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Analytical Methods

Analytical determination of flavonoids aimed to analysis of natural samples and active packaging applications

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ABSTRACT

Several HPLC and UHPLC developed methods were compared to analyse the natural antioxidants catechins and quercetin used in active packaging and functional foods. Photodiode array detector coupled with a fluorescence detector and compared with LTQ-Orbitrap-MS was used. UHPLC was investigated as quick alternative without compromising the separation, analysis time shortened up to 6-fold. The feasibility of the four developed methods was compared. Linearity up to 0.9995, low detection limits (between 0.02 and 0.7 for HPLC-PDA, 2 to 7-fold lower for HPLC- LTQ-Orbitrap-MS and from 0.2 to 2 mg L^{-1} for UHPLC-PDA) and good precision parameters (RSD lower than 0.06%) were obtained. All methods were successfully applied to natural samples. LTQ-Orbitrap-MS allowed to identify other analytes of interest too. Good feasibility of the methods was also concluded from the analysis of catechin and quercetin release from new active packaging materials based on polypropylene added with catechins and green tea.

1. Introduction

Oxidative stress and the consequent release of free oxygen radicals seem to be associated with many diseases, such as cancer, cardiovascular malfunction or inflammatory disorders (Mastelic et al., 2008). Due to their increasing incidence, considerably attention has been paid to natural substances with antioxidant activity; which inhibit or delay the reaction of oxidation.

Natural antioxidants, and specially the flavonoids catechins and quercetin have attracted considerable attention showing an important antioxidant activity (Dopico-García et al., 2011). Tea, and especially green tea (*Camellia sinensis*), is one of the most widely consumed beverages worldwide, second only to water, and is a natural source of those antioxidant compounds, which confers tea with important antioxidant and thus, health effects (Gramza & Korczak, 2005). The strong antioxidant capacity of catechins has been reported to have a protective and beneficial health effect related to anti-mutagenic, anti-diabetic, anti-inflamatory, anti-bacterial and anti-viral qualities and prevention against several kinds of cancer. Moreover, they seemed to be effective in against heart and liver diseases, slowing aging and neurodegenerative processes and enhancing weight loss, among others (Braicu, Ladomery, Chedea, Irimie, & Berindan-Neagoe, 2013; Shi & Chelegel, 2012). Quercetin also provide tea with antioxidant and mood-cognitive-enhancing properties (Nijveldt et al., 2001).

Resulting from a response to trends in consumer preferences towards mildly preserved, fresh, tasty and convenient food products with a prolonged shelf-life, their use in active packaging and functional foods has become increasingly significant. Those active agents provide active antioxidant functions to the packaging that allow the shelf-life of the packaged food to be extended or to improve the safety or sensory properties while maintaining the quality of the food (Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999). Due to that importance on current consumer demands and market trends, it is fundamental to determine release levels in food matrices using suitable analytical methods.

Therefore, efficiency, speed and cost of analysis have become of a great importance, especially, aimed to routine analysis where it is important to increase throughput and reduce analysis costs. Ways to improve resolution, speed and sensitivity of chromatographic methods have still continued under investigation.

The most developed methods for catechins determination have been established based upon reversed-phase HPLC coupled to PDA (Dalluge & Nelson, 2000; Molnár-Perl & Füzfai, 2005). The desire for higher resolution of catechins has led to consider the use of acidic mobile phases as essential. Column degradation resulting from the lack of stability of the silica-based bonded reversed-phase columns used under low pH conditions could be risky, though (Lipper et al., 2007). High flow values (~1 mL min^{-1}), high analysis times (20–90 min), complex mobile phases, determination of only some catechins or rather scarce simultaneous determination of them, and/or unsatisfactory quantifications limits and resolutions also make those methodologies little useful aimed both to routine

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analysis and real samples analysis (Dalluge & Nelson, 2000; El-Shahawi, Hamza, Bahaffi, Al-Sibaai, & Abduljabbar, 2012; Molnár-Perl & Füzfai, 2005; Qin, Li, Tu, Ma, & Zhang, 2012; Samanidou, Tsagiannidis, & Sarakatsianos, 2012; Scoparo et al., 2012; Wang et al., 2012; Wei et al., 2012; Wu, Xu, Héritier, & Adlauer, 2012).

The difficulty in interpreting some complex sample chromatograms also makes necessary to optimise the detection stage (Schieber, Keller, & Carle, 2001). To this end, other detectors have been proposed. Fluorescence (FL) in order to avoid interferences with other compounds discriminating between fluorescent and non-fluorescent overlapping peaks; electrochemical detectors (ECD), proving to be a useful completion technique to the analysis of phenolic compounds providing special selectivity related with the diversities in electro active substituents or mass spectrometer (MS) frequently used to obtain structural information after chromatographic separation, are examples of the detectors used (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013; Molnár-Perl & Füzfai, 2005; Novak, Janeiro, Seruga, & Oliveira-Brett, 2008; Novak, Šeruga, & Komorsky-Lovric, 2010; Porgali & Büyûktuncel, 2012; Qin et al., 2012; Wu et al., 2012).

Ultra-high performance liquid chromatography (UHPLC) has also been considered as a new direction of liquid chromatography (Spácil, Nováková, & Solich, 2010). Nevertheless, the very few reports targeted on the determination of catechins lacked in its application to the analysis of real samples in terms of peak resolution, sample matrix effect or detection of some aimed compounds.

Determination of quercetin and catechins in the same run has been very rarely reported, despite being present together and with a similar content and antioxidant capacity in several natural matrices (Dimitrios, 2006).

Therefore, several chromatographic methods have already been reported for a detailed characterization of the antioxidant content of some extracts. Nevertheless, the aim of the present work deal more with studying and selecting the most useful and profitable chromatographic method to evaluate the most outstanding contribution of some extracts on both their daily-intake or their use as active additives in active packaging and in functional foods. Due to their reported antioxidant capacity (Dopico-García et al., 2011; Gramza & Korczak, 2005) catechin and green tea extract, with high content of catechins, were selected in this work as active agents.

Thus, the feasibility of HPLC-PDA-FL, HPLC-PDA-LTQ Orbitrap MS and UHPLC-PDA methodologies for the routine simultaneous determination of major and minor catechins and quercetin was compared in terms of best resolution and highest sensitivity of detection. The proposed methodologies were also evaluated assaying the catechins and quercetin content in natural samples and the release levels form the active antioxidant films developed.

2. Experimental

2.1. Chemicals and standards

Methanol (>99.8%) and ethanol (>99.9%) HPLC-gradient for instrumental analysis were supplied by Merck (Darmstadt, Germany). Formic acid 98–100% puriss-p.a. was from Sigma–Aldrich (Steinheim, Germany). Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA). (−)-Epigallocatechin (EGC) (>95%), (−)-Epigallocatechin gallate (EGCG) (80%), (−)-Epicatechin gallate (ECG) (>98%), (−)-Epicatechin (EC) (>98%), (+)-Catechin hydrate (C) (>98%), (−)-Gallocatechin Gallate (GCG) (>98%), (−)-Catechin Gallate (CG) (>98%), quercetin (Quer) (>95%) and Irgafos 168 (Tris(2,4-di-tert-butylphenyl)phosphate; I168) (purity not specified) standards were purchased from Sigma–Aldrich.

2.2. Standard preparation

Individual stock standard solutions (1000 mg L^{-1}) were prepared in an aqueous solution of formic acid (pH 1.5) for catechins and ethanol for quercetin. Work standard solution containing all compounds (20 mg L^{-1} for catechins and 5 mg L^{-1} for quercetin) was prepared from individual stock standard solutions in aqueous-formic acid solution at pH 1.5. Work standard solution at concentration of 5 mg L^{-1} of catechins and 0.5 mg L^{-1} for quercetin was prepared for HPLC-MS analysis. Stability of each stock and work standard solution was tested and corroborated trough a period of time of 60 days.

2.3. Sample preparation

The following natural samples were tested. Red and white tea commercialized in infusion bag, green and black tea commercialized in bulk and cocoa, were purchased in local supermarkets. Tutsan and lemon verbena were kindly donated by Serviço de Farmacognosia, Faculdade de Farmácia, University of Porto, Portugal. Grape residues, barley shell and chestnut hedgehog were kindly obtained from Department of Chemical Engineering, University of Vigo, Spain. Hop was obtained from a local farming, and residue of beer fabrication were kindly donated by Estrella de Galicia beer company, Galicia, Spain. Samples were extracted in Milli-Q water (1 g of sample: 25 mL of water) under magnetic stirring for 10 min accordingly to common daily consumption way. Formic acid was then added (50 μL of formic acid: 1 mL of extracted sample) to each extract. The final samples were filtered through an **Acrodisc^{R} PTFE CR 13 mm, 0.2 μm filters (Waters, Mildford, MA, USA) and transferred into HPLC vials.

2.4. Preparation of antioxidant films

PP-catechin-containing films and PP-green tea-containing films were obtained by extrusion. Catechin and green tea were incorporated at two levels of concentration: 2% and 5% (w/w). Irgafos 168 at 0.2% (w/w) was added to protect films during manufacture process. Extrusion was carried out using a miniextruder equipped with twin conical co-rotating screws and a capacity of 7 cm^3 (Minilab Haake Rheomex CTW5 (Thermo Scientific)). Screw rotation rate of 40 rpm, temperature of 180 °C and 1 min of residence time were used.

2.5. Antioxidant release tests

A study of the release of the active compounds from the films was carried out by determining the specific migration from the polymer into food simulants A (10% ethanol) and D1 (50% ethanol) as specified in European Commission Regulation N° 10/2011 (The European Commision, 2011). Total immersion of rectangular strips film pieces (80 × 0.4 × 0.17 mm) in 10 mL of food simulant at 40 °C and 5 and 10 days of storage were the conditions selected. After the contact period, an aliquot of food simulant was filtered and analysed by means of HPLC. Data was statistical analysed by a one-way analysis of variance (ANOVA) test using the SPSS statistics software (SPSS Inc., Chicago, IL). Data was expressed as the mean ± standard deviation. Box plot representations were also used to display differences between groups of data.

2.6. Chromatographic conditions

2.6.1. HPLC-PDA-FL analysis

A Waters 2695 (Waters) system was used for HPLC analysis. SunFire™ C_{18} (150 × 3.0 mm, 3.5 μm) (Waters) kept at 35 °C, photodiode array detector (PDA, model 996 UV) set in the range of