, and its chromatogram (**Figure 1**). Due to this lack of pure standards of each crocetin ester, quantification was based on the equation

$$\%\ \text{of ester } i \text{ on dry basis} = \frac{\mathrm{Mw}_i(E_{1\mathrm{cm}}^{1\%}\ 440\ \mathrm{nm})A_i}{10\epsilon_{t,c}} \quad (2)$$

where Mw_i stands for the molecular weight of the crocetin ester i, $E_{1\,\mathrm{cm}}^{1\%}$ 440 nm is the coloring strength, A_i is the percentage peak area of the crocetin ester i at 440 nm, and $\epsilon_{t,c}$ is the molecular coefficient absorbance value [89000 for *trans*-crocetin esters and 63350 for *cis*-crocetin esters (*31*)]. Data reported represent the average of three sample replicates.

Picrocrocin was isolated by column chromatography on C_{18} packing material. Removal of nonpolar compounds was achieved with 30 mL of cyclohexane added to 5 g of powdered saffron for 24 h at room temperature in the dark with sporadic agitation. The organic solvent was discarded, and the residue was dried under vacuum before the addition of 60 mL of water bubbled with nitrogen. The resulting solution was magnetically stirred for 1 h at room temperature in the dark. Then, the extract was centrifuged at 4000 rpm for 10 min, and the supernatant was collected and transferred to a plastic LC column (8 cm high × 2.7 cm i.d.) filled with the C_{18} packing material. Picrocrocin was eluted with 90 mL of 10% acetonitrile/water (v/v) after elution of flavonoids with 20 mL of 2% acetonitrile/water (v/v) and, finally, the solvent was eliminated to dryness by evaporation under vacuum. A calibration curve as a function of its peak area was constructed. The chromatographic purity of the picrocrocin obtained was calculated as the percentage of the total peak area at 250 nm.

Multivariate Calibration. The data were analyzed by means of multivariate techniques, applying the Unscrambler software, version 9.2 (CAMO Process AS, Oslo, Norway). One PLSR model for each saffron compound as well as for the sum of crocetin esters was built; the outliers were identified and eliminated for each model. The data matrix X was formed by the UV–vis spectra of the saffron aqueous extract, and the vector Y contained the reference values for the corresponding crocetin glycoside or picrocrocin content as the dependent variable. The variables were centered. A new method proposed by Galvao et al. (*29*), which is a modification of the Kennard–Stone algorithm (*30*), was used to divide the pool of samples into calibration and validation subsets for multivariate modeling. The Kennard–Stone algorithm follows a stepwise procedure in which new selections are taken in regions of the space far from the samples already selected. For this purpose, the algorithm employs the Euclidean distances $d_x(p,q)$ between the x-vectors of each pair (p,q) of samples calculated as

$$d_x(p,q) = \sqrt{\sum_{j=1}^{J} [x_p(j) - x_q(j)]^2};\ p,q \in [1,N] \quad (3)$$

where $x_p(j)$ and $x_q(j)$ are instrumental responses at the jth wavelength for samples p and q, respectively. J denotes the number of wavelengths in the spectra,and N is the number of samples. The modification proposed by Galvao et al. for sample set partitioning based on joint $x-y$ distances, SPXY (*29*), consists of augmenting the distance defined with a distance in the dependent variables, $d_y(p,q)$. This distance can be calculated for each pair of samples p and q as follows:

$$d_y(p,q) = \sqrt{\sum_{k=1}^{K} [y_p(k) - y_q(k)]^2};\ p,q \in [1,N] \quad (4)$$

In this paper, a normalized $x-y$ distance was calculated as

$$d_{xy}(p,q) = \frac{d_x(p,q)}{\max_{p,q} d_x(p,q)} + \frac{d_y(p,q)}{\max_{p,q} d_y(p,q)};\ p,q \in [1,N] \quad (5)$$

where $d_x(p,q)$ and $d_y(p,q)$ are divided by their maximum values in the data set in order to assign equal importance to the distribution of the samples in the x and y spaces. A stepwise selection procedure similar to the Kennard−Stone algorithm could then be applied with $d_{xy}(p,q)$ instead of $d_x(p,q)$ alone. A Matlab code (version 7.0) was used for implementation of this method. The division of the 61 samples into calibration, validation, and prediction sets was carried out in the following manner. Initially, 11 prediction samples were extracted from the full set in a random manner to simulate the analysis of a batch of real unknown samples. The remaining 50 samples were divided into calibration and validation sets of 40 and 10 samples, respectively, by using the SPXY algorithm. To improve the statistical significance of the comparison, the extraction of the samples was repeated five times.

The root-mean-square error (RMSE) was calculated with the expression

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{r} (\hat{y}_i - y_i)^2}{r}} \quad (6)$$

where $\hat{y}_i$ is the predicted concentration value of the ith calibration sample, y_i is its real concentration, and r is the number of samples in each case. RMSE was calculated in calibration (RMSEC), validation (RMSEV), and prediction (RMSEP). The number of principal components (PCs) in the PLSR models was determined by testing on the validation set. The minimum RMSEV determined the number of PCs. Three forms to identify outliers were used (*32*): data with extreme leverage, unmodeled residuals in spectral data, and unmodeled residuals in the dependent variable.

Finally, to increase the model's precision and to study the values of the regression coefficients, a global model for each variable was constructed with the SPXY method, 48 calibration samples, and 13 validation ones.

RESULTS AND DISCUSSION

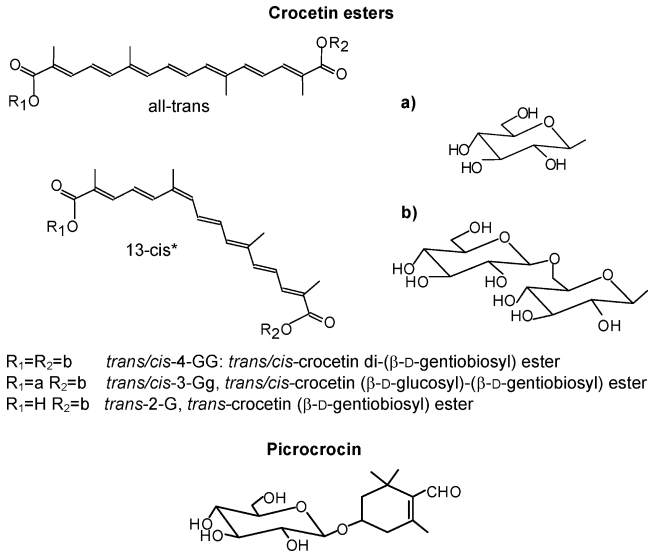
Experimental Measures. The majority of the samples, 55 of the total, fulfilled the ISO specifications for category I regarding moisture and volatile matter content, as well as the main characteristics using UV–vis spectrophotometry; only 4 samples belonged to category II and 2 to category III (**Table 1**). Such a distribution was representative of the saffron available in the Spanish market in a specific harvest, when saffron attains the highest category in most cases. **Table 1** also shows the composition in crocetin esters and picrocrocin expressed as a percentage of saffron on dry mass. The clear fragmentation patterns, retention times, UV–vis spectra,

Table 1. Classification of the Saffron Samples into ISO Categories and Saffron Sample Composition in Crocetin Esters and Picrocrocin Expressed as Grams of Compound per 100 g of Saffron on a Dry Basis

sample	ISO/TS 3632 category	*trans*-4-GG	*trans*-3-Gg	*trans*-2-G	*cis*-4-GG	*cis*-3-Gg	sum of crocetin esters	picrocrocin
M1	I	17.26	8.00	0.28	1.78	0.50	27.82	24.96
M2	I	17.41	7.35	1.04	0.72	0.31	26.83	26.57
M3	I	12.38	5.43	0.36	2.47	0.92	21.57	15.79
M4	I	12.03	5.60	0.57	1.53	0.64	20.38	12.66
M5	I	13.71	6.30	0.61	1.71	0.71	23.04	15.66
M6	I	13.85	5.70	0.56	1.37	0.62	22.10	14.81
M7	I	14.14	5.58	0.44	1.71	0.80	22.67	14.29
M10	III	8.37	3.14	0.23	1.06	0.35	13.15	6.91
M11	II	10.62	4.71	0.46	1.31	0.52	17.62	10.18
M12	III	7.45	2.49	0.19	1.03	0.41	11.57	5.08
M13	II	9.69	4.33	0.42	1.09	0.48	16.01	9.16
M17	I	15.55	7.37	0.58	0.87	0.00	24.37	20.97
M18	I	17.69	7.08	0.32	0.44	0.00	25.52	20.66
M19	I	15.56	6.92	0.35	0.42	0.31	23.56	16.08
M20	I	15.93	7.59	0.42	0.00	0.00	23.94	19.96
M21	I	16.34	7.86	0.64	0.00	0.00	24.84	19.44
M22	I	13.60	6.28	0.39	0.00	0.00	20.27	16.35
M23	II	11.83	4.98	0.29	0.25	0.00	17.36	14.96
M24	I	14.36	7.98	0.68	1.56	0.67	25.24	12.77
M25	I	14.63	7.31	0.58	1.56	0.72	24.80	11.98
M26	I	14.09	7.61	0.69	1.31	0.70	24.40	10.74
M27	I	13.09	7.30	0.68	1.20	0.67	22.94	11.31
M28	I	14.09	7.04	0.60	1.27	0.57	23.57	12.30
M29	I	14.53	7.50	0.64	1.80	0.86	25.32	12.42
M30	I	13.39	6.98	0.61	1.76	0.90	23.63	11.27
M31	I	14.36	7.43	0.65	1.39	0.66	24.49	12.21
M32	I	12.73	6.80	0.65	1.24	0.61	22.03	9.91
M33	I	13.48	6.52	0.50	1.51	0.71	22.73	12.28
M34	I	15.75	7.87	0.72	0.97	0.48	25.79	14.05
M35	I	13.75	6.89	0.61	0.90	0.43	22.60	12.44
M36	I	14.95	8.09	0.68	1.56	0.77	26.05	14.10
M37	I	13.42	7.21	0.59	1.34	0.65	23.21	12.55
M38	I	14.97	6.63	0.39	1.47	0.53	23.99	21.82
M39	I	15.53	7.34	0.35	1.86	0.70	25.77	22.28
M40	I	15.12	7.76	0.56	1.22	0.30	24.97	16.55
M41	I	12.97	5.62	0.19	3.15	1.14	23.07	9.84
M42	I	15.35	8.53	0.66	0.42	0.22	25.18	24.22
M43	I	14.20	6.37	0.25	0.92	0.44	22.18	16.05
M44	I	16.54	7.80	0.50	1.73	0.63	27.20	26.00
M45	I	14.83	7.69	0.42	2.01	0.78	25.73	16.51
M46	I	15.24	9.59	0.80	0.94	0.60	27.16	21.48
M47	I	17.38	9.61	0.74	0.57	0.29	28.59	17.25
M48	I	13.02	6.56	0.27	3.13	1.18	24.17	23.50
M49	I	14.06	9.35	0.67	1.00	0.60	25.68	25.76
M50	I	14.00	6.47	0.31	3.26	1.13	25.16	11.02
M51	I	14.40	7.40	0.29	0.85	0.60	23.52	10.55
M52	I	14.95	8.98	0.63	1.23	0.62	26.41	10.61
M53	I	15.27	7.35	0.37	2.04	0.76	25.79	12.52
M54	I	14.03	7.63	0.45	1.73	0.69	24.52	10.44
M55	I	13.71	8.17	0.48	1.54	0.82	24.71	15.70
M56	I	14.88	6.94	0.28	1.74	0.74	24.59	13.82
M57	I	15.26	8.00	0.48	1.32	0.48	25.53	20.36
M58	I	17.63	8.35	0.73	0.00	0.00	26.72	15.26
M59	I	19.03	7.93	0.43	0.00	0.00	27.39	17.95
M60	I	14.00	4.47	0.11	2.94	0.91	22.42	14.92
M61	I	17.81	7.66	0.38	0.47	0.29	26.61	13.64
M62	I	18.75	7.87	0.24	0.21	0.33	27.41	17.06
M63	I	15.85	7.23	0.31	0.54	0.38	24.30	13.14
M64	II	11.98	4.08	0.12	1.18	0.32	17.69	10.16
M65	I	16.65	7.55	0.40	0.56	0.52	25.68	20.50
M66	I	17.54	7.15	0.33	0.00	0.00	25.01	26.10

and their comparison with previous papers (*12, 28, 33*) allowed the identification of five crocetin esters and picrocrocin (**Figure 2**). To abbreviate the names of crocetin esters, the nomenclature used by Carmona et al. (*12*) has been adopted: first, the reference to the isomeric cis and trans forms has been written with a hyphen separating the total number of glucose moieties at both extremes of the base molecule. Then, the glucose moiety distribution has been indicated as (t) triglucoside, (n) neapolitanoside, (G) gentiobioside, or (g) glucoside. The name of the base structure, crocetin esters, was removed, because it is the same in all compounds.

The three major crocetin esters were, in decreasing order of mean values, *trans*-4-GG > *trans*-3-Gg > *cis*-4-GG. It is noteworthy that the first one alone was found to represent >60% of the total crocetin ester content of the aqueous saffron extract, whereas these three accounted for >95% of the total esters



R_1=R_2=b *trans/cis*-4-GG: *trans/cis*-crocetin di-(β-D-gentiobiosyl) ester
R_1=a R_2=b *trans/cis*-3-Gg, *trans/cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester
R_1=H R_2=b *trans*-2-G, *trans*-crocetin (β-D-gentiobiosyl) ester

Figure 2. Structures of saffron compounds under discussion. (*) In the case of crocetin esters with cis configuration, the position of the substituents R_1 and R_2 could not be exactly determined in relation to the C_{13-14} bond.

recorded. Despite calculating $E_{1\,\mathrm{cm}}^{1\%}$ at 330 nm for the classification of the samples into the ISO categories, safranal was not determined by HPLC due to its low water solubility.

The chromatographic purity of the picrocrocin obtained was 96%. The calibration curve of the picrocrocin concentration, *c* (mg/L), as a function of its peak area, *a*, exhibited good linear regression in the range of 2–315 mg/L: $c = 0.0290\,a + 0.5194$, *R* value = 0.999 for a total of six data points. The heterogeneity of saffron samples (of different coloring strengths and submitted to different dehydration processes), together with the lack of standards for each crocetin ester, has led to a wide range of results concerning the composition of saffron in the literature, making comparisons difficult. On comparing our composition results (**Table 1**) to those obtained by Alonso et al. (*2*) for Spanish saffron from the La Mancha region, we obtained higher contents of *trans*-4-GG, *trans*-3-Gg, and picrocrocin (approximately 3 times more) but very similar ones of *cis*-4-GG and *cis*-3-Gg. Contents of picrocrocin ranging from 0.79 to 13.9% have been previously reported (*2, 34*), even though we found samples up to 26.6% of picrocrocin.

Before multivariate calibration, linear correlations between the content expressed as percentage on a dry basis of the main *trans*- and *cis*-crocetin esters (*trans*-4-GG, *trans*-3-Gg, *trans*-2-G, *cis*-4-GG, and *cis*-3-Gg), the sum of crocetin esters, coloring strength ($E_{1\,\mathrm{cm}}^{1\%}$ 440 nm), $E_{1\,\mathrm{cm}}^{1\%}$ 257 nm, and $E_{1\,\mathrm{cm}}^{1\%}$ 330 nm were studied. The highest Pearson correlation coefficients, *R*, were found between the coloring strength and the following determinations: sum of crocetin esters ($R = 0.994$), % of *trans*-3-Gg ($R = 0.929$), and $E_{1\,\mathrm{cm}}^{1\%}$ 257 nm ($R = 0.925$).

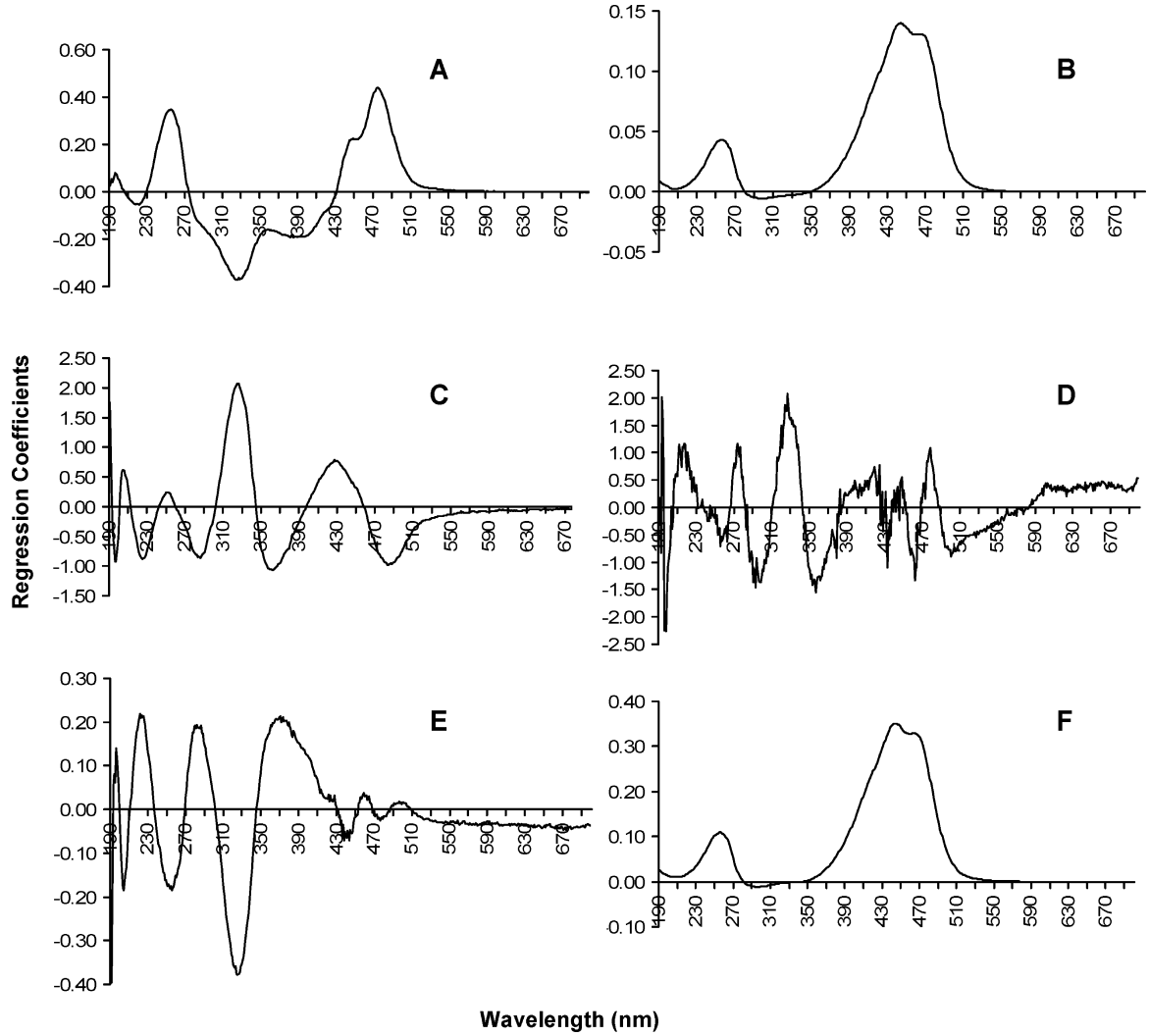


Figure 3. Regression coefficients of the PLSR models for *trans*-4-GG (**A**), *trans*-3-Gg (**B**), *cis*-4-GG (**C**), *cis*-3-Gg (**D**), *trans*-2-G (**E**), and the sum of crocetin esters (**F**).

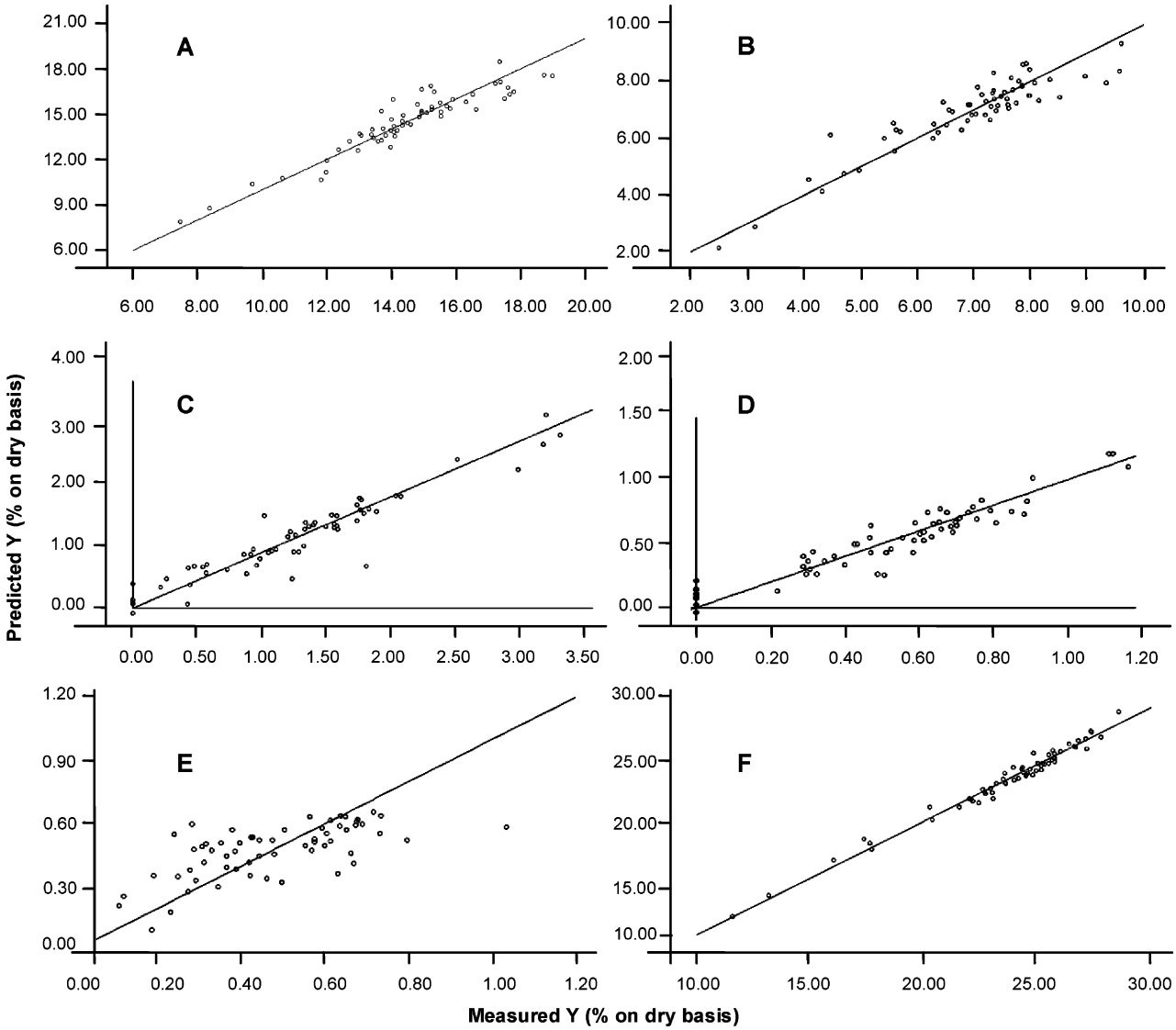


Figure 4. PLSR model predicted values of *trans*-4-GG (**A**), *trans*-3-Gg (**B**), *cis*-4-GG (**C**), *cis*-3-Gg (**D**), *trans*-2-G (**E**), and the sum of crocetin esters (**F**) versus measured ones.

Multivariate Calibration. *PLSR.* In multivariate calibration problems involving complex matrices, it can be difficult to reproduce the composition variability of real samples by means of optimized experimental design. In such cases, a representative calibration set must be extracted from a pool of real samples. Moreover, validation samples should also be selected to assess the quality of the model. Random sampling is a popular technique because of its simplicity and because a group of data randomly extracted from a larger set follows the statistical distribution of the entire set. However, random sampling does not guarantee the representativity of the set, nor does it prevent extrapolation problems. An alternative to random sampling is the Kennard−Stone algorithm (*30, 35*), which covers the multidimensional space in a uniform manner by maximizing the Euclidean distances between the instrumental response vectors of the selected samples. Therefore, this algorithm, with modifications, was used to split samples into calibration and validation sets.

First, an attempt was made to build a PLSR2, that is, a joint model for all of the compounds studied. Because the results were unsatisfactory, it was decided to develop a different PLSR1 model for each one. In the models built with 11 prediction samples and having calibration and validation data sets composed of 40 and 10 samples, respectively, the RMSEP values found, which represented the standard error of the prediction for unknown samples, were low enough for the models to be applied in practice. In all models, except for picrocrocin and *cis*-3Gg, the values of RMSEP were lower than or similar to RMSEC values. This proves the robustness and prediction capability of these models.

In the definitive PLSR model for *trans*-4-GG, the optimum model dimension determined by the minimum RMSEV was two. These two PCs accounted for 97.8% of the variability in the data set and 87.9% of the *trans*-4-GG composition variability (% explained *Y*). In **Figure 3A** the regions of the UV–vis spectrum with higher regression coefficients for this model, and therefore more informative, are shown. It was found that the wavelengths with the highest regression coefficients were at 475, 328, and 255 nm, not coinciding with the maxima of the *trans*-4-GG spectrum. It is quite interesting to point out that, although the spectrum of *trans*-4-GG does not have a maximum at 330 nm, the model gave importance to this region. Almost all of the regression coefficients except those for the region approximating 330 nm were positive. This means that the lower the absorbance is around 330 nm in the spectrum and the higher it is in the rest of the spectrum, the higher the content of *trans*-4-GG. **Figure 4A** shows the goodness of fit model for *trans*-4-GG, presented by plotting its predicted values versus its

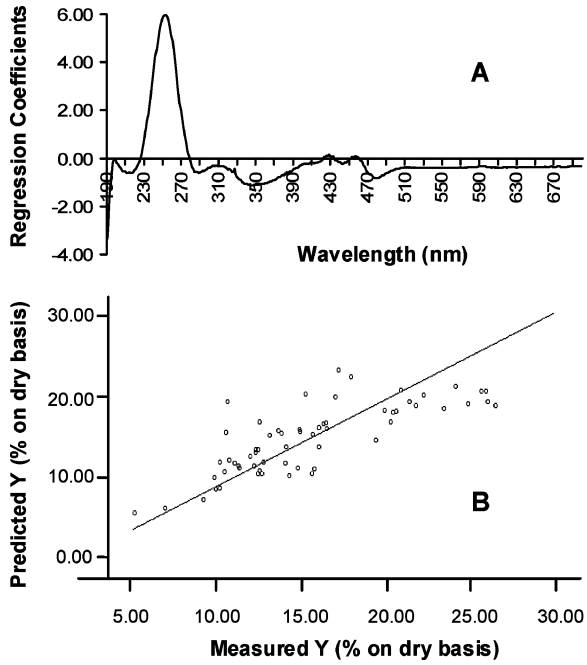


Figure 5. Regression coefficients (**A**) and predicted values of the PLSR model for picrocrocin versus measured ones (**B**).

measured ones. Good results were obtained for the RMSE, which was 0.824 in the calibration (RMSEC), and 0.470 in the validation (RMSEV).

The PLSR model for *trans*-3-Gg presented just one latent variable that accounted for 96.4% of the variability in the data set and 86.2% of the *trans*-3-Gg composition variability. In **Figure 3B** the regions of the UV–vis spectrum with higher regression coefficients are shown. The wavelengths with the highest regression coefficients were at 444, 460, and 255 nm, being very close to the maxima of the *trans*-3-Gg UV–vis spectrum. However, unlike the *trans*-4-GG model, the region around 330 nm was almost not taken into consideration. **Figure 4B** shows the predicted values for *trans*-3-Gg versus the measured ones. The RMSEC was 0.568, whereas RMSEV was 0.452.

The optimum number of PCs for the *cis*-4-GG PLSR model was five, with these accounting for 99.8% of the variability in the data set but explaining only 74.6% of the reference set. This result was understandable as the content in *cis*-4-GG was <10 times that of *trans*-4-GG and <5 times that of *trans*-3-Gg. Its higher variation coefficient could have been responsible for this behavior. In **Figure 3C**, the UV–vis regions with higher regression coefficients are shown. It was found that the wavelengths with the highest regression coefficients were at 326, 363, 485, and 197 nm. Only the maximum at 326 nm coincided with one maximum of the *cis*-4-GG UV–vis spectrum. Differing from the *trans*-4-GG and *trans*-3-Gg models, the correlation coefficients for the region around 330 nm were positive, being in accordance with the fact that *cis*-4-GG has a maximum at 327 nm. The RMSEC was 0.423, whereas the RMSEV was 0.254. **Figure 4C** shows the predicted values for *cis*-4-GG versus the measured ones.

In the PLSR model built for the content of *cis*-3-Gg, the number of PCs was nine, accounting for 99.9% of the variability in the data set, whereas the result for explained *Y* was 90.0%. **Figure 3D** shows that the highest positive regression coefficients were at 327, 193, and 478 nm, whereas the highest negative ones were at 198, 293, and 462 nm. As in the previous model explained, but different from the *trans*-4-GG and *trans*-3-Gg models, the correlation coefficients for the region around 330 nm were positive. **Figure 4D** shows the predicted values for *cis*-3-Gg versus the measured ones. The RMSEC was 0.100, whereas RMSEV was 0.083.

Despite being in the same magnitude order as *cis*-3-Gg, it was not possible to obtain such a good regression model for *trans*-2-G (**Figures 3E** and **4E**). With five PCs the model could explain 99.8% of *X* variability but only 42.1% of *Y* variability. When the model was generated from the sum of crocetin ester data, only one PC was found to explain 96.4% of the variability in the UV–vis spectrum variability and 98.0% of the crocetin ester composition variability. The wavelengths with the highest regression coefficients were exactly the same as those for *trans*-3-Gg (**Figure 3F**), being close to the maxima of the *trans*-crocetin ester spectrum. The region between 280 and 360 nm was not significant in this model. **Figure 4F** shows the predicted values for the sum of crocetin esters versus the measured ones. The RMSEC was 0.469, whereas the RMSEV was 0.339.

The optimum number of PCs in the model built for picrocrocin was four. The region of the saffron spectrum between 240 and 270 nm showed a maximum regression coefficient at 253 nm, practically coinciding with the maximum of the picrocrocin spectrum, whereas the 330–380 nm region showed the lowest weights and negative regression coefficients (**Figure 5A**). The percentage of the variability in the data set for this parameter was 99.8% but only 61.1% of the *Y* variability. **Figure 5B** shows the predicted values of the PLSR model for picrocrocin versus measured ones. Apart from the R^2 value of the *trans*-2-G, the R^2 value of picrocrocin model was the lowest among all of models herein described. The RMSEC was 3.405, whereas RMSEV was 1.892. This result gives evidence that the maximum at 257 nm of the saffron spectrum is due not only to picrocrocin but also to the possible interference of flavonoids and crocetin esters. Better results were reported for NIR by Zalacain et al. (*28*) in reference to the picrocrocin correlation model.

These models are really simple tools that, included or associated with spectrophotometer software, will afford small enterprises the opportunity to achieve quick monitoring of the quality of its products, with only appropriate and periodically checked calibration.

In conclusion, the PLS method based on spectrophotometric data has proved to be a valid tool for determining the main components of saffron spice. Seven PLS1 models have been obtained, and six of them were successfully applied to the determination of *trans*-4-Gg, *trans*-3-Gg, *cis*-4-GG, *cis*-3-Gg, sum of crocetin glycosides, and picrocrocin from saffron. The best predictions were obtained with the sum of the crocetin ester model, followed by the model corresponding to the *cis*-3-Gg, *trans*-4-GG, and *trans*-3-Gg, whereas the worst predictions were found with the picrocrocin and *trans*-2-G models. Nonetheless, with these models we managed to get better correlations with the detailed composition of saffron than by using the UV–vis parameters established by the ISO. These models may considerably enhance quality control in saffron enterprises without the liability of further investments in additional instruments, allowing a more efficient use of their spectrophotometers on a large number of samples.

ABBREVIATIONS USED

Abbreviations in nomenclature were adopted from Carmona et al. (*21*): *trans*-4-GG, *trans*-crocetin di-(β-D-gentiobiosyl) ester; *trans*-3-Gg, *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; *trans*-2-G, *trans*-crocetin (β-D-gentiobiosyl) ester; *cis*-

4-GG, *cis*-crocetin di-(β-D-gentiobiosyl) ester; *cis*-3-Gg, *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester.

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Supporting Information Available: Pearson correlation coefficients (R) between the different parameters studied; RMSE results obtained for PLSR prediction models of main saffron components with 11, 40, and 10 prediction, calibration, and validation samples, respectively; and principal characteristics of PLSR prediction models of the main saffron components with 48 and 13 calibration and validation samples, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

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Pearson Correlation Coefficients (R) between the Different Parameters Studied.

	$E^{1\%}_{1cm}$ at 257 nm	$E^{1\%}_{1cm}$ at 330 nm	$E^{1\%}_{1cm}$ at 440 nm	*trans*-4-GG	*trans*-3-Gg	*trans*-2-G	*cis*-4-GG	*cis*-3-Gg	Sum of crocetin esters	Picrocrocin
$E^{1\%}_{1cm}$ at 257 nm	1.000	-0.114[a]	0.925	0.896	0.852	0.294	-0.204[a]	-0.185[a]	0.916	0.712
$E^{1\%}_{1cm}$ at 330 nm	-0.114[a]	1.000	-0.096[a]	-0.287	-0.151[a]	-0.041[a]	0.686	0.676	-0.036[a]	-0.271
$E^{1\%}_{1cm}$ at 440 nm	0.925	-0.096[a]	1.000	0.904	0.929	0.445	-0.126[a]	-0.041[a]	0.994	0.567
trans-4-GG	0.896	-0.287	0.904	1.000	0.752	0.224	-0.351	-0.331	0.891	0.617
trans-3-Gg	0.852	-0.151[a]	0.929	0.752	1.000	0.565	-0.235[a]	-0.090[a]	0.899	0.490
trans-2-G	0.294	-0.041[a]	0.445	0.224[a]	0.565	1.000	-0.212[a]	-0.058[a]	0.393	0.158[a]
cis-4-GG	-0.204[a]	0.686	-0.126[a]	-0.351	-0.235[a]	-0.212[a]	1.000	0.909	-0.029[a]	-0.208[a]
cis-3-Gg	-0.185[a]	0.676	-0.041[a]	-0.331	-0.090[a]	-0.058[a]	0.909	1.000	0.042[a]	-0.285
Sum of crocetin esters	0.916	-0.036[a]	0.994	0.891	0.899	0.393	-0.029	0.042[a]	1.000	0.557
Picrocrocin	0.712	-0.271	0.567	0.617	0.490	0.158[a]	-0.208	-0.285	0.557	1.000

[a] No significative correlation α=0.05

RMSE Results Obtained for PLSR Prediction Models of Main Saffron Components with 11, 40 and 10 Prediction, Calibration and Validation Samples Respectively.

	RMSEC	RMSEV	RMSEP
trans-4-GG	0.815	0.547	0.578
trans-3-Gg	0.511	0.353	0.427
trans-2-G	0.151	0.119	0.159
cis-4-GG	0.445	0.238	0.274
cis-3-Gg	0.137	0.104	0.175
Sum of crocetin esters	0.501	0.265	0.297
Picrocrocin	3.183	2.703	3.764

Principal Characteristics of PLSR Prediction Models of Main Saffron Components with 48 and 13 Calibration and Validation Samples Respectively.

	Number of Pcs	% Explained X	% Explained Y	RMSEC	RMSEV
trans-4-GG	2	97.84	87.94	0.824	0.470
trans-3-Gg	1	96.37	86.21	0.568	0.452
trans-2-G	5	99.76	42.08	0.149	0.118
cis-4-GG	5	99.79	74.60	0.423	0.254
cis-3-Gg	9	99.95	90.07	0.100	0.083
Sum of crocetin esters	1	96.37	98.01	0.469	0.339
Picrocrocin	4	99.81	61.10	3.405	1.892

5.6. Extracción en fase sólida para la determinación de la picrocrocina en el control de calidad del azafrán especia (*Crocus sativus* L.)

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Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.)

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El objetivo de este trabajo fue estudiar la aplicación de extracción en fase sólida (SPE) para eliminar las interferencias de otros compuestos, principalmente ésteres de crocetina, en la determinación por espectrofotometría UV-vis del contenido de picrocrocina del azafrán especia. Se pretendía obtener un método que pudiera ser aplicado de forma rutinaria durante el control de calidad de la especia por lo que, además de utilizar espectrofotometría UV-vis, requería la utilización de pequeñas cantidades de muestra, rapidez y sencillez.

Se estudiaron y optimizaron algunos de los parámetros que influían en el procedimiento de SPE, como la concentración inicial del extracto, el volumen del mismo a introducir en el cartucho de SPE y los eluyentes necesarios para la separación de la picrocrocina. Además el método se diseñó de manera que pudiera realizarse a la vez que otras determinaciones generalizadas en el control de calidad, como la del poder colorante según ISO/TS 3632 (2003).

El método finalmente obtenido es el siguiente:

Preparación del extracto de azafrán

El extracto de azafrán se prepara tal y como propone la Especificación Técnica ISO/TS 3632 para la determinación de las principales características del azafrán.

- Se pesan 0,5 g de azafrán molido y tamizado y se echan en un matraz aforado de un L con aproximadamente 900 mL de agua destilada.
- Se mete un agitador magnético en el matraz y se agita durante una hora protegiéndolo de la luz.
- Pasada esa hora se saca el agitador magnético y se enrasa el matraz.

Si el procedimiento se realiza a la vez que la determinación del poder colorante, se puede utilizar el mismo extracto, con el consiguiente ahorro de tiempo y trabajo.

Acondicionamiento del cartucho C_{18}

Se utilizan los cartuchos C_{18} Sep-pak Plus de Waters.

- Se pasan por el cartucho 2 mL de acetonitrilo.
- Después se pasan 5 mL de agua destilada.
- Se vuelven a pasar 2 mL de acetonitrilo, seguidos de 5 mL agua destilada.

Separación de la picrocrocina

- Se toma, con una pipeta, 1 mL del sobrenadante del extracto que queda al centrifugar durante 5 minutos a 4000 rpm unos 25 mL del extracto preparado.
- Se pasa el mL lentamente por el cartucho a un flujo aproximado de 1 mL min^{-1}.
- Se lava el cartucho con 10 mL de agua destilada.
- Se eluye la picrocrocina con una disolución de acetonitrilo en agua al 12 % (v/v) recogiendo un volumen de eluído de 10 mL.

Además se pueden eluir los ésteres de crocetina con 10 mL de acetonitrilo.

Medida y cuantificación de la picrocrocina

- Se mide la absorbancia de la fracción recogida a 250 nm en una cubeta de 1 cm de paso óptico.
- Se calcula $E_{1cm}^{1\%}$ 250 nm.

Propusimos la expresión de los resultados como $E_{1cm}^{1\%}$ para el uso del método en el control de calidad porque es una forma con la que las empresas del sector que utilizan la normativa ISO están familiarizadas. Sin embargo, su conversión a % en masa seca de azafrán fue necesaria para comparar con los resultados obtenidos por HPLC. Esta conversión se realizó aplicando la siguiente ecuación:

$$\text{Picrocrocina (\% en masa seca)} = \frac{(E_{1\,cm}^{1\%}\,250\text{ nm}) \times Mr \times 10}{\varepsilon} \times F \qquad (1)$$

donde, la masa molecular relativa de la picrocrocina es Mr = 330, el coeficiente de extinción molar de la picrocrocina en acetonitrilo/agua 12% (v/v) a 250 nm es $\varepsilon = 9936$ L cm^{-1} mol^{-1} y F es un factor que considera la recuperación de la picrocrocina tras el proceso de SPE. Según los resultados de los estudios de recuperación que se comentarán más adelante, el valor de F fue de 1,1.

Se realizó una validación interna del procedimiento siguiendo las directivas de Eurachem (Eurachem, 1998) con 20 muestras procedentes de Grecia, Irán, Italia y España. De estas muestras, 17 cumplían los requerimientos de humedad y materia volátil, $E_{1cm}^{1\%}$ 440 nm, $E_{1cm}^{1\%}$ 330 nm y $E_{1cm}^{1\%}$ 257 nm establecidos para la categoría I de ISO, mientras que el resto pertenecían a categoría II. Los resultados indicaron que el método era selectivo, pues los cromatogramas a 250 nm de la fracción que contenía la picrocrocina prácticamente no presentaban más picos que el correspondiente a esta sustancia. También se obtuvieron buenos resultados ($R > 0.999$) para la linealidad del método desarrollado en el rango de trabajo, 60,00-0,60 mg L^{-1}. Los valores de

recuperación de picrocrocina tras el paso por el cartucho fueron del 89,8 ± 2,3% por lo que, como se ha mencionado anteriormente, se consideró F = 1,1 (Ecuación 1).

Se comprobó la exactitud del método determinando la proximidad entre los resultados del contenido de picrocrocina obtenidos por SPE y los determinados por HPLC mediante inyección directa del extracto de azafrán. Con la excepción de una de las muestras, no se encontraron diferencias significativas en la comparación de los valores medios obtenidos por ambos procedimientos mediante la t de Student. Además, los resultados de los dos procedimientos estaban correlacionados significativamente (R = 0,9866; α = 0,01). Se observó que $E_{1cm}^{1\%}$ 250 nm tenía mayor capacidad para ordenar las muestras por su mayor o menor contenido en picrocrocina que otros parámetros citados en la bibliografía por su relación con la picrocrocina como $E_{1cm}^{1\%}$ 257 nm o ΔEpic (ISO/TS 3632, 2003; Corradi y Micheli, 1979b).

Respecto a la precisión del método desarrollado, se obtuvieron valores satisfactorios de repetibilidad (RSD = 5,6%) y de precisión intermedia (RSD = 9,3%), siendo los límites de repetibilidad y reproducibilidad, expresados como % de picrocrocina en masa seca de azafrán, de L_r = 2,5 y L_R = 4,6 respectivamente. La sensibilidad del método, determinada a través de los límites de detección y cuantificación también era adecuada para su aplicación: LOD = 0,30 mg L^{-1} de picrocrocina (0,6% en masa seca de azafrán) y LOQ = 0,63 mg L^{-1} de picrocrocina (1,3% en masa seca de azafrán). Este último valor es aproximadamente 100 veces más pequeño que el presentado por Lechtenberg y col. (2008).

No se observaron diferencias en los resultados del método según el origen ni la categoría comercial del azafrán.

Los buenos resultados obtenidos con el método desarrollado, así como su sencillez y rapidez podrían justificar más estudios (como, por ejemplo, una validación externa) y su consideración cuando se aborden modificaciones de ISO 3632.

5.6. Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.)

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Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus* L.)

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Solid phase extraction for picrocrocin determination in the quality control of saffron spice (*Crocus sativus*, L.)

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Abstract

The application of solid phase extraction (SPE) in the determination of picrocrocin by UV-vis spectrophotometry has been studied in order to develop a rapid and low-cost method that can be used in the industry for routine quality control of saffron spice. Parameters influencing the SPE procedure, such as concentration of the initial extract, sample size and eluents, were studied and optimized. Twenty one different saffron spice samples from Greece, Iran, Italy and Spain were used in the intra-laboratory validation of the SPE method. The results indicated the selectivity, trueness, linearity, precision (repeatability: RSD < 6%, intermediate precision: RSD < 10%), good recovery (about 90%) and sensitivity (*LOD* = 0.30 mg L^{-1}; *LOQ* = 0.63 mg L^{-1}). The method also proved valid for overcoming the limitations of $E_{1cm}^{1\%}$ 257 nm due to crocetin esters in the determination of picrocrocin.

Keywords: Solid phase extraction (SPE); Picrocrocin; Crocetin esters; Saffron spice; UV-vis spectrophotometry; RP-HPLC

1. Introduction

Saffron spice, made up of the dried stigmas of *Crocus sativus* L., is chiefly used in food due to its colouring, flavouring and aromatic properties. Saffron quality in the international trade has been mainly determined by specifications recommended by the ISO 3632 standard, whose latest revision has given rise to the Technical Specification ISO/TS 3632 (2003). This classifies saffron into three categories with regard to a large number of physical and chemical parameters that define saffron quality: microscopic characteristics, presence of flower waste, moisture and volatile matter content, ash content, $E_{1cm}^{1\%}$ 440 nm (colouring strength), $E_{1cm}^{1\%}$ 330 nm, $E_{1cm}^{1\%}$ 257 nm, etc. These last three parameters are historically related to the content of crocetin esters, safranal and picrocrocin, respectively. The crocetin esters are a group of water-soluble carotenoids that derive from crocetin ($C_{20}H_{24}O_4$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid), where glucose, gentiobiose, neapolitanose or triglucose are the sugar moieties and where trans- or cis-configuration is found. This group of compounds is responsible for colour and colouring properties of saffron spice and represents between 0.5 and 32.4% of the dry basis of saffron spice (Alonso, Salinas, Garijo, & Sánchez, 2001). Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound in the volatile fraction of saffron representing around 70% (Carmona, Zalacain, Salinas, & Alonso, 2007; Tarantilis, & Polissiou, 1997; Rödel & Petrzika, 1991; Zarghami & Heinz, 1971). Picrocrocin (4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexane-1-carboxaldehyde), with reported contents in saffron spice from 0.8 to 26.6% on a dry basis (Iborra, Castellar, Canovas & Manjón, 1992; Alonso et al., 2001; Sánchez, Carmona, Zalacain, Carot, Jabaloyes & Alonso, 2008b), is considered responsible for saffron's bitter taste.

The systematic use of the specifications recommended by the ISO 3632 has managed to classify saffron in world trade by its colouring strength, provided that the remaining requirements are fulfilled. Consequently, this classification has led to the existence of a spectrophotometre in almost all saffron companies. The colouring strength is representative of the crocetin ester content. However, the determination of picrocrocin through the parameter $E_{1cm}^{1\%}$ 257 nm shows a problem of selectivity since other compounds of saffron extract, primarily crocetin esters, also

have absorbance at this wavelength due to the glycoside bonds, causing interferences in measurement (Sanchez et al., 2008b; Carmona, Zalacain, Sánchez, Novella, & Alonso, 2006; Orfanou, & Tsimidou, 1996; Tarantilis, Polissiou, & Manfait, 1994).

Up to now, other techniques such as thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) have been used to quantify picrocrocin, with this last technique considered as being the most effective (Tarantilis, Tsoupras, & Polissiou, 1995; Alonso et al., 2001; Lozano, Castellar, Simancas, & Iborra, 1999; Sujata, Ravishankar, & Venkataraman, 1992). Near-infrared (NIR) spectroscopy has also been applied to the determination of saffron chemical composition (Zalacain et al., 2005b). However, all of these methods can hardly be used for routine industrial work to monitor raw materials, processes or final products since they are time consuming and, in the case of HPLC or NIR spectroscopy, require equipment that is seldom found in small or medium-size companies that process and package saffron spice. Thus, there is a real interest in the development of rapid methods for routine quality control of saffron using UV-vis spectral information (Zalacain et al., 2005a; Sánchez et al., 2008b). Definitively, the main requirements that such methods should fulfil are the following: a) to overcome the limitations of $E_{1\text{cm}}^{1\%}$ 257 nm to determine picrocrocin from saffron by avoiding interferences from other saffron compounds, mainly crocetin esters; b) low cost, which first implies no investment in additional equipment and secondly, the use of small quantities of the sample; c) rapidity and straight-forwardness.

Solid phase extraction (SPE) is one of the most common and least expensive purification techniques and is considered as a convenient approach for sample preparation in food analysis. In the last few years much research has gone into SPE application to the analysis of major and minor components of foods (Grigoriadou, Androulaki, Psomiadou, & Tsimidou, 2007; Puoci, Curcio, Cirillo, Iemma, Spizzirri, & Picci, 2008). In saffron analysis, SPE is applied to the detection of adulterations by artificial colorants (ISO, 2003; Zalacain et al., 2005a). However, it has only been marginally applied to saffron component purification (Sujata et al., 1992; Escribano, Alonso, Coca-Prados, & Fernández, 1996; Carmona, Sánchez, Ferreres, Zalacain, Tomás-Barberán, & Alonso, 2007).

The purpose of this work was to study the application of SPE in order to avoid crocetin ester interferences in the determination of picrocrocin by UV-vis spectrophotometry during the routine quality control of saffron spice. Parameters influencing the SPE procedure such as concentration of the initial extract, sample size and eluents were studied and optimized. The SPE method was validated and applied to different saffron spice samples from Greece, Iran, Italy and Spain.

2. Materials and methods

2.1. Samples and chemicals

Twenty one saffron spice samples in filaments were used. These were obtained directly from the producers and packers with a guarantee of their origin and freedom from fraud (Table 1). They came from Greece, Iran, Italy and Spain, and were harvested during the years 2004, 2005 or 2006. All Spanish samples from the 2006 harvest were of the Protected Designation of Origin "Azafrán de La Mancha." Picrocrocin purified according to the method described below was used for calibration curves. C_{18} adsorbent (125×10^{-8} cm pore size, 55-105 μm particle size) for picrocrocin isolation was from Waters (Milford, MA). HPLC-grade acetonitrile and cyclohexane were used from Scharlau (Barcelona, Spain). Ultra high purity water was produced using a Milli-Q System from Millipore (Bedford, MA) and PTFE filters (11 mm, 0.45 μm) were also purchased from Millipore. C_{18} SPE cartridges (Sep-pak plus™, 125×10^{-8} cm pore size, 55-105 μm particle size, 360 mg sorbent weight) were supplied by Waters, (Milford, MA).

2.2. Picrocrocin isolation

Picrocrocin was extracted from saffron and isolated by column chromatography using a C_{18} adsorbent. For extraction, 30 mL of cyclohexane were added to 5 g of powdered saffron and the suspension was left in the dark at room temperature for 24 h with sporadic agitation. Then the organic solvent was discarded and the solid residue was dried under reduced pressure. Sixty mL of nitrogen-saturated water were added to the thus treated saffron and the resulting suspension was stirred for 1 h in the dark at room temperature. Then the extract was centrifuged at 4000 rpm for 10 min and the supernatant was collected and loaded onto the previously conditioned C_{18} column (8 cm high × 2.7 cm i.d.). Picrocrocin was eluted with 90 mL of 10% acetonitrile/water (v/v) after the elution of flavonoids with 20 mL of 2% acetonitrile/water

(v/v). Finally, the solvent was eliminated by evaporation to dryness under reduced pressure and the purified picrocrocin was kept at -20 ºC until its utilization. The chromatographic purity of the obtained picrocrocin was 96%, calculated as the percent of the total peak area at 250 nm.

2.3. Saffron extract preparation

Aqueous extracts of 2.5 g L^{-1} and 0.5 g L^{-1} concentration were prepared with ultra high purity water by stirring the suspension in the dark at room temperature for 1 h, as specified by the ISO (2003). The former were used in the study of the retention and elution of saffron components in the SPE cartridges, while the latter were used for the determination of the main characteristics of saffron and in the optimized SPE procedure. The extracts were centrifuged at 4000 rpm for 5 min before being loaded into the SPE cartridges.

2.4. Solid phase extraction procedure

The C_{18} SPE cartridges were conditioned with 2 mL of acetonitrile, followed by 5 mL of water, 2 mL of acetonitrile and 5 mL of water before the application of the saffron extract. Four mL of a saffron aqueous extract (2.5 g L^{-1}) were loaded into the SPE cartridge. Then, to study the retention and elution characteristics of the cartridge, this was washed successively with water, 5% and 15% acetonitrile/water (v/v). For each solvent, eluted fractions of 3 mL were collected and their absorbances at 250 and 440 nm were measured until they were nearly zero. SPE procedure was optimised with respect to sample size: 7 mL, 4 mL, 2 mL and 1 mL of a 0.5 g L^{-1} extract; and elution solvent: 5, 10, 12 and 15% of acetonitrile/water (v/v).

The optimised SPE procedure for picrocrocin purification was as follows: 1 mL of the 0.5 g L^{-1} saffron extract was added to the SPE cartridge, it was washed with 10 mL water, and then picrocrocin was eluted with acetonitrile/water 12% (v/v) up to collecting 10 mL into a volumetric flask being measured. Finally, to check the composition of all fractions, crocetin esters were eluted with 10 mL of acetonitrile.

2.5. Spectrophotometric analysis

Spectral characteristics of aqueous saffron extracts and eluted fractions were monitored by scanning from 190 to 700 nm using a Perkin-Elmer Lambda 25 spectrophotometer (Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin-Elmer). Colouring strength ($E_{1cm}^{1\%}$ 440 nm), $E_{1cm}^{1\%}$ 257 nm and $E_{1cm}^{1\%}$ 330 nm were determined according to ISO (2003). $E_{1cm}^{1\%}$ 250 nm was assessed from the absorbance at 250 nm of the fraction containing picrocrocin. Δ*E*pic was calculated from spectral data of saffron extracts as defined by Corradi and Micheli (1979):

$$\Delta Epic = E_{257}^{1\%_{000}} - E_{297}^{1\%_{000}} \quad (1)$$

All analyses were done in duplicate and two measurements were also taken for each replicate.

2.6. RP-HPLC-DAD analysis

Twenty μL of each sample (aqueous saffron extracts and eluted fractions) were injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) operating with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column, thermostated at 30 °C. Eluents were water (A) and acetonitrile (B) with the following gradient: 20% B, 0–5 min; 20–80% B, 5–15 min; 80% B, 15–20 min; 20% B, 20–30 min. The flow rate was 0.8 mL min^{-1}. The DAD detector (Hewlett Packard, Waldbronn, Germany) was set at 250 and 440 nm for picrocrocin and crocetin ester detection, respectively. All analyses were done in duplicate and two measurements were also taken for each replicate.

2.7. Calibration solutions of picrocrocin

From the purified picrocrocin, solutions of seven different concentrations: 0.51, 2.56, 5.12, 10.24, 25.60, 51.20 and 256.00 mg L^{-1} were prepared in water while solutions of six concentrations: 0.60, 3.00, 6.00, 12.00, 30.00 and 60.00 mg L^{-1} were prepared in acetonitrile/water 12% (v/v). Quantification of picrocrocin was accomplished with the use of the calibration curves calculated by linear regression analysis. Quantification of crocetin esters was done as previously reported by Sánchez, Carmona, Ordoudi, Tsimidou, & Alonso, G. L. (2008).

2.8. Validation of the SPE method

Intra-laboratory method validation was carried out according to Eurachem Guidelines (Eurachem, 1998). The selectivity of the method to determine picrocrocin was studied by measuring the SPE fraction that contained picrocrocin with HPLC and checking that no additional peaks appeared in the

chromatograms. Accuracy was studied as two components: trueness and precision. Trueness was assessed as the closeness of agreement between the average content of picrocrocin for the same samples obtained after SPE, and the reference value of picrocrocin content determined by direct injection of saffron extracts in the HPLC chromatograph. With regard to precision, two parameters were determined: repeatability and intermediate precision and they were stated in terms of relative standard deviation (RSD). Repeatability was checked for the sample numbered as 15 (Table 1) analysed five times by the same analyst within the same day. The repeatability limit, L_r, at the 95% confidence level was calculated as:

$$L_r = 1.96 \times \sqrt{2} \times \sigma_r \quad (2)$$

where σ_r is the standard deviation measured under repeatability conditions.

Intermediate precision was determined for the same saffron sample that was used to calculate repeatability. This sample was analysed by different analysts on three separate days. The reproducibility limit, L_R, at the 95% confidence level was calculated as:

$$L_R = 1.96 \times \sqrt{2} \times \sigma_R \quad (3)$$

where σ_R is the standard deviation measured under reproducibility conditions.

Besides sample 15, samples numbered as 1, 2, 9, 13 and 19 (Table 1) were used in the recovery study of picrocrocin in order to broaden the concentration range. Limit of detection (*LOD*) was calculated as the picrocrocin concentration corresponding to the mean value of 10 independent sample blanks measured once each plus three times their standard deviation. As it was not possible to have a sample of saffron without picrocrocin, water was used as a blank sample. Limit of quantification (*LOQ*) was calculated as the picrocrocin concentration corresponding to the mean value of 10 independent sample blanks measured once each, plus 10 times their standard deviation.

Linearity for both the UV-vis method and the HPLC one was determined by plotting signal response versus picrocrocin concentration in water and acetonitrile/water 12% v/v in the range of 256.00-0.51 mg L^{-1} using at least six levels of calibration.

Data were subjected to a comparison of means with a Student *t* test ($\alpha = 0.01$) and analysis of variance (ANOVA) using the SPSS 15.0 statistical program for Windows (SPSS Inc., IL, USA).

3. Results and discussion

3.1. Evaluation of SPE cartridge behaviour

A 2.5 g L^{-1} extract was prepared with the saffron sample numbered as 21 (Table 1) from the Designation of Origin "Azafrán de La Mancha". In Figure 1 the retention and elution characteristics of the SPE cartridges loading 4 mL of the saffron extract are shown. They were monitored at 250 and 440 nm because these are wavelengths close to picrocrocin and crocetin ester absorbance maxima, respectively. It was observed that very few substances were eluted with water. The compounds that absorb at 250 nm (Figure 1A) started to elute with acetonitrile/water 5% (v/v) and after 73 mL of this solvent, the absorbance of the eluted fractions was nearly zero, suggesting that most of picrocrocin had been eluted. An increase of absorbance at 250 nm followed by a steady decrease until reaching a plateau was observed when acetonitrile/water 15% (v/v) was added, showing the picrocrocin that remained in the cartridge. Nearly 200 mL of acetonitrile/water 15% (v/v) were necessary for complete elution of crocetin esters (Figure 1B). These results suggested a reduction of the extract concentration and a light increase in the proportion of acetonitrile for the elution of picrocrocin in order to use lower elution volumes and increase picrocrocin recovery, which are crucial factors for a rapid and low cost method.

3.2. Optimisation of the SPE procedure

The concentration of the extract to be loaded into the SPE cartridge was established as 0.5 g L^{-1} so that the same extract used in the determination of the colouring strength could be used in the proposed method, with the subsequent saving of time and work.

Regarding the sample size, the maximum one that the SPE cartridge was able to hold was 7 mL, but there was not a good separation of picrocrocin and crocetin esters. With 4 mL the separation improved, although too much eluent was still necessary to elute all the picrocrocin. Two and 1 mL showed the best results, and we decided to use the latter in order to shorten the time of analysis. A volume of 10 mL of water was sufficient for the clean-up step, while 10 mL of acetonitrile/water 12% (v/v) were necessary to manage the elution of picrocrocin without that of crocetin esters.

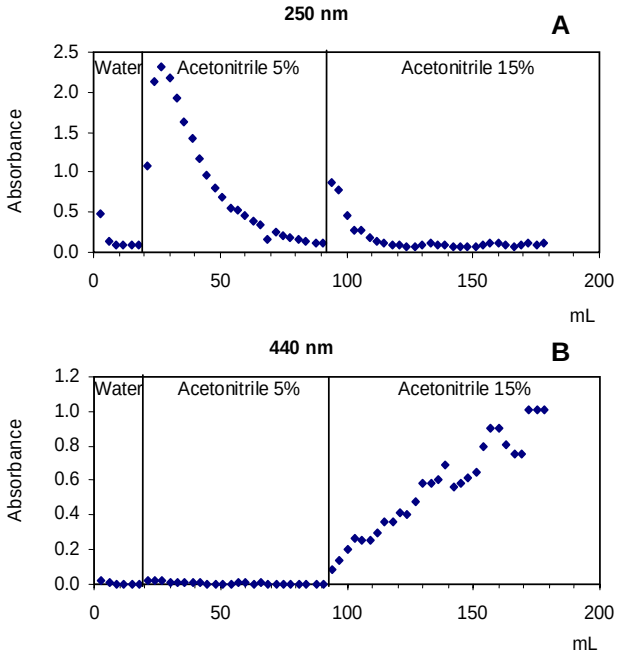

Figure 1. Retention and elution characteristics of the SPE cartridges. Absorbance at 250 nm (A) and 440 nm (B) measured in 3 mL fractions after loading 4 mL of a 2.5 g L^{-1} saffron extract.

Lower acetonitrile concentrations (5%) resulted in too many losses of picrocrocin in the SPE cartridge, as previously mentioned, while higher ones (15%) resulted in a contamination of the picrocrocin fraction with crocetin esters. In Figure 2 the UV-vis spectra of the two eluted fractions containing the picrocrocin and crocetin esters respectively are shown and compared to that of the saffron extract from which they came. As could be observed when comparing the spectrum of the picrocrocin fraction to that of crocetin esters, both crocetin esters and picrocrocin absorb at 257 nm. Consequently, absorbance of saffron extracts at this wavelength is influenced not only by picrocrocin content but also by crocetin ester composition.

Another point to be taken into account is that $E_{1cm}^{1\%}$ 440 nm and $E_{1cm}^{1\%}$ 257 nm are measured, according to ISO (2003), after a dilution 1/10 of a 0.5 g L^{-1} extract. Therefore, the 10 mL volume of the eluted picrocrocin fraction also supposed a dilution of 1/10 with respect to the loaded extract (1 mL). This allows for the use of the same factor which multiplies the corresponding absorbance to express the results of colouring strength ($E_{1cm}^{1\%}$ 440 nm), $E_{1cm}^{1\%}$ 257 nm and also the results obtained with the SPE method developed. Our proposal is to express the content of picrocrocin as $E_{1cm}^{1\%}$ 250 nm of the fraction in which it is contained, firstly because the companies are familiarized with the $E_{1cm}^{1\%}$, and secondly, because the conversion to % on a dry basis of saffron, once known the molar absorption coefficient, ε, of picrocrocin is immediate:

$$\%\ \text{of picrocrocin on dry basis} = \frac{(E_{1cm}^{1\%}\,250\,\text{nm}) \times \text{Mw} \times 10}{\varepsilon} \times \text{F} \qquad (4)$$

where Mw is the molecular weight of picrocrocin, and F is a factor included in order to correct the results according to the recovery of picrocrocin in the SPE procedure.

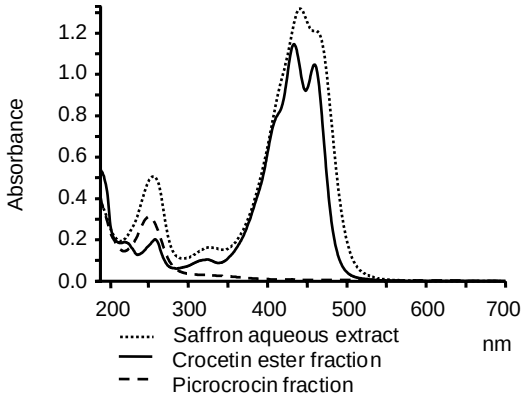

Figure 2. UV-vis spectra of a saffron extract and its corresponding crocetin ester and picrocrocin fractions after SPE.

3.3. Validation of the SPE method

Table 1 displays the main saffron quality characteristics according to ISO (2003) of the 20 samples used for the intra-laboratory validation. A total of 17 samples fulfilled the specifications for the best category: category I, regarding moisture and volatile matter content, as well as the main characteristics using UV-vis spectrophotometry, whereas the rest of the samples belonged to category II. The moisture and volatile matter content of the samples studied ranged from 6.0 to 10.4%, the colouring strength ranged from 162.4 to 284.3, $E_{1cm}^{1\%}$ 330 nm ranged from 29.4 to 47.0 and $E_{1cm}^{1\%}$ 257 nm from 70.0 to 101.9.

The sample composition in crocetin esters and picrocrocin is shown in Table 2. As previously reported by Sanchez et al. (2008a), the samples with the highest or the lowest crocetin ester contents were the samples having the highest or the lowest $E_{1cm}^{1\%}$ 440 nm as well, i.e. samples 12 and 19, respectively (Table 1).

Table 1.
Origin, harvest and quality characteristics of the saffron samples according to ISO/TS 3632 (2003).

Sample	Origin	Harvest	Moisture and volatile matter content (%)	$E^{1\%}_{1cm}$ 440 nm (mean ± SD)[a]	$E^{1\%}_{1cm}$ 330 nm (mean ± SD)	$E^{1\%}_{1cm}$ 257 nm (mean ± SD)	Category
1	Greece	2005	8.3	234.4 e ± 2.0	41.3 i ± 0.6	83.4 e ± 1.1	I
2	Greece	2005	8.5	273.0 i,j ± 1.6	36.4 c,d,e,f ± 0.8	99.2 i,j ± 1.4	I
3	Greece	2005	8.9	255.7 g ± 1.1	39.7 g,h,i ± 0.5	93.7 f,g ± 1.4	I
4	Greece	2005	8.2	259.7 g,h ± 6.4	35.5 b,c,d ± 0.9	94.9 f,g,h ± 2.3	I
5	Greece	2005	10.4	162.6 a ± 4.6	41.3 i ± 1.1	71.4 a,b ± 2.0	II
6	Iran	2006	8.7	233.7 e ± 2.3	40.4 h,i ± 0.4	86.3 e ± 1.0	I
7	Iran	2006	9.6	267.0 h,i ± 3.1	37.8 e,f,g,h ± 2.4	97.7 h,i ± 3.1	I
8	Iran	2006	8.8	170.5 b ± 4.2	41.6 i ± 1.0	74.1 b,c ± 1.8	II
9	Iran	2006	6.9	232.9 d,e ± 1.7	37.7 d,e,f,g ± 4.5	93.5 f,g ± 4.5	I
10	Iran	2005	7.2	199.7 c ± 4.9	38.5 f,g,h ± 0.9	79.4 d ± 1.9	I
11	Italy	2006	8.4	266.1 h,i ± 8.2	33.8 b ± 1.8	97.2 g,h,i ± 3.5	I
12	Italy	2006	9.0	284.3 k ± 7.0	35.0 b,c ± 0.9	101.9 j ± 2.5	I
13	Italy	2006	8.8	199.1 c ± 2.1	36.1 b,c,d,e ± 0.8	79.4 d ± 0.7	I
14	Italy	2006	10.1	225.6 d ± 7.7	41.8 i ± 1.8	92.2 f ± 3.5	I
15	Spain	2006	6.1	258.5 g ± 7.2	29.9 a ± 1.0	93.4 f,g ± 2.5	I
16	Spain	2006	6.4	276.1 j ± 6.8	30.9 a ± 0.8	95.4 f,g,h ± 2.4	I
17	Spain	2006	6.4	230.7 d,e ± 1.2	47.0 j ± 0.1	76.3 c,d ± 0.2	I
18	Spain	2006	6.0	245.2 f ± 6.0	40.0 g,h,i ± 1.0	93.3 f ± 2.3	I
19	Spain	2004	7.7	162.4 a ± 6.9	40.4 h,i ± 1.6	70.0 a ± 3.1	II
20	Spain	2004	8.0	198.9 c ± 5.5	38.8 g,h,i ± 1.5	84.2 e ± 1.1	I
21	Spain	2006	6.1	259.3 ± 3.1	29.4 ± 1.4	99.2 ± 2.3	I

[a] For samples 1-20, the same letter in a column indicates non significant differences according to Duncan's test at the 0.05% level. Sample 21 was used for the evaluation of SPE cartridge behaviour.

However, our results revealed that there was no coincidence between the sample with the highest or lowest content of picrocrocin (sample 4 or sample 17, respectively) (Table 2) and those with the highest or the lowest $E^{1\%}_{1cm}$ 257 nm, samples 12 or 19 respectively (Table 1). In addition, these last two samples were also the samples having the highest or lowest $E^{1\%}_{1cm}$ 440 nm, showing the influence of crocetin esters in $E^{1\%}_{1cm}$ 257 nm. Although Corradi et al. (1979) proposed the measurement of saffron's bitter taste by means of ΔEpic (equation 1), there was no agreement between the sample with the highest picrocrocin content (sample 4) and the highest ΔEpic (sample 12), nor between the sample with the lowest picrocrocin content (sample 17) and the sample with the lowest ΔEpic (sample 5) (Table 2). All these lacks of coincidence seemed to corroborate the effect of other components apart from picrocrocin in $E^{1\%}_{1cm}$ 257 nm and ΔEpic. Results of $E^{1\%}_{1cm}$ 250 nm are presented in Table 2 and, unlike $E^{1\%}_{1cm}$ 257 nm or ΔEpic, these results demonstrated the higher capacity of $E^{1\%}_{1cm}$ 250 nm to order the samples according to the content of picrocrocin determined by HPLC. Moreover, the regression analysis of $E^{1\%}_{1cm}$ 257 nm, ΔEpic and $E^{1\%}_{1cm}$ 250 nm versus picrocrocin content (%) gave the equations:

$$E^{1\%}_{1cm}\ 257\ \text{nm} = 1.49\ (\text{picrocrocin content, \%}) + 58.66 \quad r = 0.8964; \tag{5}$$

$$\Delta E\text{pic} = 0.02\ (\text{picrocrocin content, \%}) + 0.21 \quad r = 0.9241; \tag{6}$$

$$E^{1\%}_{1cm}\ 250\ \text{nm} = 2.69\ (\text{picrocrocin content, \%}) + 0.68 \quad r = 0.9866; \tag{7}$$

showing the good results for $E^{1\%}_{1cm}$ 250 nm in relation to the other two parameters.

The proposed method showed a good selectivity to determine picrocrocin. Its corresponding peak at retention time 5.84 ± 0.03 min was practically the only one present in the chromatograms of the picrocrocin fraction obtained by SPE (Figure 3).

Table 2.

Composition in crocetin glycosides and picrocrocin; Δ*E*pic; and $E_{1cm}^{1\%}$ 250 nm of the saffron samples used in the validation.

Sample	Total crocetin glycosides (% on dry basis, mean ± SD)[a]	HPLC Picrocrocin (% on dry basis, mean ± SD)	Δ*E*pic (mean ± SD)	$E_{1cm}^{1\%}$ 250 nm
1	24.7 d,e ± 0.7	17.5 f ± 0.5	0.552 e,f ± 0.004	48.5 e,f ± 2.5
2	28.4 g,h,i ± 0.9	26.1 j,k ± 0.8	0.749 k ± 0.006	73.2 j,k ± 3.7
3	26.4 f ± 0.8	23.3 h ± 0.7	0.671 h,i ± 0.008	61.4 g,h ± 3.1
4	27.6 g ± 0.8	27.0 k ± 0.8	0.723 j ± 0.018	71.4 j,k ± 3.6
5	17.8 a ± 0.5	12.3 c ± 0.4	0.396 a ± 0.012	33.5 c ± 1.7
6	24.5 c,d ± 0.7	16.3 d,e ± 0.5	0.568 f ± 0.007	45.0 e ± 2.3
7	27.9 g,h ± 0.8	19.8 g ± 0.7	0.715 j ± 0.022	46.8 e ± 2.3
8	18.1 a ± 0.5	11.6 b,c ± 0.4	0.412 a ± 0.010	29.9 b,c ± 1.5
9	24.2 c ± 0.7	22.3 h ± 0.7	0.652 h ± 0.004	58.9 g ± 3.0
10	20.9 b ± 0.6	15.3 d ± 0.5	0.501 c ± 0.012	39.1 d ± 2.0
11	28.6 h,i,j ± 0.9	26.2 j,k ± 0.8	0.745 k ± 0.024	75.8 k ± 3.8
12	29.4 j ± 0.9	24.7 i ± 0.7	0.794 l ± 0.021	65.5 h,i ± 3.3
13	20.8 b ± 0.6	16.8 e,f ± 0.5	0.522 d ± 0.004	45.2 e ± 2.3
14	23.7 c ± 0.7	19.8 g ± 0.6	0.609 g ± 0.021	52.0 f ± 2.4
15	26.2 e,f ± 0.8	26.3 k ± 0.8	0.744 k ± 0.018	69.6 i,j ± 3.5
16	27.9 g,h ± 0.8	22.7 h ± 0.7	0.758 k ± 0.019	59.6 g ± 3.0
17	24.0 c ± 0.7	7.1 a ± 0.2	0.437 b ± 0.002	20.8 a ± 1.1
18	26.1 e,f ± 0.8	25.2 i,j ± 0.8	0.676 i ± 0.017	66.6 i ± 3.4
19	17.2 a ± 0.5	10.7 b ± 0.3	0.417 a,b ± 0.017	28.5 b ± 1.4
20	20.6 b ± 0.6	19.8 g ± 0.6	0.543 e ± 0.001	47.0 e ± 2.4

[a]The same letter in a column indicates non significant differences according to Duncan's test at the 0.05% level.

As shown in Table 3, the method also had a good linearity with $r > 0.999$ for both HPLC and UV-vis analyses. Quantification with HPLC and UV-vis data was based on the calibration equations displayed. The HPLC calibration curve in water was used to quantify the picrocrocin by direct injection of a 0.5 g L^{-1} saffron extract while in the rest of determinations the sample underwent a 1/10 dilution before measuring. For that very reason its range was higher than in the rest of the calibration curves. The ε of picrocrocin obtained in acetonitrile/water 12% (v/v) was 9936 ± 113 L cm^{-1} mol^{-1} and totally agreed with the reported value of 9927 L cm^{-1} mol^{-1} (Sanchez, Carmona, Zalacain, & Alonso, 2005), whereas ε of picrocrocin in water was 10482 ± 53, and also was in accordance with the value of 10515 L cm^{-1} mol^{-1} reported by Sanchez et al. (2005) and the value of 10100 L cm^{-1} mol^{-1} reported by Bucheker and Eugster (1973).

Although for routine quality control in the industry, our proposal is the expression of results as $E_{1cm}^{1\%}$ 250 nm, it was necessary for the results to be stated as % on dry basis in order to compare the proposed method with the HPLC reference. With regard to HPLC data, it was only necessary to relate the picrocrocin concentration obtained through calibration curves with the saffron concentration in the extract. In the case of data from the proposed method, equation 4 could be applied. Recovery results ranged from 87.9 ± 1.8 for sample 9, to 93.1 ± 1.9 for sample 2 (Complete results are shown in the Supplementary Data). Taking into account the mentioned ε of picrocrocin in acetonitrile/water 12% (v/v) and the satisfactory recovery results of approximately 90%, therefore a factor of F = 1.1, equation 4 was applied as:

$$\%\ \text{of picrocrocin on dry basis} = (E_{1cm}^{1\%}\,250\,\text{nm}) \times 0.366 \quad (8)$$

The comparison of means with the Student *t* test for checking the trueness resulted in no significant differences in the content of picrocrocin determined by both methods in any of the samples studied except in sample 7. In addition, the results from the two methods correlated significantly at a 0.01 level; y = 0.9849x - 0.2647, (y = % of picrocrocin determined with the proposed SPE method, x = % picrocrocin determined with HPLC method); $r = 0.9866$. If it was assumed that $E_{1cm}^{1\%}$ 257 nm

and Δ*E*pic are only due to picrocrocin, their corresponding content of picrocrocin as % on a dry basis could be assessed with the ε obtained, but they would be clearly overestimated from $E_{1cm}^{1\%}$ 257 nm and sometimes over and sometimes underestimated from Δ*E*pic.Neither origin nor commercial grade of saffron was found to differentiate method performance as exemplified in the case of recovery (Supplementary data).

Table 3.
Linearity of picrocrocin analysis by HPLC and UV-vis spectrophotometry.

Regression curve data: picrocrocin concentration (mg L^{-1}) = $ax^a + b$				
	Slope, a (mean ± SD)	Intercept, b (mean ± SD)	*r*	Linearity range (mg L^{-1})
HPLC (water)	0.0496 ± 0.0004	0.2709 ± 0.0667	0.9998	256.00-0.51
HPLC (acetonitrile 12%)	0.0453 ± 0.0001	0.1563 ± 0.0021	0.99995	60.00-0.60
UV-vis (water)	31.548 ± 0.193	0.030 ± 0.037	0.9998	51.20-0.51
UV-vis (acetonitrile 12%)	33.209 ± 0.200	0.123 ± 0.025	0.9998	60.00-0.60

[a]x = picrocrocin peak area in HPLC determinations or absorbance at 250 nm in UV-vis determinations.

An RSD = 5.6% indicated that the repeatability of the procedure was satisfactory. From the repeatability standard deviation it was useful to calculate the L_r (equation 2) which enabled the analyst to decide whether the difference between duplicate analyses of a sample, determined under repeatability conditions, was significant. Its value was 2.5 expressed as % picrocrocin on a dry basis and 7.6 expressed as $E_{1cm}^{1\%}$ 250 nm. Intermediate precision determined by different analysts on three separate days was also found satisfactory (RSD = 9.3%). The L_R was calculated according to equation 3 and indicated the same as L_r but under reproducibility conditions. Its value was 4.6 expressed as % picrocrocin on a dry basis and 13.7 expressed as $E_{1cm}^{1\%}$ 250 nm. These values found were in consonance with those reported by Sánchez, (1996) with regard to repeatability and reproducibility of $E_{1cm}^{1\%}$ 257 nm: 2.8% and 8.5%, respectively. More recently, Lechtenberg, Schepmann, Niehues, Hellenbrand, Wünsch, & Hensel (2008) have reported values of 8.9% for repeatability and of 9.0% for intermediate precision in the analysis of picrocrocin by HPLC.

The proposed method also showed a good sensitivity, the *LOD* was 0.30 mg L^{-1} of picrocrocin (0.6% on a dry basis of saffron) and the *LOQ* was 0.63 mg L^{-1} of picrocrocin (1.3% on a dry basis of saffron). This value was approximately 100 times lower than the value reported by Lechtenberg et al., 2008.

4. Conclusions

The SPE procedure developed and validated in this study gave good results for determining the content of picrocrocin in saffron spice samples from UV-vis spectral information. The procedure was found to be accurate, reproducible and sensitive enough for this application in samples from different countries.

Furthermore, its common points with the ISO determinations in saffron, the short time necessary to carry it out and its simplicity make the proposed procedure of particular interest for routine quality control in the industry. The results obtained suggest that this SPE method could even be included in the present ISO 3632/TS.

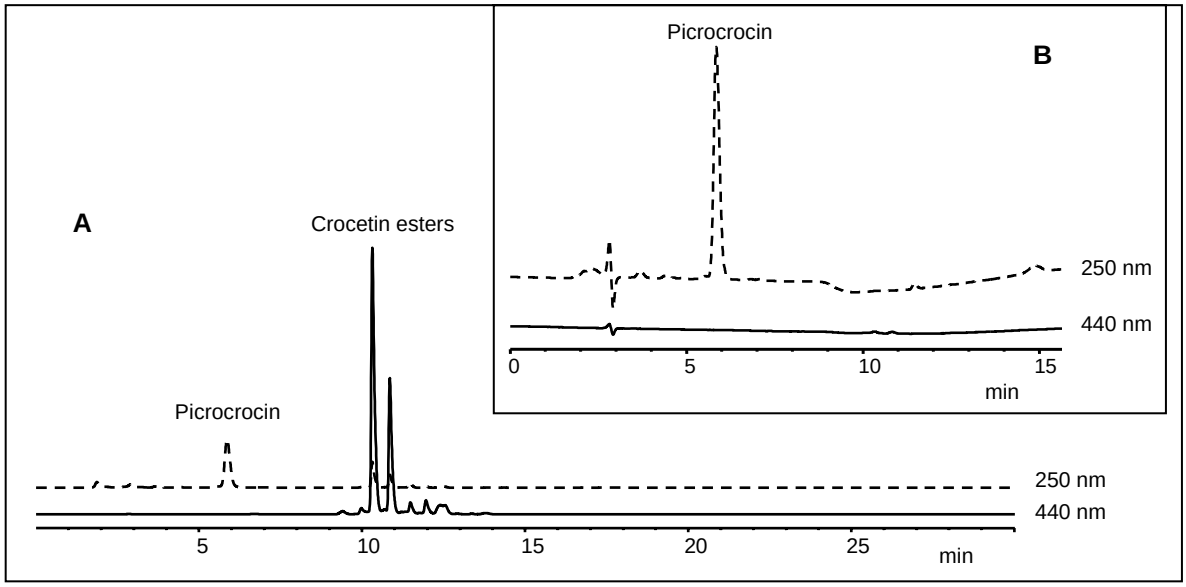

Figure 3. Chromatograms at 250 and 440 nm of a saffron extract (A) and its corresponding picrocrocin fraction from SPE (B)

Supplementary Data

Recovery of picrocrocin for the SPE procedure.

Acknowledgements

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Supplementary data.

Recovery of picrocrocin for the SPE procedure.

Sample	Picrocrocin by HPLC method (mg L^{-1})[a]	Picrocrocin by SPE method (mg L^{-1})[a]	Recovery[a] (%)
1	8.01 ± 0.24	7.39 ± 0.37	92.3 ± 1.9
2	11.96 ± 0.36	11.14 ± 0.56	93.1 ± 1.9
9	10.35 ± 0.31	9.11 ± 0.46	88.0 ± 1.8
13	7.68 ± 0.23	6.85 ± 0.34	89.2 ± 1.8
15	12.24 ± 0.37	10.76 ± 0.52	87.9 ± 1.8
19	4.94 ± 0.15	4.37 ± 0.22	88.4 ± 1.8
Total[b]			89.8 ± 2.3

[a]Mean value ± standard deviation (SD). [b]Mean of the recoveries from the six samples studied ± SD.

5.7. Efecto de la ultrafiltración por centrifugación en la composición de los extractos acuosos del azafrán especia (*Crocus sativus* L.)

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Effect of Centrifugal Ultrafiltration on the Composition of Aqueous Extracts of Saffron Spice (*Crocus sativus* L.)

ANA M. SÁNCHEZ,[†] MANUEL CARMONA,[†] MARIN PRODANOV,[‡] AND GONZALO L. ALONSO*,[†]

En este artículo se estudiaron las modificaciones que produce la ultrafiltración por centrifugación en la composición de ésteres de crocetina, picrocrocina y glicósidos de kaempferol de los extractos acuosos del azafrán especia, mediante dispositivos de filtración para pequeños volúmenes de muestra.

Tal y como puede verse en la Tabla 1 del artículo, se utilizaron 16 dispositivos de cuatro casas comerciales diferentes, con membranas de celulosa regenerada o de polietersulfona cuyo corte molecular nominal (MWCO) estaba comprendido entre 1 y 100 kDa.

Los perfiles de filtración, es decir, la representación del volumen de filtrado en función del tiempo de centrifugación mostraron buenos resultados para la mayoría de los dispositivos en 20 minutos de centrifugación.

Los resultados indicaron distintos grados de separación entre los ésteres de crocetina y la picrocrocina, según la membrana utilizada. Las mejores separaciones de

estos compuestos se consiguieron con los dispositivos designados como 1, 3, 4, 7, 8, 9 y 10 (Tabla 1 del artículo). Estos dispositivos permitían el paso de la picrocrocina a través de la membrana manteniendo gran parte de los ésteres de crocetina en la fracción retenida. De entre ellos, los cuatro últimos dispositivos mencionados pertenecían al grupo de los dispositivos con mayores recuperaciones de picrocrocina en el filtrado y a la vez, con mayores rechazos de ésteres de crocetina, demostrando ser una buena opción para su separación. Todos ellos tenían membranas de celulosa regenerada, salvo el dispositivo 8 que la tenía de polietersulfona. Los mayores MWCO de estos cuatro dispositivos respecto a los dispositivos 1, 3 y 4 hacían a sus membranas más apropiadas para posteriores estudios de ultrafiltración a nivel de planta piloto. Sin embargo, por su pequeño MWCO, el dispositivo 1 y su membrana podrían ser considerados para un segundo paso de ultrafiltración, una vez que el extracto hubiera sido parcialmente purificado de ésteres de crocetina con otra membrana y así, obtener picrocrocina prácticamente libre de ésteres de crocetina.

En la mayoría de los filtrados obtenidos, se produjo una purificación parcial de la picrocrocina respecto a los glicósidos de kaempferol, pues en la mayoría de los dispositivos las recuperaciones de estos compuestos fueron menores que las de la picrocrocina (sólo en los dispositivos 1, 4 y 16 fueron iguales).

Los dispositivos 7, 9 y 10 también presentaron los mejores resultados para concentrar ésteres de crocetina en las fracciones retenidas. Además, 9 y 10 mostraron de forma destacada una precipitación de estos compuestos que podría ser utilizada para su purificación. La concentración de picrocrocina sólo se observó en las fracciones retenidas de los dispositivos 9 y 15, pero sus recuperaciones en el filtrado fueron del orden del 95% por lo que muy poca masa de picrocrocina permanecía en la fracción retenida. La concentración de los glicósidos de kaempferol se consiguió en las fracciones retenidas de los dispositivos 8, 12, 7, 9, 10, 14, 13 y 15 por orden creciente de factor de concentración. De estos dispositivos, las menores recuperaciones en el filtrado

se encontraron en los dispositivos 7-9, por lo que fueron los más interesantes para la concentración de los glicósidos de kaempferol.

5.7. Effect of centrifugal ultrafiltration on the composition of aqueous extracts of saffron spice (*Crocus sativus* L.)

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The purpose of this research was to study the effect of centrifugal ultrafiltration (UF) on the composition of aqueous extracts of saffron spice. The contents of seven crocetin esters, picrocrocin, and two kaempferol glycosides were analyzed by UV−vis and HPLC in the filtrate and retentate fractions from 16 centrifugal filter devices with regenerated cellulose (RC) and polyethersulfone (PES) membranes ranging from 1−100 kDa nominal molecular weight cutoff (MWCO). The separation of crocetin esters from picrocrocin and their concentration with centrifugal UF have been demonstrated. A great heterogeneity of results regarding devices with equal MWCO was found that could not be related to the membrane material or manufacturer. Four devices of 5 and 10 kDa MWCO, three of which had RC membranes, showed the best results. The device having the lowest MWCO also showed a potential to obtain picrocrocin without crocetin esters and could be considered in successive UF steps. The less polar crocetin esters were rejected better than the others.

KEYWORDS: Saffron (*Crocus sativus* L.); carotenoids; crocetin esters; picrocrocin; kaempferol glycosides; centrifugal ultrafiltration; regenerated cellulose; polyethersulfone

INTRODUCTION

Mainly, three groups of compounds stand out in the composition of aqueous extracts of saffron (*Crocus sativus* L.) spice (**Figure 1**): (1) a group of water-soluble carotenoids responsible for saffron color and coloring capacity that consists of various esters of crocetin ($C_{20}H_{24}O_4$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid), where glucose, gentiobiose, neapolitanose, or triglucose are the sugar moieties (*1*) and where trans- or cis-configuration is found; (2) picrocrocin (4-(β-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) and its related compounds; (3) flavonoids such as kaempferol glycosides, which are thought to contribute to the bitter taste of saffron together with the previous group (*1–4*). Saffron spice also contains safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde), the major compound of its volatile fraction, although its solubility in water is very low (*5*). Until now, separation and purification procedures for water-soluble saffron components consisted of column chromatography (*6, 7*), preparative or analytical HPLC (*8, 9*), multilayer coil countercurrent chromatography (*3*), and TLC (*10, 11*). An attractive alternative to these methods is ultrafiltration (UF) because of its mild operating conditions and relatively high selectivity. However, there are no studies on its application to saffron extracts, even though there are enough differences in molecular weight (Mw) among the principal compounds responsible for saffron's color and taste to consider their partial or total separation with UF technologies.

UF is widely used in the agro-food industry for recovering peptides, proteins, polysaccharides, and other biopolymers of animal or vegetal origin (*12–15*). It is particularly suitable for the separation of suspended solids in liquid foods and as a preliminary step to other processes, such as concentration by reverse osmosis or the deacidification and debittering of fruit juices (*16*). This technique offers the food industry the advantages of a chemical-free separation treatment, the possible diafiltration of the retentate, and maximum protection of the sample against external factors.

New applications for separation, concentration, or purification in saffron analysis and the saffron industry could arise from careful research into the behavior of saffron components when they are subjected to UF processes. The relevance of UF in saffron chemistry lies in its potential for component separation and concentration together with a better handling of labile and easily oxidized components such as crocetin esters. From the technological point of view, this field of research might contribute to the introduction of modifications in the color, taste, or functional properties of saffron extracts by changing the proportion of its components.

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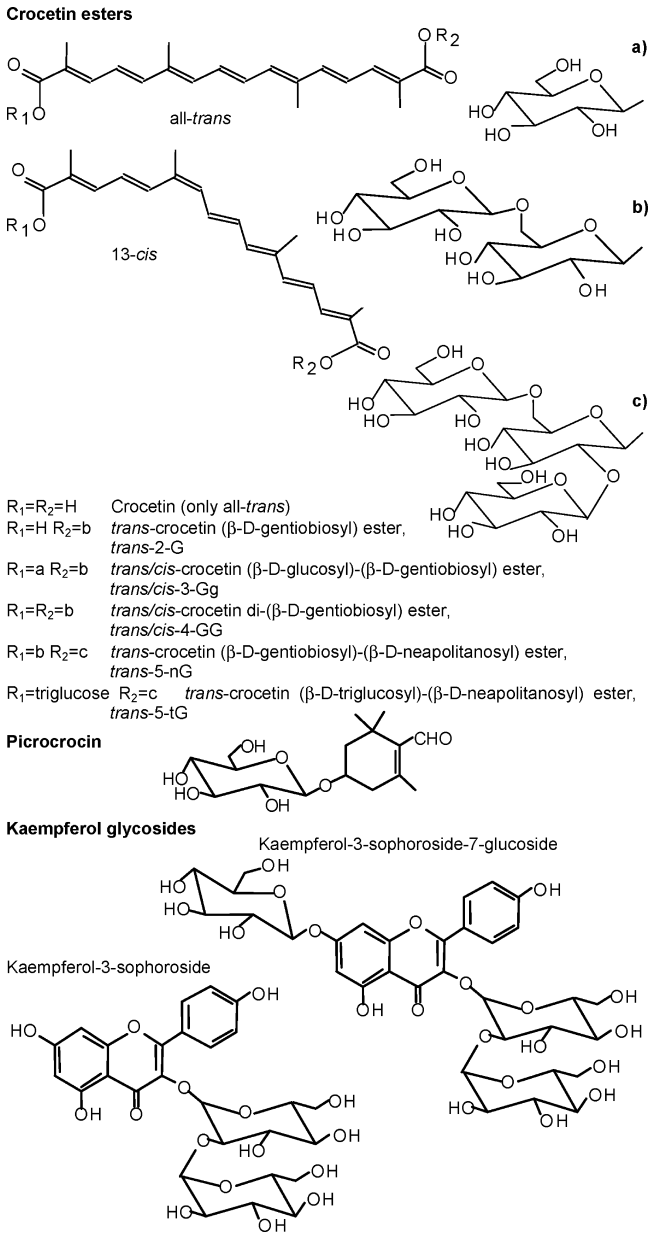

Figure 1. Structures of saffron compounds under discussion. In the case of crocetin esters with *cis*-configuration, the position of the substitutes R_1 and R_2 could not be exactly determined in relation to the C_{13-14} bond.

The performance of a given UF membrane depends on several factors including transmembrane pressure, cross-flow velocity, concentration of dissolved solids, fouling characteristics, and nominal molecular weight cutoff (MWCO) (*15*). The MWCO is a term associated with pore size and is used for describing the separating capabilities of a UF membrane. It refers to the Mw of a solute, such as a globular protein, which is 90% rejected by the membrane under standard conditions (*13*). But MWCO may not be valid for all solutes since it depends on molecular dimensions and behavior, making a test of each membrane necessary for the solutes of interest.

In the application of UF to saffron, the vast supply of membranes available on the market and their high cost, together with the high price of the spice, make small scale procedures necessary to select the most appropriate membranes using small volumes of saffron extract. This can be carried out by using centrifugal filter devices. These filter devices are widely used for the concentration, purification and desalting of protein and nucleic acid solutions, but they have never been applied to the study of saffron extracts.

The purpose of this work was to study the effect of centrifugal UF on the composition of crocetin esters, picrocrocin, and kaempferol glycosides in aqueous extracts of saffron spice, in order to determine the possibility of concentration or purification of these components and to select the most appropriate membranes to attain it. Special attention was paid to comparing the performance of regenerated cellulose and polyethersulfone membranes ranging from 1 kDa to 100 kDa MWCO.

MATERIALS AND METHODS

Samples and Standards. Saffron spice (*Crocus sativus* L.) was used from the 2006 harvest of the Protected Designation of Origin Azafrán de La Mancha. Rutin hydrate (95%) was purchased from Sigma-Aldrich (Steinheim, Germany).

Centrifugal Filter Devices. The centrifugal filter devices under study and their technical specifications are listed in **Table 1**. A total of 16 different filter devices were used, designated from 1 to 16. They came from three manufacturers (Millipore, Bedford, MA; Pall, Ann Arbor, MI; and Sartorius, Goettingen, Germany) and had five different membranes (Omega, Amicon, Ultracel, Vivaspin and Biomax) made of polyethersulfone (PES) or regenerated cellulose (RC) with an MWCO ranging from 1 to 100 kDa.

Saffron Extract Preparation. To reduce the coextraction of nonpolar compounds, 1 g of powdered saffron was extracted twice with 20 mL of cyclohexane. Each extraction was carried out at room temperature in the dark for 5 h with sporadic agitation. Then the organic solvent was discarded, and the solid residue was dried under reduced pressure. Five-hundred milligrams of the thus treated saffron powder was extracted with 1 L of ultra high purity water by stirring the suspension in the dark at room temperature for 1 h. Next, to obtain the initial extract for the centrifugal UF treatment, the extract was clarified by centrifugation at 4280 × *g* (7000 rpm) for 20 min and successive dead-end microfiltration through 0.8, then 0.45 and then 0.2 μm pore size cellulose acetate membrane filters from Albet (Barcelona, Spain). This aqueous saffron extract preparation was carried out just before each treatment in order to avoid storage and degradation of crocetin esters.

Centrifugal UF Treatment. First, in order to set centrifugation time, the filtration profile, that is to say filtrate volume versus centrifugation time, was studied with 10 mL feed volume of aqueous saffron extract and the centrifuge's maximum rcf: 3220 × *g* (4000 rpm), in a swinging bucket at 20 °C for 10, 15, 20, 30, 40, 50, and 60 min. Once the centrifugal conditions were selected (3220 × *g*, 20 min), experiments were conducted in triplicate for each device. The only exceptions were devices 4 and 5 (**Table 1**), which were centrifuged in a fixed rotor at 2687 × *g* (4300 rpm) for 20 min, due to their dimensions and maximum rcf.

Spectrophotometric Analysis. Spectroscopic characteristics of aqueous saffron extracts, filtrate and retentate fractions were monitored by scanning from 190 to 700 nm using a Perkin-Elmer Lambda 25 spectrophotometer (Norwalk, CT, USA) with UV WinLab 2.85.04 software (Perkin-Elmer). Saffron quality characteristics, moisture and volatile matter content, coloring strength ($E^{1\%}_{1cm}$ 440 nm), $E^{1\%}_{1cm}$ 257 nm, and $E^{1\%}_{1cm}$ 330 nm were determined according to ISO 3632/TS (*17*). The picrocrocin molar absorption coefficient in aqueous solution was determined by following the Beer−Lambert law. All analyses were done in triplicate.

RP-HPLC-DAD Analysis. Forty microliters of each sample were injected into an Agilent 1100 HPLC chromatograph (Palo Alto, CA) operating with a 150 mm × 4.6 mm i.d., 5 μm Phenomenex (Le Pecq Cedex, France) Luna C_{18} column, thermostated at 30 °C. Eluents were water (A) and acetonitrile (B), with the following gradient: 20% B, 0−5 min; 20−80% B, 5−15 min and 80% B, 15−20 min. The flow rate was 0.8 mL/min. The DAD detector (Hewlett-Packard, Waldbronn, Germany) was set at 250, 330, and 440 nm for picrocrocin, kaempferol glycoside and crocetin ester detection, respectively. All analyses were carried out in triplicate. The measurements of the extract used as feed

Table 1. Technical Specifications of the Centrifugal Membrane Filter Devices under Study

no.	centrifugal filter device trade name	membrane trade name	membrane material[a]	MWCO[b] (kDa)	active membrane area (cm^2)	maximum rcf (g)	manufacturer[c]
1	Macrosep	Omega	PES	1	10.00	5000	Pall
2	Macrosep	Omega	PES	3	10.00	5000	Pall
3	Vivaspin−20	Vivaspin	PES	3	6.00	5000	Sartorius
4	Centriplus	Amicon YMT	RC	3	2.34	3000	Millipore
5	Centriprep	Ultracel YM-3	RC	3	2.84	3000	Millipore
6	Vivaspin−20	Vivaspin	PES	5	6.00	5000	Sartorius
7	Amicon Ultra-15	Ultracel	RC	5	7.60	4000	Millipore
8	Centricon Plus-20	Biomax	PES	5	9.50	4000	Millipore
9	Amicon Ultra-15	Ultracel	RC	10	7.60	4000	Millipore
10	Centricon Plus-20	Ultracel PL	RC	10	9.50	4000	Millipore
11	Macrosep	Omega	PES	10	10.00	5000	Pall
12	Vivaspin−20	Vivaspin	PES	10	6.00	5000	Sartorius
13	Vivaspin−20	Vivaspin	PES	30	6.00	5000	Sartorius
14	Vivaspin−20	Vivaspin	PES	50	6.00	5000	Sartorius
15	Amicon Ultra-15	Ultracel	RC	50	7.60	4000	Millipore
16	Centricon Plus-20	Ultracel PL	RC	100	9.50	4000	Millipore

[a] PES, polyethersulfone; RC, regenerated cellulose. [b] MWCO: nominal molecular weight cutoff. [c] Pall, (Ann Arbor, MI), Sartorius (Goettingen, Germany), Millipore (Bedford, MA).

Table 2. Molecular Weight (Mw), Composition (Mean and Standard Deviation: SD, $n = 3$) in Kaempferol Glycosides, Picrocrocin, and Crocetin Esters, UV−Vis Maxima and Retention Times (RT) of the Initial Saffron Extract

compound	Mw	mean content ± SD (mg/L)	mean content ± SD (% on dry basis)	UV−vis λmax (nm)	RT (min)
kaempferol-3-sophoroside-7-glucoside[a]	772	7.78 ± 0.37	1.66 ± 0.08	265, 321sh, 345	2.9
kaempferol-3-sophoroside[a]	610	8.00 ± 0.82	1.71 ± 0.18	266, 295sh, 350	5.6
total kaempferol glycosides		15.78 ± 0.45	3.37 ± 0.11		
picrocrocin	330	86.21 ± 7.81	18.43 ± 1.54	250	5.9
trans-5-tG	1139	1.72 ± 0.11	0.34 ± 0.02	263, 443, 467	9.6
trans-5-nG	1139	1.69 ± 0.60	0.33 ± 0.12	263, 422sh, 440, 467sh	10.0
trans-4-GG	977	66.35 ± 3.12	13.04 ± 0.70	262, 442, 465	10.3
trans-3-Gg	815	40.76 ± 2.91	8.01 ± 0.54	262, 441, 465	10.9
trans-2-G	653	5.57 ± 0.72	1.10 ± 0.15	259, 434, 459	11.5
cis-4-GG	977	6.71 ± 0.27	1.32 ± 0.06	262, 327, 435, 458	12.0
cis-3-Gg	815	2.67 ± 1.05	0.53 ± 0.21	262, 325, 434, 458	12.7
total crocetin glycosides		125.48 ± 2.56	24.66 ± 0.68		

[a] Kaempferol glycoside content expressed as equivalent mass of rutin.

for centrifugal filter devices were made at the same time as the measurements of the filtrate and retentate fractions in order to minimize variations in the results due to crocetin ester degradation.

Identification and Quantification of Saffron Components. Identification of crocetin esters, picrocrocin, and kaempferol glycosides by HPLC-DAD-MS was carried out as previously described (*1, 4*). Total crocetin esters were first determined by UV−vis spectrophotometry, using their absorbance at 440 nm. The results were expressed as the percent of *trans*-4-GG, as reported by Basker et al. (*18*) but using the molar absorption coefficient determined by Speranza (*8*). Because of the lack of pure standards of each crocetin ester, their quantification was based on the following equation (*19, 20*):

$$\%\ \text{of crocetin ester } i \text{ on dry basis} = \frac{\mathrm{Mw}_i(E^{1\%}_{1\,\mathrm{cm}}\ 440\ \mathrm{nm})A_i}{10\varepsilon_{\mathrm{t,c}}} \qquad (1)$$

where $\mathrm{Mw_i}$ stands for the molecular weight of the crocetin ester i, $E^{1\%}_{1\mathrm{cm}}$ 440 nm is the coloring strength, A_i is the percent peak area of the crocetin ester i at 440 nm and $\varepsilon_{\mathrm{t,c}}$ is the molar absorption coefficient value (89000 for *trans*-crocetin esters and 63350 for *cis*-crocetin esters) (*8*). For comparative purposes, besides the results for each crocetin ester, the total crocetin ester content was assessed with HPLC data.

Quantification with UV−vis data was based on the determined picrocrocin molar absorption coefficient (10515 L cm^{-1} mol^{-1}). Quantification with HPLC data was based on calibration curves of the picrocrocin concentration, c, (mg/L) as a function of its peak area, a, in the range of 2−315 mg/L: $c = 0.0354\ a + 0.0018$, $r^2 = 0.999$, for a total of six data points. Picrocrocin was purified according to the procedure described below.

Quantification of kaempferol glycosides referred to a rutin standard, whose concentration, d (mg/L), as a function of its HPLC peak area, b, also exhibited good linear regression in the 5−100 mg/L range ($d = 0.0882b + 0.0021$, $r^2 = 0.996$, for 10 data points). The content of kaempferol glycosides was expressed as equivalent mg of rutin/100 mg of dry saffron.

Picrocrocin Isolation. Picrocrocin was extracted from saffron and isolated by column chromatography by using a C_{18} adsorbent (125×10^{-8} cm pore size, 55−105 μm particle size) from Waters (Milford, MA). For extraction, 30 mL of cyclohexane (HPLC-grade from Scharlau, Barcelona, Spain) were added to 5 g of powdered saffron, and the suspension was left in the dark at room temperature for 24 h with sporadic agitation. Then the organic solvent was discarded, and the solid residue was dried under reduced pressure. Sixty milliliters of nitrogen-saturated water were added to the thus treated saffron, and the resulting suspension was stirred for 1 h in the dark at room temperature. Then the extract was centrifuged at 4000 rpm for 10 min, and the supernatant was collected and loaded on the previously conditioned C_{18} column (8 cm high × 2.7 cm i.d.). Picrocrocin was eluted with 90 mL of 10% acetonitrile/water (v/v) after the elution of flavonoids with 20 mL of 2% acetonitrile/water (v/v). Finally, the solvent was eliminated by evaporation to dryness under reduced pressure, and the purified picrocrocin was kept at −20 °C until its utilization. The chromatographic purity of the obtained picrocrocin was 96%, calculated as the percent of the total peak area at 250 nm.

Centrifugal Filter Membrane Performance. Performance of the centrifugal filter membranes was expressed in terms of the parameters described below. Volume concentration ratio (*VCR*) is defined as:

$$VCR = \frac{V_O}{V_R} \quad (2)$$

where V_0 is the volume of the feed extract, and V_R is the volume of the retentate fraction. Rejection (R) of any solute is defined as the variation (<0 = an increase, >0 = a decrease) in the solute concentration, expressed as a percentage of its concentration in the feed extract, which is observed in the filtrate fraction:

$$R\,(\%) = \left(1 - \frac{C_F}{C_O}\right) \times 100 \quad (3)$$

where C_F is the concentration of the solute in the filtrate fraction, and C_0 is the concentration of the solute in the feed extract. Filtrate, retentate, and total recoveries (%) were calculated by a direct weighing procedure, considering the density of the aqueous solutions as equal to 1 g/mL and using the measured concentrations as follows:

$$\text{Filtrate recovery}\,(\%) = \left(\frac{V_F \times C_F}{V_O \times C_O}\right) \times 100 \quad (4)$$

$$\text{Retentate recovery}\,(\%) = \left(\frac{V_R \times C_R}{V_O \times C_O}\right) \times 100 \quad (5)$$

$$\text{Total recovery}\,(\%) = \text{Retentate recovery}\,(\%) + \text{Filtrate recovery}\,(\%) \quad (6)$$

$$\text{Losses}\,(\%) = 100 - \text{Total recovery}\,(\%) \quad (7)$$

where V_F is the filtrate volume, V_0 is the initial extract volume (feed volume), V_R is the retentate fraction volume, C_F is the concentration of the solute in the filtrate fraction, C_0 is the concentration of the solute in the initial extract, and C_R is the concentration of the solute in the retentate fraction.

Nomenclature for Crocetin Esters. Abbreviations were adopted from Carmona et al. (*1*): *trans*-5-tG, *trans*-crocetin (β-D-triglucosyl)-(β-D-gentiobiosyl) ester; *trans*-5-nG, *trans*-crocetin (β-D-neapolitanosyl)-(β-D-gentiobiosyl) ester; *trans*-4-GG, *trans*-crocetin di-(β-D-gentiobiosyl) ester; *trans*-3-Gg, *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester; *trans*-2-G, *trans*-crocetin (β-D-gentiobiosyl) ester; *cis*-4-GG, *cis*-crocetin di-(β-D-gentiobiosyl) ester; and *cis*-3-Gg, *cis*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester.

Statistics. Data were subjected to an analysis of variance (ANOVA) using the SPSS 15.0 statistical program for Windows (SPSS Inc.).

RESULTS AND DISCUSSION

Saffron Quality Characteristics and Chemical Composition of the Initial Saffron Extract. Results indicated that the commercial saffron sample used belonged to ISO category I (*17*): 8.1% moisture and volatile matter content; $E^{1\%}_{1cm}$ 440 nm = 239.2 ± 3.1 (coloring strength); $E^{1\%}_{1cm}$ 257 nm = 91.3 ± 1.2; $E^{1\%}_{1cm}$ 330 nm = 25.8 ± 0.5.

It was observed that the cyclohexane removed from the sample preparation was yellow; therefore, after this process the $E^{1\%}_{1cm}$ 440 nm, $E^{1\%}_{1cm}$ 257 nm, and $E^{1\%}_{1cm}$ 330 nm aqueous extracts were measured. The results indicated that after cyclohexane extraction, the $E^{1\%}_{1cm}$ 440 nm of the aqueous extract remained the same (239.1 ± 5.6), while $E^{1\%}_{1cm}$ 257 nm and $E^{1\%}_{1cm}$ 330 nm were slightly higher (95.5 ± 1.0 and 27.8 ± 0.4, respectively). The similarities in $E^{1\%}_{1cm}$ 440 nm indicated an extraction of nonwater-soluble pigments such as α and β-carotene, lycopene, or zeaxanthin, whose presence in saffron has already been described (*21, 22*), rather than a removal of crocetin esters.

The composition of the initial saffron extract in kaempferol glycosides, picrocrocin and crocetin esters is shown in **Table 2**. It was expressed as mg/L and as a percentage on a dry basis so that the comparison to previously reported data could be made. In addition, this table shows Mw, UV−vis maxima and retention times (RT) for the above-mentioned compounds. The initial concentration of kaempferol glycosides in the extract determined by HPLC was ∼16 mg/L, while the picrocrocin concentration was ∼86 mg/L, and the one for crocetin esters was ∼125 mg/L. The main kaempferol glycosides found were kaempferol-3-sophoroside-7-glucoside and kaempferol-3-sophoroside, which showed fairly similar contents, ∼1.7 mg of rutin/100 mg of dry saffron each. These results were higher than the data reported in previous research: 0.258 and 0.312 mg of rutin/100 mg of dry saffron, respectively (*4*). The picrocrocin content of the sample used here (18.4%) was also higher than what Alonso et al. reported (0.79−12.94%) (*23*), and than results by Iborra et al. (*10*) (13.9%). Crocetin esters were the major compounds, comprising ∼25%. Among them, *trans*-4-GG was the most abundant (13.04%), followed by *trans*-3-Gg (8.01%), *cis*-4-GG (1.32%), *trans*-2-G (1.10%), *cis*-3-Gg (0.53%), *trans*-5-tG (0.34%), and *trans*-5-nG (0.33%). Alonso et al. (*23*) also reported lower values for *trans*-4-GG (0.46−12.12%) in Spanish saffron, but results from the present study for *trans*-3-Gg, *cis*-4-GG and *cis*-3-Gg remained within the ranges previously given in ref *23* for the above-mentioned crocetin esters (0.01−9.44%; 0.04−8.53%; 0.01−2.26%, respectively).

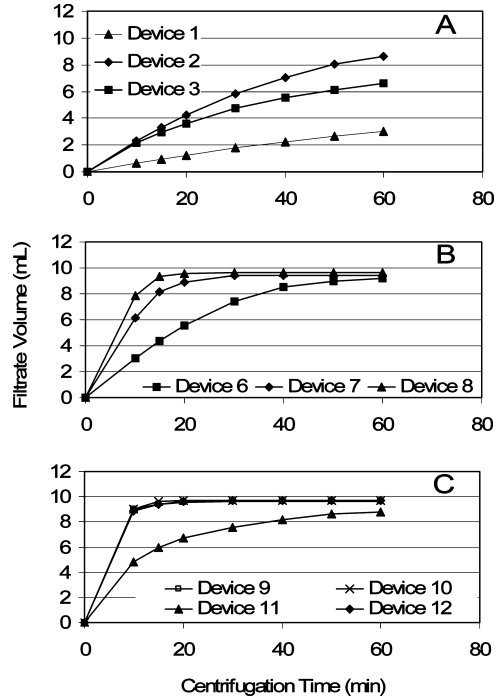

Figure 2. Filtration profile for saffron extracts from ≤10 kDa MWCO devices centrifuged at 3220 × *g*. (**A**) 3 kDa MWCO; (**B**) 5 kDa MWCO; (**C**) 10 kDa MWCO.

Filtration Profile for Aqueous Saffron Extracts. Filtration profiles from the centrifugal filter devices have been established by manufacturers for several well-known materials (bovine serum albumin, cytochrome *c*, etc.). However, actual performance depends on the nature of the specific solute under study, making a study with saffron extracts indispensable. The filtration profile for saffron extracts consisted of an increasing filtrate volume as centrifugation time increased until a plateau was reached. **Figure 2** shows the filtration profile from ≤10 kDa MWCO devices centrifuged at 3220 × *g*. When using centrifugal devices from 1 to 3 kDa MWCO (**Figure 2A**), filtrate volume did not reach the plateau in 60 min and was on the increase as MWCO increased. Also, differences were found between devices 2 and 3 (both of 3 kDa MWCO). Device 2 showed higher filtrate volumes, especially after 20 min of centrifugation, probably due to its higher active membrane area or the kind of membrane. In 5 kDa MWCO devices (**Figure**

Table 3. Volume Concentration Ratio (*VCR*) and Mean ± Standard Deviation, $n = 3$

centrifugal filter device	*VCR*[a]	centrifugal filter device	*VCR*
1	1.2 a ± 0.1	9	43.4 f ± 8.7
2	1.7 a ± 0.1	10	76.0 g ± 1.3
3	1.8 a ± 0.1	11	2.6 a ± 0.1
4	1.6 a ± 0.3	12	37.4 e ± 1.9
5	1.4 a ± 0.2	13	31.8 d ± 1.6
6	2.8 a ± 0.3	14	91.6 h ± 4.6
7	14.3 b ± 4.4	15	88.7 h ± 15.4
8	20.6 c ± 4.6	16	15.1 b ± 0.8

[a] The same letter in the *VCR* columns indicates nonsignificant differences according to Duncan's test at the 0.05% level.

2B), it was found that the filtrate volumes were ordered from lower to higher active membrane areas, and all but device 6 had reached a stable filtrate volume at about 20 min. Comparing their characteristics, the highest similarities were found in devices from the same manufacturer, rather than in devices with the same membrane material. In spite of having different membrane materials and active membrane areas, the filtration profiles of all 10 kDa MWCO devices (**Figure 2C**) were the same, except for device 11, which showed lower filtrate volumes. For the same type of device and therefore, the same active membrane area, it was observed that device 8 (5 kDa MWCO, PES membrane) and device 10 (10 kDa MWCO, RC membrane) had equal filtration profiles for saffron extracts. It seemed that PES was more permeable than RC in this type of device. In light of these results and so that all devices would have the same experimental conditions, 20 min was selected as the centrifugation time. Regarding the filtration profiles that are not presented in **Figure 2**, devices 4 and 5 showed filtration profiles similar to device 2, and device 13 was very similar to device 12. Filtration profiles of devices 14 and 15 reached maxima filtrate volumes in the early min of centrifugation, while devices 16 and 7 had similar filtration profiles.

Volume Concentration Ratio (*VCR*). A vast range of *VCR* was reached with the membranes studied, from ~1 to ~92 (**Table 3**). The lower limit of this range corresponded mainly to devices with MWCO membranes under 5 kDa. These devices (from device 1 to 5) and devices 11 and 6 did not present significant differences in *VCR*. However, the highest *VCR* value was found for devices 14 and 15, which had 50 kDa MWCO. There was a great heterogeneity in the *VCR* of devices sharing the same MWCO membrane, even when the membrane material was the same but the manufacturer was different. This could be seen when comparing devices 6 and 8, both with a 5 kDa MWCO and a PES membrane, or devices 11 and 12, both having a 10 kDa MWCO and a PES membrane. As for the membrane material (= MWCO, = manufacturer), PES gave slightly higher *VCR* results than RC (comparing devices 7 and 8). However, the low magnitude of the difference could be due rather to its higher active membrane area. In general, from the comparison of devices 1, 2 and 11 or 3, 6, 12, 13 and 14 or 7, 9 and 15, it was found that *VCR* increased with increasing MWCO (= membrane material, = manufacturer and type of device). Sometimes the differences were not significant, however, and in devices 12 and 13, the tendency was inverted.

Performances of Each Membrane Determined by UV−Vis Spectrophotometry versus Those Determined by HPLC. Results indicated that total crocetin ester *R* and recoveries were very similar for both means of quantification, and therefore, they could be used equally. These similarities were in consonance with the good correlation found between UV−vis and HPLC results (*20*). However, higher values of picrocrocin *R* and lower or equal picrocrocin recoveries were found in most cases with UV−vis data than with HPLC data. This may be due to the fact that not only picrocrocin, but also other compounds, such as crocetin esters or flavonoids, absorb at 250 nm. Consequently, HPLC results will be shown and used throughout this discussion, but not UV−vis results, which can be seen in the Supporting Information.

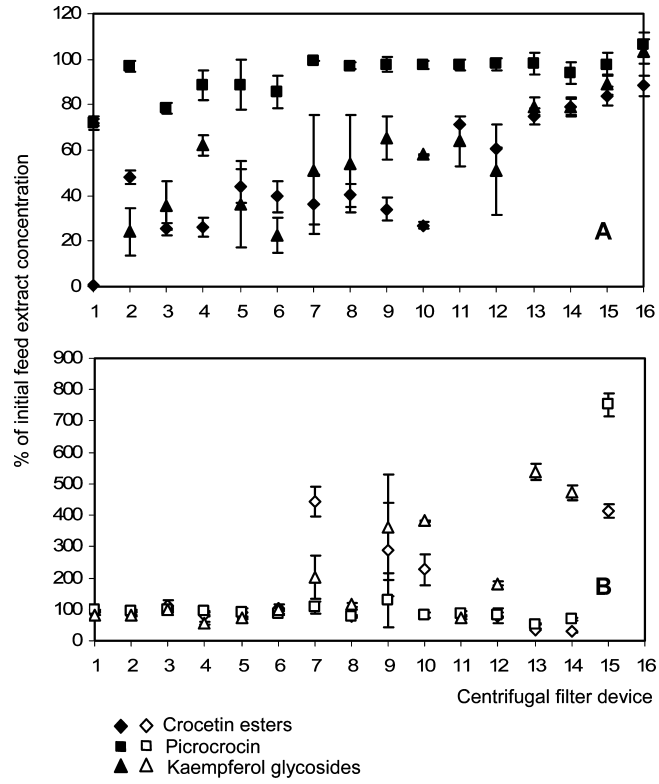

Figure 3. Concentration of crocetin esters, picrocrocin, and kaempferol glycosides compared to their concentrations in the saffron feed extract, in filtrate (**A**) and retentate (**B**) fractions.

Crocetin Ester Composition in the Filtrate and Retentate Fractions. The concentration of the main components in filtrate and retentate fractions compared to their concentrations in the saffron feed extract is presented in **Figure 3**. Crocetin ester concentration in the filtrate fractions (**Figure 3A**) decreased to a greater or lesser extent depending on the centrifugal filter device, especially in those with ≤10 kDa MWCO. The filtrate fractions from devices 13−16 underwent the lowest concentration changes. The magnitude of these decreases of the crocetin ester *R* (**Table 4**) varied from ~12% for the membrane with the highest MWCO, corresponding to device 16, to ~99% for device 1, which contained the membrane with the lowest MWCO. However, *R* did not always follow a decreasing order with increasing MWCO. Moreover, significant differences were found in *R* from devices having the same MWCO, as happened in the 3 kDa MWCO and the 10 kDa groups of devices. In the former group, two subgroups of *R* values were distinguished that were not connected either with their membrane material or manufacturer. The first subgroup consisted of devices 2 and 5; the second one, of devices 3 and 4. In the latter group, three subgroups of *R* values were found corresponding to devices 9−10, 11, and 12. They depended on the manufacturer but not on the membrane material since both devices with PES membranes showed different *R* values. Within the same type of devices, there was a general trend of decreasing crocetin ester *R* as MWCO rose, especially when dealing with the same

Table 4. Rejection (*R*), Filtrate Recovery (%), and Losses of Crocetin Esters, Mean ± Standard Deviation, $n = 3$

centrifugal filter device	crocetin esters[a]		
	R (%)[b]	filtrate recovery (%)[b]	losses (%)[b]
1	99.4 j ± 0.1	0.1 a ± 0.1	21.1 a,b ± 2.9
2	52.4 e ± 3.4	18.3 c ± 0.9	32.8 c,d ± 3.0
3	74.7 i ± 2.5	10.3 b ± 1.3	26.9 b,c ± 9.3
4	73.9 h,i ± 4.1	4.5 a,b ± 1.1	29.3 b,c ± 1.7
5	55.8 e,f ± 7.4	17.8 c ± 2.8	32.4 c,d ± 0.2
6	60.9 e,f,g ± 7.3	24.5 c,d ± 5.6	38.6 d ± 8.3
7	63.9 f,g ± 13.0	32.9 e,f ± 12.9	35.1 c,d ± 7.4
8	59.9 e,f,g ± 4.9	38.3 f,g ± 5.9	59.1 e ± 6.5
9	66.0 g,h ± 5.0	32.8 e,f ± 5.1	62.3 e ± 5.2
10	73.1 h,i ± 1.3	26.1 d,e ± 1.3	70.9 f ± 0.7
11	28.6 c ± 0.1	42.4 g ± 0.7	28.1 b,c ± 0.1
12	39.3 d ± 0.2	58.1 h ± 0.1	40.4 d ± 0.2
13	25.1 c ± 0.1	71.5 i ± 0.1	27.4 b,c ± 0.1
14	21.2 b,c ± 0.2	76.7 i,j ± 0.1	23.0 a,b ± 0.1
15	16.2 a,b ± 0.1	82.9 j ± 0.1	16.6 a ± 0.1
16	11.7 a ± 0.1	81.1 j ± 0.1	n.a.[c]

[a] Sum of individual crocetin esters determined by HPLC. [b] The same letter in a column indicates nonsignificant differences according to Duncan's test at the 0.05% level. [c] Data not available.

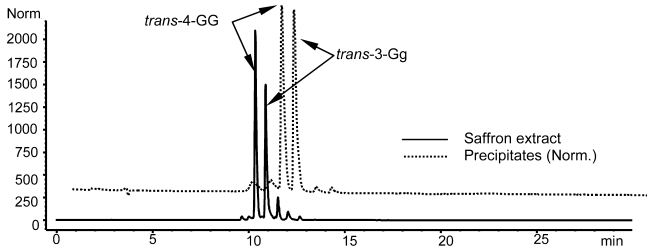

Figure 4. Chromatograms at 440 nm of the saffron extract and the precipitates of crocetin esters.

membrane. Device 8 (5 kDa, PES membrane) was the exception, having lower crocetin ester *R* than device 10 (10 kDa, RC membrane). Besides device 1, which had the only filtrate fraction nearly free from crocetin esters, devices 3, 4, and 10 presented the highest reductions in crocetin ester concentrations from filtrate fractions (*R* ~74%), followed by devices 6, 7, 8, and 9, which showed reductions of ~60%. They came from three different manufacturers; devices 1, 3, 6, and 8 were made of PES, while the others were made of RC. As previously stated, devices with MWCO higher than 10 kDa showed the lowest crocetin ester *R*, which was too low for consideration in the separation of crocetin esters from picrocrocin and kaempferol glycosides. Regarding mass balance, the highlighted devices had crocetin ester filtrate recoveries lower than 40%; this parameter was just 0.1% in device 1 (**Table 4**). Retentate results (**Figure 3B**) showed that higher concentrations of crocetin esters were found than in the initial extract only in devices 7, 9, 10, and 15. Because of the low retentate volume and some problems with its recuperation, it was not possible to analyze the retentate fraction of device 16. Devices 7, 9, 10, and 15 showed concentration factors for crocetin esters of 4.4, 2.9, 2.3, and 4.1, respectively. However, a phenomenon of crocetin ester precipitation that was not included in the results shown in **Figure 3B** was observed in the retentate fractions of devices 2−4 and 6−11. The precipitates were washed with water, redissolved in 50% acetonitrile/water v/v, and analyzed under chromatographic conditions (see Materials and Methods) (**Figure 4**). A mixture of crocetin esters was found, which had almost the same proportion of *trans*-4-GG and *trans*-3-Gg. The magnitude of precipitated crocetin esters and/or their adsorption on membranes were considered losses and were estimated through the difference between 100% and total recovery. Loss values of the initial crocetin ester mass ranged from nearly 17 to 71%, and the highest values were found in device 10 followed by devices 9 and 8 (**Table 4**). These results indicated the possibility of purifying the main crocetin esters through concentration and precipitation, which should be further studied.

With regard to each crocetin ester, it was found that the less polar ones (see RT in **Table 2**) and therefore cis-isomers were better rejected by the majority of membranes. The proportion of these esters was changed in the retentate and filtrate fractions as reported, for example, by Kalbasi et al. (*24*), for monomeric and polymeric anthocyanin fractions. The device 1 membrane totally rejected all crocetin esters, and only small quantities of *trans*-4-GG were detected in its filtrate fraction; therefore, they are not shown in **Figure 5**. **Figure 5A** shows the *R* results of the two main *trans*- and *cis*-crocetin esters. Devices 6, 7, and 8, all having 5 kDa MWCO and previously emphasized for their high crocetin ester *R*, showed a tendency to reject the less polar crocetin esters better than devices 3, 4, 9, and 10. In this way, -3-Gg forms were better rejected than -4-GG forms, although sometimes the differences were not significant. Apart from device 1, the *R* for *trans*-5-tG, *trans*-5-nG, and *trans*-4-GG in the various devices ranged from 0 to ~70%, whereas the *R* for *trans*-3-Gg, *trans*-2-G, *cis*-4-GG, and *cis*-3-Gg were ≥20, ≥50, ≥54, and ≥66%, respectively. Furthermore, *trans*-5-tG, *trans*-5-nG, and *trans*-4-GG were recovered in the filtrate at a higher proportion than the rest of the crocetin esters for all the devices under study. **Figure 5B**, shows the recovery results of the two main *trans*- and *cis*-crocetin esters. The greatest losses were found in *trans*-2-G, *cis*-4-GG, and *cis*-3-Gg for devices 8−10 and 12. **Figure 5C** shows the losses found in the two main *trans*- and *cis*-crocetin esters.

Picrocrocin Composition in the Filtrate and Retentate Fractions. The picrocrocin concentration in the filtrate fractions was almost equal to its concentration in the feed extract (**Figure 3A**). A narrow range of picrocrocin *R* values were found, from 0.5% (device 16) to 28% (device 1) (**Table 5**). Heterogeneous results were observed, the same as for crocetin esters, with significant differences among devices with the same MWCO membrane. These differences could not be related to the membrane material or manufacturer, making membrane tests necessary. For example, picrocrocin *R* from device 2 was lower than the other values found for devices with 3 kDa membranes, while the value from device 6 was higher than the other values from devices with 5 kDa membranes. Within the same type of device, a general trend of picrocrocin *R* to decrease or remain stable was observed as the MWCO rose. When attention is focused on picrocrocin filtrate recoveries from devices 7 through 16, except for device 11, there were values of ≥90%, picrocrocin being the best recovered component, having the highest filtrate and total recovery. This trend for filtrate recovery within the same type of device as the MWCO increased was the same as for picrocrocin *R*. Picrocrocin losses reached as high as 16% (devices 5 and 6), which is a much lower result than that for crocetin esters. The only picrocrocin concentration in the retentate fractions (**Figure 3B**) that was clearly higher than in the initial extract was from device 15. However, the retentate fraction from device 13 had only 53% of the initial picrocrocin concentration.

Kaempferol Glycoside Composition in the Filtrate and Retentate Fractions. Like in crocetin esters, the total kaempferol glycoside concentration in the filtrate fractions (**Figure 3A**) decreased depending on the centrifugal filter device, with the lowest concentration changes corresponding to the filtrate

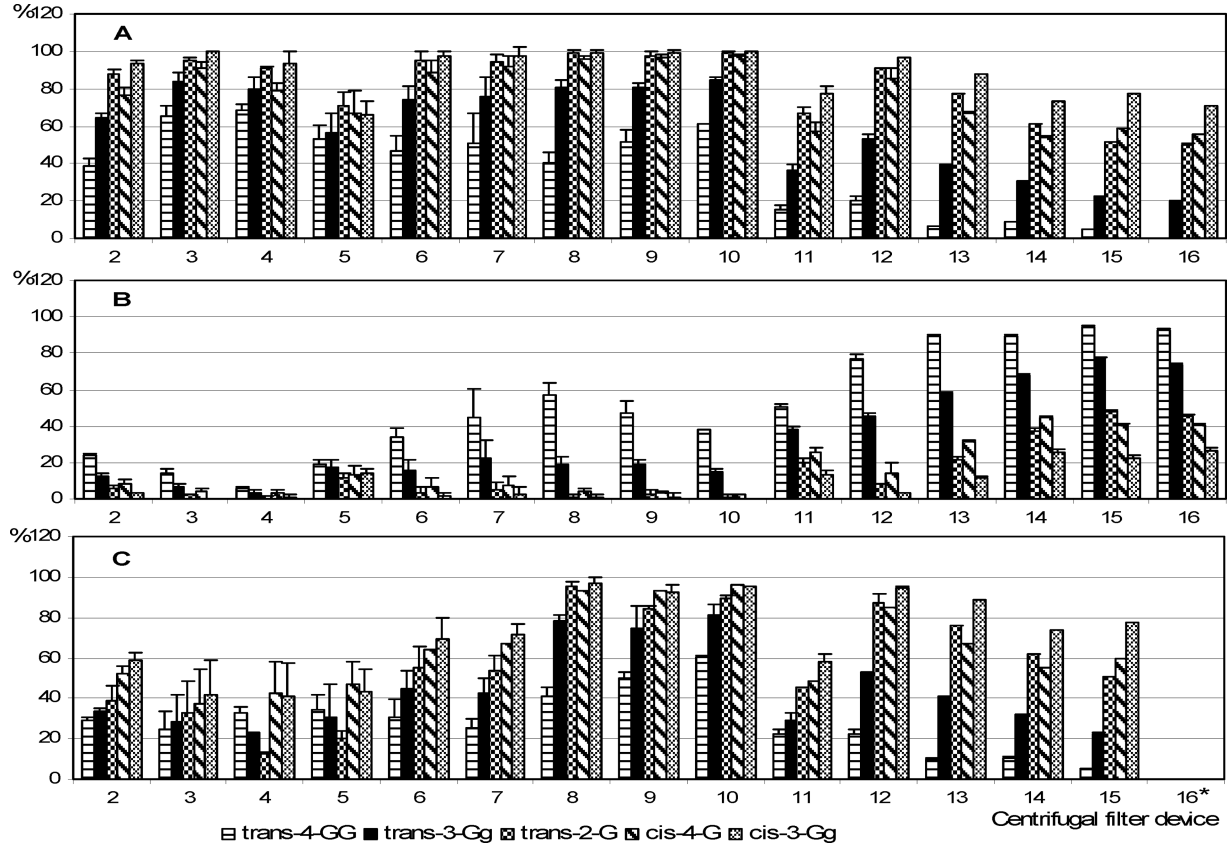

Figure 5. (**A**) Rejection, *R* (%); (**B**) filtrate recovery (%), and (**C**) losses (%) of the two main *trans*-crocetin esters and *cis*-crocetin esters individually. *Data not available.

Table 5. Rejection (*R*), Filtrate Recovery (%), and Losses of Picrocrocin, Mean ± Standard Deviation, $n = 3$

centrifugal filter device	picrocrocin[a]		
	R (%)	filtrate recovery(%)	losses (%)
1	28.1 d ± 1.6	8.4 a ± 0.5	8.0 b ± 1.6
2	3.3 a,b ± 1.4	36.7 c ± 1.2	7.2 a,b ± 3.3
3	13.5 c ± 8.0	35.1 c ± 6.4	8.1 b ± 5.4
4	11.5 b,c ± 3.7	15.2 b ± 8.9	8.7 b ± 1.5
5	11.4 b,c ± 6.4	35.7 c ± 4.1	15.5 c ± 0.2
6	14.6 c ± 4.8	52.7 d ± 7.3	16.0 c ± 6.7
7	0.8 a ± 0.1	89.8 e ± 2.3	2.7 a ± 1.5
8	3.0 a,b ± 1.0	92.3 e,f ± 2.4	5.6 a,b ± 1.2
9	2.3 a,b ± 1.8	94.2 e,f ± 2.2	4.1 a,b ± 2.0
10	2.7 a,b ± 1.0	94.2 e,f ± 2.0	4.7 a,b ± 2.1
11	2.7 a,b ± 1.4	57.7 d ± 2.4	8.9 b ± 0.5
12	2.2 a,b ± 1.6	93.6 e,f ± 3.2	4.7 a,b ± 2.9
13	2.1 a,b ± 0.1	93.3 e,f ± 0.1	5.5 a,b ± 0.5
14	6.3 a,b,c ± 0.1	91.3 e,f ± 0.1	7.9 b ± 0.2
15	2.4 a,b ± 0.1	96.5 f ± 0.2	2.5 a ± 0.1
16	0.5 a ± 0.4	97.8 f ± 0.1	n.a.[b]

[a] The same letter in a column indicates nonsignificant differences according to Duncan's test at the 0.05% level. [b] Data not available.

Table 6. Rejection (*R*), Filtrate Recovery (%), and Losses of Kaempferol Glycosides, Mean ± Standard Deviation, $n = 3$

centrifugal filter device	kaempferol glycosides[a]		
	R (%)[b]	filtrate recovery (%)[b]	losses (%)[b]
1	27.2 d ± 1.1	8.5 a ± 0.1	21.4 b,c ± 1.9
2	65.4 i ± 1.1	13.2 a,b ± 0.1	40.0 f ± 1.9
3	58.4 h ± 3.3	16.7 b,c ± 0.7	27.5 c,d ± 3.0
4	37.8 e,f ± 4.4	10.5 a ± 0.2	43.4 f ± 4.2
5	44.6 g ± 2.2	22.2 c ± 1.1	30.8 d,e ± 3.5
6	69.8 i ± 3.5	17.5 b,c ± 0.9	41.1 f ± 2.9
7	35.8 e ± 8.6	58.8 e ± 9.0	26.7 c,d ± 9.1
8	36.1 e ± 1.3	58.1 e ± 2.1	36.5 e,f ± 2.2
9	29.8 d ± 3.9	67.9 f ± 4.3	26.3 c,d ± 4.2
10	42.0 f,g ± 0.4	56.2 e ± 0.2	38.8 f ± 0.3
11	25.0 c,d ± 1.3	45.2 d ± 2.3	25.8 c,d ± 3.7
12	28.7 d ± 1.4	68.5 f ± 3.4	28.7 c,d ± 3.6
13	20.8 c ± 1.0	75.5 g ± 3.8	7.6 a ± 4.6
14	20.9 c ± 1.0	77.0 g ± 3.9	17.7 b ± 4.1
15	10.9 b ± 0.5	88.1 h ± 4.4	6.4 a ± 4.7
16	3.0 a ± 0.2	94.9 i ± 4.7	n.a.[c]

[a] Kaempferol glycoside content expressed as equivalent mass of rutin. [b] The same letter in a column indicates nonsignificant differences according to Duncan's test at the 0.05% level. [c] Data not available.

fractions from devices 13−16. The results for total kaempferol glycoside *R* (**Table 6**) reached values up to ∼70% (device 6) and did not decrease with increasing MWCO. Within the same type of devices, a decreasing kaempferol glycoside *R* was observed as the MWCO rose only in the group of devices 7, 9, and 15. The highest kaempferol glycoside filtrate recoveries were found from device 16 (∼95%) and the lowest, from devices 1, 2, and 4 (∼10%). Losses of kaempferol glycosides ranged from ∼6% (device 15) to ∼43% (device 4). Once more, the heterogeneity of results is noteworthy in relation to devices with the same MWCO membrane that could not be related to the membrane material or manufacturer. In the retentate fractions (**Figure 3B**), half of the devices were able to concentrate kaempferol glycosides. These devices, in an increasing order of concentration factors, were 8, 12, 7, 9, 1, 14, 13, and 15. Kaempferol glycoside concentration from the latter device was 4334 ± 217% of the initial concentration in the feed extract (data not shown in **Figure 3B** due to the scale).

Regarding each kaempferol glycoside (**Figure 6**), the highest kaempferol-3-sophoroside-7-glucoside *R* came from devices 2 and 6 (∼70%) and the lowest from devices 11−16. In addition,

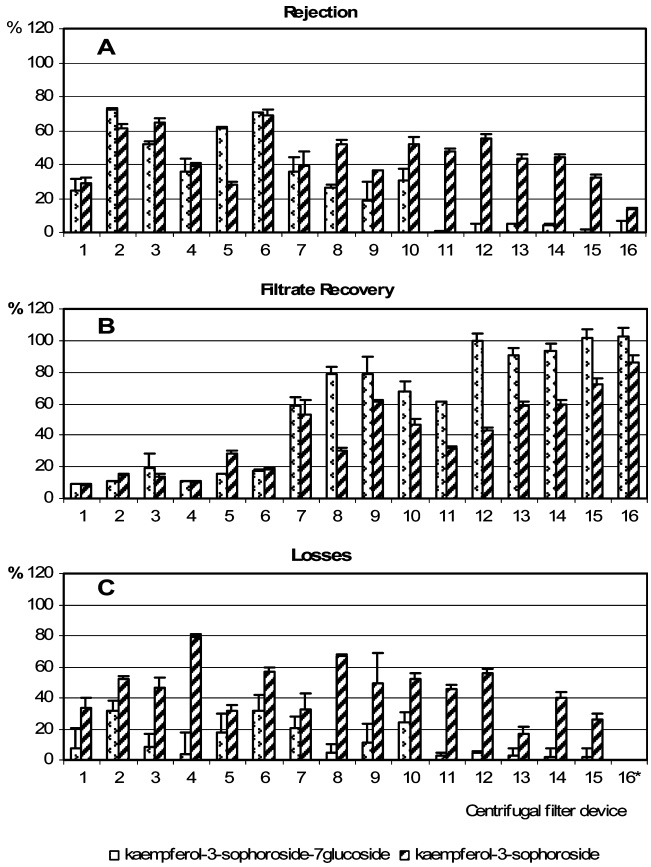

Figure 6. (**A**) Kaempferol glycoside rejection, *R* (%); (**B**) kaempferol glycoside filtrate recovery (%), and (**C**) kaempferol glycoside losses (%).*Data not available.

the highest kaempferol-3-sophoroside *R* were from devices 2, 3, and 6 (between 61 and 69%) and the lowest, from device 16 (~14%). No significant differences were found in the *R* of either quantified kaempferol glycoside with devices 1, 4, 6, and 7, but kaempferol-3-sophoroside-7-glucoside showed higher *R* than kaempferol-3-sophoroside with devices 2 and 5. On the contrary, lower *R* values from the former kaempferol glycoside were found with the remaining devices. Nearly total filtrate recoveries of kaempferol-3-sophoroside-7-glucoside were found for devices 12, 15, and 16, whereas the lowest values were found for devices 1, 2, and 4 (~10%). However, for kaempferol-3-sophoroside, filtrate recoveries extended from 8% for device 1 to 86% for device 16. In comparing both kaempferol glycosides, kaempferol-3-sophoroside-7-glucoside had similar (devices 1−4 and 6−7) or higher (devices 8−16) filtrate recoveries than kaempferol-3-sophoroside, with the sole exception of device 5. The highest losses of kaempferol-3-sophoroside-7-glucoside came from devices 2, 6, and 10 (~30%) and the lowest losses, from devices 11−15 (~5%). The highest loss of kaempferol-3-sophoroside came from device 4 (~79%) and the lowest, from device 13 (~17%). Kaempferol-3-sophoroside-7-glucoside had similar (devices 5 and 7) or lower losses (the rest of the devices) than kaempferol-3-sophoroside.

Membrane Selection for Concentration or Purification of Saffron Components. First, UF can be used for the separation of crocetin esters from picrocrocin. Different degrees of purification were obtained depending on the membrane employed. The best results corresponded to devices 1, 3, 4, 7, 8, 9, and 10, which had the highest coefficient between picrocrocin and crocetin ester filtrate recoveries since they let picrocrocin pass through the membrane while keeping a great deal of crocetin esters in their retentate fractions. The four latter devices were in the group of devices with the highest picrocrocin filtrate recoveries and the highest crocetin ester *R* as well. Therefore, they are a good choice for this separation. All of them had RC membranes except for device 8, which had a PES membrane. Besides, their higher MWCO in relation to the membranes in devices 1, 3, and 4 makes them more suitable for further studies in a pilot plant UF unit. However, device 1 can especially be considered for a second UF step, once the extract has been partially purified of crocetin esters, in order to obtain picrocrocin nearly free from crocetin esters.

Filtrate recoveries of kaempferol glycosides in devices 1, 4, and 16 were equal to or, in the remaining devices, lower than filtrate recoveries of picrocrocin; thus, a partial purification of picrocrocin was produced in most filtrate fractions.

Devices 7, 9, and 10 were also the most suitable ones for crocetin ester concentration in their retentate fractions. Devices 9 and 10 showed high losses of these compounds due to precipitation, making them also suitable for the purification of crocetin esters. Picrocrocin concentration was observed only in the retentate fractions from devices 9 and 15, but their picrocrocin filtrate recoveries were ~95% with a very low mass of picrocrocin remaining in the retentate fractions. Kaempferol glycoside concentration was attained in the retentate fractions of devices 8, 12, 7, 9, 10, 14, 13, and 15, in this order according to the increase in their concentration factors. Of these devices, the lowest kaempferol glycoside filtrate recoveries were found in devices 7−9, which consequently showed the most interesting results from this viewpoint.

In summary, this is the first time that the application of centrifugal UF to saffron spice is reported. Filtration profiles showed good results for most devices in short centrifugation times, which is very interesting from an analytical point of view. Results show that centrifugal UF modified the proportion of main saffron components in a vast range, making it possible to tailor crocetin ester, picrocrocin, and kaempferol glycoside proportions or even purify them by selecting the appropriate membrane. The possibility of using successive UF steps has also been inferred. Results from this research establish the first basis of knowledge for the application of UF to aqueous saffron extracts, and, although further studies will be conducted in a pilot plant UF unit, these promising results from commercially available membranes prompted the consideration of new UF applications in the analysis and modification of the principal saffron components with the subsequent repercussion on their associated properties.

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Supporting Information Available: Rejection (*R*), filtrate recovery (%) and losses of crocetin esters and picrocrocin determined from UV−vis data, mean ± standard deviation, $n = 3$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information Table. Rejection (*R*), Filtrate Recovery (%) and Losses of Crocetin Esters and Picrocrocin determined from UV-Vis data, Mean ± Standard Deviation, n = 3.

	crocetin esters[a]			picrocrocin[a]		
centrifugal filter device	*R* (%)	filtrate recovery (%)	losses (%)	*R* (%)	filtrate recovery (%)	losses (%)
1	98.9 k ± 0.3	0.1 a ± 0.1	14.6 a ± 2.68	42.9 g ± 6.5	6.7 a ± 0.7	7.5 a ± 2.9
2	51.1 e ± 2.8	18.6 c ± 0.9	27.5 c,d ± 3.38	18.4 d,e ± 0.6	31.0 c ± 0.4	12. 8 c ± 3.4
3	75.2 j ± 1.6	10.1 b ± 0.9	22.4 a,b,c ± 9.7	21.4 e,f ± 2.0	31.9 c ± 0.3	3.9 c ± 3.4
4	72.8 h,i,j ± 5.0	4.7 a,b ± 1.3	18.2 a,b ± 2.8	19.6 d,e ± 3.0	13.8 b ± 1.7	8.7 b ± 2.4
5	54.5 e,f ± 7.1	18.4 c ± 2.7	28.9 c,d ± 0.3	20.6 e,f ± 2.1	32.1 c ± 0.6	13.9 c ± 4.2
6	61.4 f,g ± 5.7	23.9 c,d ± 6.8	32.9 d ± 8.6	15.5 d ± 0.3	51.9 d ± 2.8	6.9 d ± 3.3
7	65.6 g,h ± 11.9	31.3 e,f ± 11.8	32.9 d ± 8.06	20.8 e,f ± 5.5	71.8 e ± 7.0	10.1 e ± 5.1
8	62.4 g ± 4.0	35.9 f ± 5.1	60.1 f ± 5.75	23.1 e,f ± 2.9	73.2 e ± 5.4	21.7 e ± 7.5
9	67.4 g,h,i ± 4.0	31.5 e,f ± 4.2	61.2 f ± 4.15	21.5 f ± 0.7	75.7 e ± 1.0	19.1 e ± 2.6
10	73.8 i,j ±0.5	25.5 d,e ± 0.6	69.4 g ± 0.10	25.1 f ± 1.9	72.6 e ± 1.6	24.6 e ± 2.0
11	25.6 c ± 1.0	44.2 g ± 1.4	22.4 a,b,c ± 1.6	9.6 c ± 2.0	53.7 d ± 2.0	10.2 d ± 2.2
12	41.2 d ± 1.5	56.3 h ± 1.7	41.2 e ± 1.3	4.9 b ± 1.1	91.1 g,h ± 1. 5	4.9 g ± 0.1
13	28.8 c ± 0.1	67.9 i ± 0.1	30.2 c,d ± 0.2	10.8 c ± 0.1	85.1 f ± 0.1	11.4 f ± 0.7
14	22.7 b,c ± 0.1	75.3 j ± 0.1	23.8 b,c ± 0.1	9.5 c ± 0.1	88.1 f,g ± 0.1	8.9 f,g ± 0.3
15	17.7 a.b ± 0.1	81.4 j ± 0.2	17.4 a,b ± 0.1	3.4 a,b ± 0.1	95.5 h ± 0.2	1.4 h ± 0.1
16	12.7 a ± 0.1	80.2 j ± 0.1	n.a.[b]	0.3 a ± 0.1	95.7 h ± 0.2	n.a.[b]

[a]The same letter in a column indicates non significant differences according to Duncan's test at the 0.05% level. [b]Data not available.

CAPÍTULO 6. CONCLUSIONES

CHAPTER 6. CONCLUSIONS

6. CONCLUSIONES

De los estudios realizados en esta tesis doctoral se derivan los siguientes logros y conclusiones:

1. Los extractos acuosos del azafrán especia presentan, al menos, 15 ésteres de crocetina distintos y se diferencian de los de gardenia en la presencia del *trans* di-(β-D-glucosil) éster de crocetina, en que tienen mayor proporción del *trans* (β-D-glucosil)-(β-D-gentiobiosil) éster de crocetina y menor proporción del *cis* di-(β-D-gentiobiosil) y del *trans* (β-D-gentiobiosil) éster de crocetina. Se han identificado tentativamente por primera vez cinco de estos ésteres de crocetina.

2. En los mismos análisis que para los ésteres de crocetina, se ha caracterizado la picrocrocina y los compuestos glicosilados relacionados con ella, identificando tentativamente en extractos acuosos de azafrán dos nuevos compuestos. De todos estos compuestos, el único encontrado tanto en azafrán como en gardenia fue el 2-metil 6-oxo-2,4-heptenato de O-β-D-gentiobiósido, que junto al compuesto conocido como genipósido conforman el perfil cromatográfico de gardenia a 250 nm.

3. La fracción de flavonoides del azafrán está formada por glicósidos de kaempferol, entre los que hemos identificado tentativamente el kaempferol-3-*O*-soforósido-7-*O*-glucósido, el kaempferol-3-*O*-soforósido, el kaempferol-3,7,4'-triglucósido, un kaempferol tetrahexósido y un kaempferol-3-dihexósido.

4. Existen diferencias significativas en el contenido en glicósidos de kaempferol de azafranes procedentes de España, Grecia, Irán y Marruecos. El análisis discriminante

ha permitido separar las muestras por su contenido en kaempferol-3-*O*-soforósido, poniendo de manifiesto la capacidad de estos flavonoides como marcadores del origen del azafrán especia.

5. Los estudios cinéticos de cada uno de los ésteres de crocetina en extractos acuosos indicaron sus semejanzas y diferencias de estabilidad ante tratamientos térmicos entre 5 y 70 ºC en oscuridad. En la mayoría de los casos, la degradación de los ésteres de crocetina seguía una cinética de primer orden.

6. La reacción de degradación de los ésteres de crocetina es la misma en extractos acuosos de azafrán y en una fracción enriquecida en ésteres de crocetina. Sin embargo, se ve afectada por factores externos que modifican los parámetros cinéticos y termodinámicos, haciendo que la degradación sea más pronunciada conforme aumenta la temperatura y cuando ha habido un proceso de purificación.

7. Los cambios en la proporción de cada uno de los ésteres de crocetina y en la picrocrocina, junto con la aparición de algunos productos de degradación, justifican las modificaciones que experimenta el espectro UV-vis de los extractos cuando se someten a tratamiento térmico. Así mismo, el poder colorante refleja de forma global los cambios que experimentan los ésteres de crocetina, estando los valores de sus parámetros cinéticos y termodinámicos situados entre los máximos y mínimos calculados para estos compuestos. Por el contrario, el parámetro $E_{1\text{cm}}^{1\%}$ 257 nm no refleja la evolución de la picrocrocina.

8. No se ha encontrado relación alguna entre la estabilidad de cada éster de crocetina y su estructura, es decir, el número de moléculas de glucosa que posee o la forma en que están unidas entre sí.

9. Los estudios cinéticos de la picrocrocina entre 5 y 100 ºC indican menor velocidad de degradación que los ésteres de crocetina, pudiéndose ajustar principalmente a cinéticas de orden dos, aunque también se han encontrado buenos ajustes para orden cero y uno, según la temperatura estudiada.

10. Se ha observado un importante efecto de la filtración sobre los parámetros cinéticos y termodinámicos de la degradación de la picrocrocina, favoreciendo su estabilidad.

11. Se han generado varios modelos PLS1 satisfactorios a partir del espectro UV-vis de extractos acuosos de azafrán para la determinación del contenido en los ésteres de crocetina *trans*-di-(β-D-gentiobiosil), *trans*-(β-D-glucosil)-(β-D-gentiobiosil), *cis*-di-(β-D-gentiobiosil) y *cis*-(β-D-glucosil)-(β-D-gentiobiosil), para el contenido total de crocinas y para la picrocrocina.

12. Se ha desarrollado un método de extracción en fase sólida y medida por espectrofotometría UV-vis del contenido de picrocrocina para el control de calidad del azafrán especia. Este método permite eliminar las interferencias causadas por los ésteres de crocetina en la medida del contenido de picrocrocina. En la validación interna, el método ha mostrado buenos resultados de selectividad, exactitud, linealidad, precisión, recuperación y sensibilidad. Además, es válido para muestras de distinto origen y categoría comercial y permite una mejor ordenación de las muestras por su contenido en picrocrocina que $E_{1\text{cm}}^{1\%}$ 257 nm y que Δ*E*pic.

13. Se ha caracterizado el funcionamiento respecto a los ésteres de crocetina, picrocrocina y flavonoides del azafrán de distintos dispositivos comerciales de ultrafiltración por centrifugación con membranas de MWCO desde 1 a 100 kDa. Los mejores resultados para separar y concentrar los compuestos se han obtenido con dispositivos de 5 y 10 kDa, la mayoría de los cuales poseían membranas de celulosa regenerada. La membrana de 1 kDa permitía la obtención de picrocrocina libre de ésteres de crocetina. Comparando el funcionamiento respecto a cada uno de los ésteres de crocetina, se ha observado un mayor rechazo de los menos polares.

14. Se ha establecido el umbral de percepción gustativa de la picrocrocina en 10 mg L^{-1} a un nivel de significación del 1%.

6. CONCLUSIONS

From the studies carried out in this doctoral thesis, the following conclusions can be made:

1. The aqueous extracts of saffron spice contain at least 15 different crocetin esters and can be differentiated from those of gardenia in the presence of *trans*-crocetin di-(β-D-glucosyl) ester, by their greater proportion of *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester and smaller proportion of *cis*-crocetin di-(β-D-gentiobiosyl) ester and *trans*-crocetin (β-D-gentiobiosyl) ester. For the first time, five of these compounds have been tentatively identified.

2. In the same analyses as those used for the crocetin esters, picrocrocin was characterised, along with related glycosylated compounds, with two new compounds from extracts of saffron being tentatively identified. Of all the compounds analysed, the only one found in both saffron and gardenia was O-β-D-gentiobiosyl ester of 2-methyl 6-oxo-hepta-2,4-dienoic acid, which, together with the compound known as geniposide makes up the chromatographic profile of gardenia at 250 nm.

3. The flavonoid fraction of saffron is made up of glycosides of kampferol, among which we have tentatively identified the kaempferol-3-*O*-sophoroside-7-*O*-glucoside, the kaempferol-3-*O*-sophoroside, the kaempferol-3,7,4'-triglucoside, a kaempferol tetrahexoside and a kaempferol-3-dihexoside.

4. Saffrons from Spain, Greece, Iran and Morocco show significant differences in the kampferol glycosides they contain. A discriminant analysis separated the samples by their respective kaempferol-3-*O*-sophoroside contents, underlining the ability of these flavonoids to act as markers of the origin of saffron spice.

5. Kinetic studies of the crocetin esters in the aqueous extracts identified the similarities in their respective stabilities in the face of heat treatments between 5 and 70 ºC in darkness. In most cases, a first order kinetic was followed.

6. The degradation reaction of the crocetin esters was the same in aqueous extracts of saffron and in a crocetin ester rich fraction. However, it was affected by external factors that modified its kinetic and thermodynamic parameters, degradation becoming more pronounced as temperature increased and when the compounds had undergone a purification process.

7. The changes in the proportion of each crocetin ester and picrocrocin, together with the appearance of some degradation products would explain the changes observed in the UV-vis spectra of the extracts upon thermal treatment. Moreover, the colouring strength reflects in a general way the changes in the degradation rates of the crocetin esters, the values of their kinetic and thermodynamic parameters falling between the maximum and minimum values calculated for these esters. On the contrary, the parameter $E_{1cm}^{1\%}$ 257 nm evolved differently from picrocrocin.

8. No relation was found between the stability of each particular crocetin ester and its structure, i.e., the number of glucose molecules that they possess or the way in which they are bound.

9. Kinetic studies of picrocrocin between 5 and 100 ºC pointed to lower degradation rates than in crocetin esters, which could be mainly fitted to a second-order kinetic model, though good fits were also found in zero and first-order models according to the temperature.

10. Filtration had a substantial effect on the kinetic and thermodynamic parameters of picrocrocin degradation, contributing to its stability.

11. Several PLS1 models were generated from the UV-vis spectra of the aqueous extracts, which gave satisfactory results for determining the *trans*-di-(β-D-gentiobiosyl), *trans*-(β-D-glucosyl)-(β-D-gentiobiosyl), *cis*-di-(β-D-gentiobiosyl), *cis*-(β-D-glucosyl)-(β-D-gentiobiosyl) esters of crocetin, and the total crocetin ester and picrocrocin contents.

12. A solid phase extraction method and UV-vis spectrophotometric measurement of the picrocrocin content have been proposed for quality control of saffron spice. The method would eliminate the interferences caused by crocetin esters in the UV-vis spectrophotometric measurement of the picrocrocin content. The method proposed showed good selectivity, accuracy, linearity, recovery and sensitivity in an internal validation. The method is also valid for samples of different origins and commercial categories, permitting better grading of saffron samples according to the picrocrocin content rather than the $E_{1cm}^{1\%}$ 257 nm or Δ*E*pic values.

13. The performance of several commercial centrifugal filter devices with membranes of 1-100 kDa was tested for separation and concentration of crocetin esters, picrocrocin and flavonoids of saffron. The best results for separating and concentrating the compounds were obtained with devices with 5 and 10 kDa membranes, most of which were equipped with regenerated cellulose membranes. The 1 kDa membrane permitted picrocrocin to be obtained free of crocetin esters. Comparing the performance for each crocetin ester, greater rejection of the less polar ones was generally observed.

14. The taste perception threshold of picrocrocin was set at 10 mg L^{-1} at a 1% significance level.

CAPÍTULO 7. BIBLIOGRAFÍA

CHAPTER 7. BIBLIOGRAPHY


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