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Analytical Methods

Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS

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ABSTRACT

The food and agricultural products processing industries generate substantial quantities of phenolics-rich subproducts, which could be valuable natural sources of polyphenols. In oranges, the peel represents roughly 30% of the fruit mass and the highest concentrations of flavonoids in citrus fruit occur in peel. In this work we have carried out the characterisation and quantification of citrus flavonoids in methanolic extracts of bitter and sweet orange peels using CE–ESI–IT–MS. Naringin (m/z 579.2) and neohesperidin (m/z 609.2) are the major polyphenols in bitter orange peels and narirutin (m/z 579.2) and hesperidin (m/z 609.2) in sweet orange peels. The proposed method allowed the unmistakable identification, using MS/MS experiments, and also the quantification of naringin (5.1 ± 0.4 mg/g), neohesperidin (7.9 ± 0.8 mg/g), narirutin (26.9 ± 2.1 mg/g) and hesperidin (35.2 ± 3.6 mg/g) in bitter and sweet orange peels. CE coupled to MS detection can provides structure-selective information about the analytes. In this work we have developed a CE–ESI–IT–MS method for the analysis and quantification of main phenolic compounds in orange peels.

1. Introduction

Polyphenols are amongst the most popular antioxidants and many natural sources are being suggested for their recovery (Tura, 2002). Crud extract of fruits, herbs, vegetables, cereals, nuts and other plant material rich in phenolics are increasingly of interest in the food industry (Sang et al., 2002). Citrus is a common term and genus of flowering plants in the family Rutaceae, originating in tropical and subtropical areas in southeast Asia. Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids (a kind of terpenes) contained in the peel, they are also good sources of vitamin C and flavonoids. Cultivated Citrus may be derived from as few as four ancestral species. Numerous natural and cultivated origin hybrids include commercially important fruit such as the orange, grapefruit, lemon, some limes, and some tangerines. Oranges are one of the most popular fruits in the world. Orange processing in the United States produces ~700.000 tons of peel as byproduct solids annually (Winter, 1995). Plant material wastes from these industries contain high levels of phenolic compounds. Importantly, most of this phytonutrient is found in the orange peel and inner white pulp, rather than in its liquid orange centre, so this beneficial compound is too often removed by the processing of oranges into juice. Polyphenols compounds have health-related properties, which are based on their antioxidant activity including anticancer, antiviral and antiinflammatory activities (Bouskela, Cyrino, & Lerond, 1997; Tanaka et al., 1997). The group of flavonoids is a widely distributed group of polyphenolic compounds according to the above fact. Flavonoids in orange peel are comprised primarily of flavanone glycosides (narirutin 4′-O-glucoside, eriocitrin, narirutin, hesperidin, isosakuranetin rutinoside), polymethoxylated flavone aglycons (sinensetin, hexa-O-methylquercetagetin, nobiletin, hexa-O-methylgossypetin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, tetra-Omethylscutellarein, tangeritin and 5-hydroxy-3,7,8,3′,4′-pentamethoxyflavone) (Horowitz & Gentili, 1977), flavone glycosides (diosmin, isorhoifolin, rutin) (Kanes, Tisserat, Berhow, & Vandercook, 1993) and C-glycosylated flavones (6,8-di-C-glucosylapigenin) (Manthley & Grohmann, 2001). Naritutin, hesperidin, naringin and neohesperidin (Fig. 1) are the most abundant flavonoids in the edible part of many species of citrus fruits (Kawai, Tomono, Katase, Ogawa, & Yano, 1999). As is well documented naritutin and hesperidin have been determined in common sweet orange (Ooghe, Ooghe, Detavernier, & Huyghebaert, 1994), and it is worthwhile referring to the recovery of hesperidin and naringin from orange peel (El-Nawawi, 1995), which is considered to be the most popular source, recovery of naringin from bitter orange (Calvarano, 1996).

Even though the characterisation of phenolic compounds in orange has been successfully carried out using HPLC (Anagnostopoulou, Kefalas, Kokkalou, Assimopoulou1, & Papageorgiou1, 2005; Belajová & Suhaj, 2004; Justesen, Knuthsen, & Leth, 1998; Kanaze,

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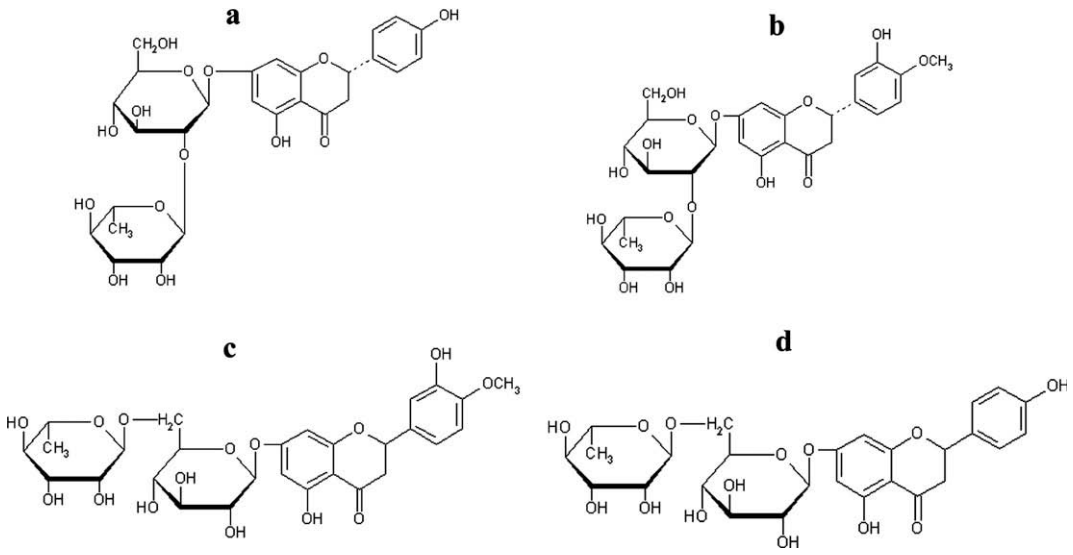

Fig. 1. Chemical structures of: (a) naringin, (b) neohesperidin, (c) hesperidin and (d) narirutin.

Gabrieli, Kokkalou, Georgarakis, & Niopas, 2003; Theodoridis et al., 2006). Capillary electrophoresis (CE) has become an alternative or complement to chromatographic separations because it needs no derivatization step, requires only small amounts of sample and buffer and has proved to be a high-resolution technique (Arráez-Román, Gómez-Caravaca, Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2006). The hyphenation of CE as analytical separation technique coupled to mass spectrometry (MS) as detection system can provide important advantages in food analysis because of the combination of the high separation capabilities of CE and the power of MS as identification and confirmation method (Arráez-Román et al., 2007; Gómez-Romero et al., 2007; Simó, Barbas, & Cifuentes, 2005). In general, if a separation technique is coupled with MS the interpretation of the analytical results can be more straightforward (Brocke, Nicholson, & Bayer, 2001; Macià, Borrull, Calull, & Aguilar, 2004; Schmitt-Kopplin & Frommberger,

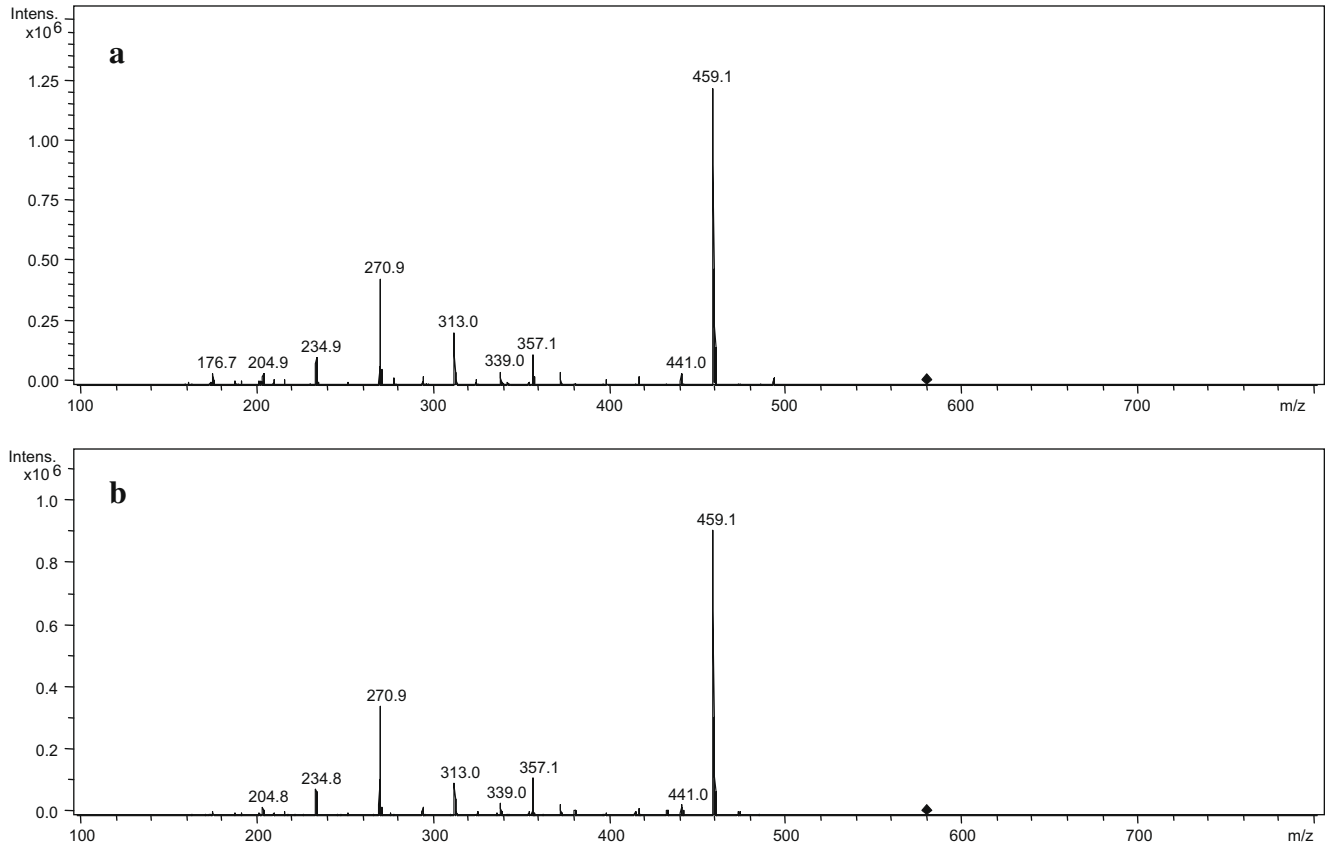

Fig. 2A. (a) MS/MS naringin (m/z 579.2) standard, (b) MS/MS naringin (m/z 579.2) in bitter orange peel sample.

2003). Furthermore, MS/MS experiments using a ion trap (IT) can be used to obtain fragment ions of structural relevance for identifying target compounds in a highly complex matrix. In this sense, electrospray ionisation (ESI) has emerged as a highly useful technique which allows direct coupling with electrophoretic separation techniques (Smith & Udseth, 1996).

The aim of this present work has been to develop a simple CE–ESI–IT–MS method for the identification and quantification of main phenolic compounds in orange peel due to these compounds are the most abundant components in all the orange parts and present a high concentration (El-Nawawi, 1995; Horowitz & Gentili, 1977).

2. Material and methods

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade and used as received. Boric acid, purchased from Sigma–Aldrich (St. Louis, MO), and ammonium hydroxide from Merck (Darmstadt, Germany) were used for preparing the CE running buffers at different concentrations and pH values. Buffers were prepared by weighing the appropriate amount of boric acid at the concentrations indicated and adding ammonium hydroxide (0.5 M) to adjust the pH. The buffers were prepared with doubly deionized water, stored at 4 °C and brought to room temperature before use. Doubly deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA). 2-Propanol HPLC grade used in the sheath flow, methanol, ethanol, hexane, DMSO and sodium hydroxide, used for capillary cleaning procedures before each analysis, were obtained from Panreac (Barcelona, Spain) and triethylamine from Aldrich (Steinheim, Germany). All solutions were filtered through a 0.45 μm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary. Naringin, neohesperidin, narirutin and hesperidin standards used for MS/MS experiments and calibration curves were obtained from Extrasynthese (Lyon, France).

2.2. CE–ESI–IT–MS apparatus

The analyses were made in a P/ACE™ System MDQ (Beckman Instruments, Fullerton, CA, USA), CE apparatus equipped with an UV–Vis detector and coupled to the MS detector by an orthogonal electrospray interface (ESI). The system comprises a 0–30 kV high-voltage built in power supplier.

All capillaries (fused-silica) used were obtained from Beckman Coulter Inc. (Fullerton, CA, USA) and had an inner diameter (i.d.) of 50 μm. A detection window was created at 10 cm for the UV detector and 100 cm was the total length (corresponding to the MS detection length). The instrument was controlled by a PC running the 32 Karat System software from Beckman.

MS and MS/MS experiments were performed on a Bruker Daltonics Esquire 2000™ ion-trap mass spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal coaxial sheath-flow electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). This triple tube ESI–MS interface provides both a coaxial sheath liquid make-up flow and a nebulization gas to assist droplet formation. The drying gas and the nebulization gas were both nitrogen. The coaxial sheath liquid and the electrical contact at the electrospray needle tip were delivered by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA).

For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation

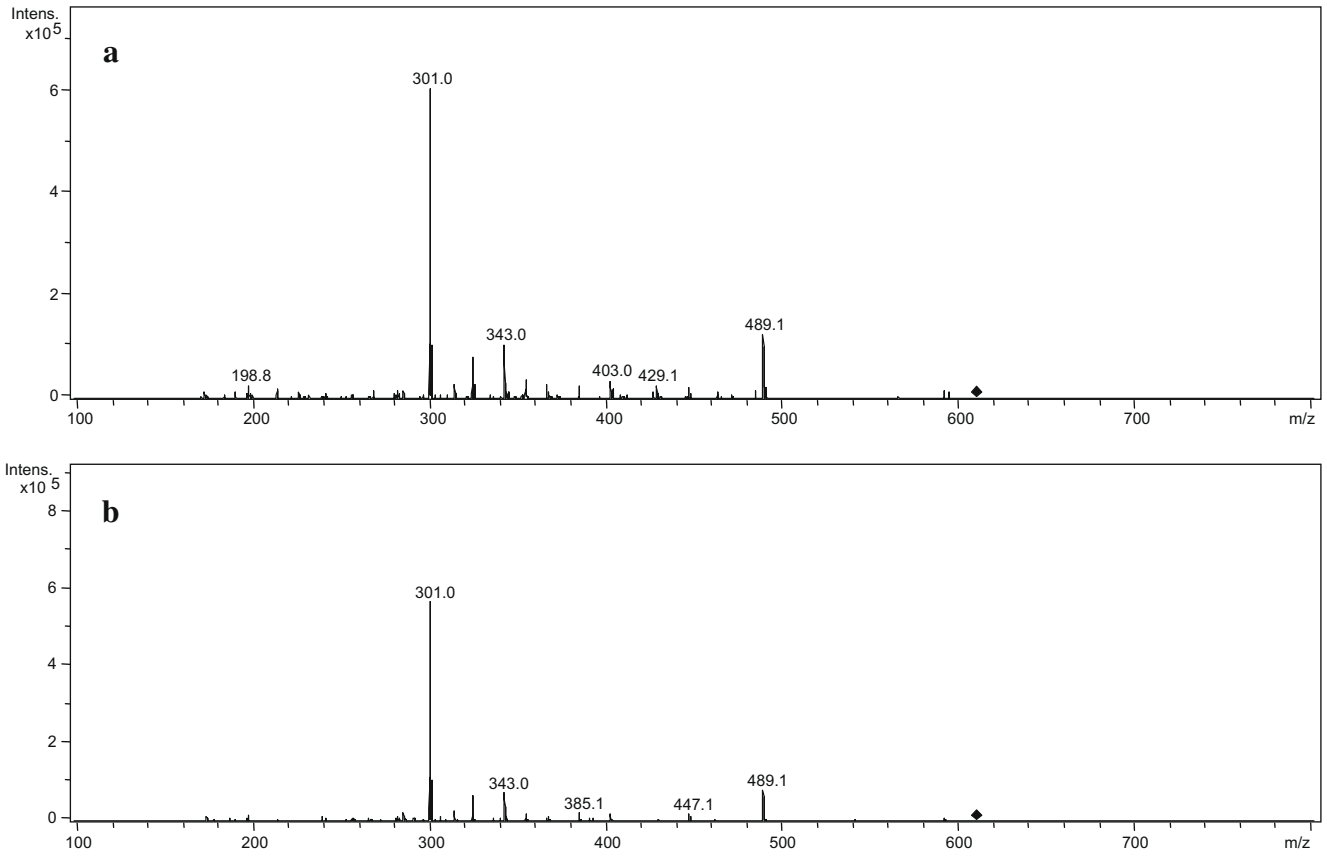

Fig. 2B. (a) MS/MS neohesperidin (*m*/*z* 609.2) standard (b) MS/MS neohesperidin (*m*/*z* 609.2) in bitter orange peel sample.

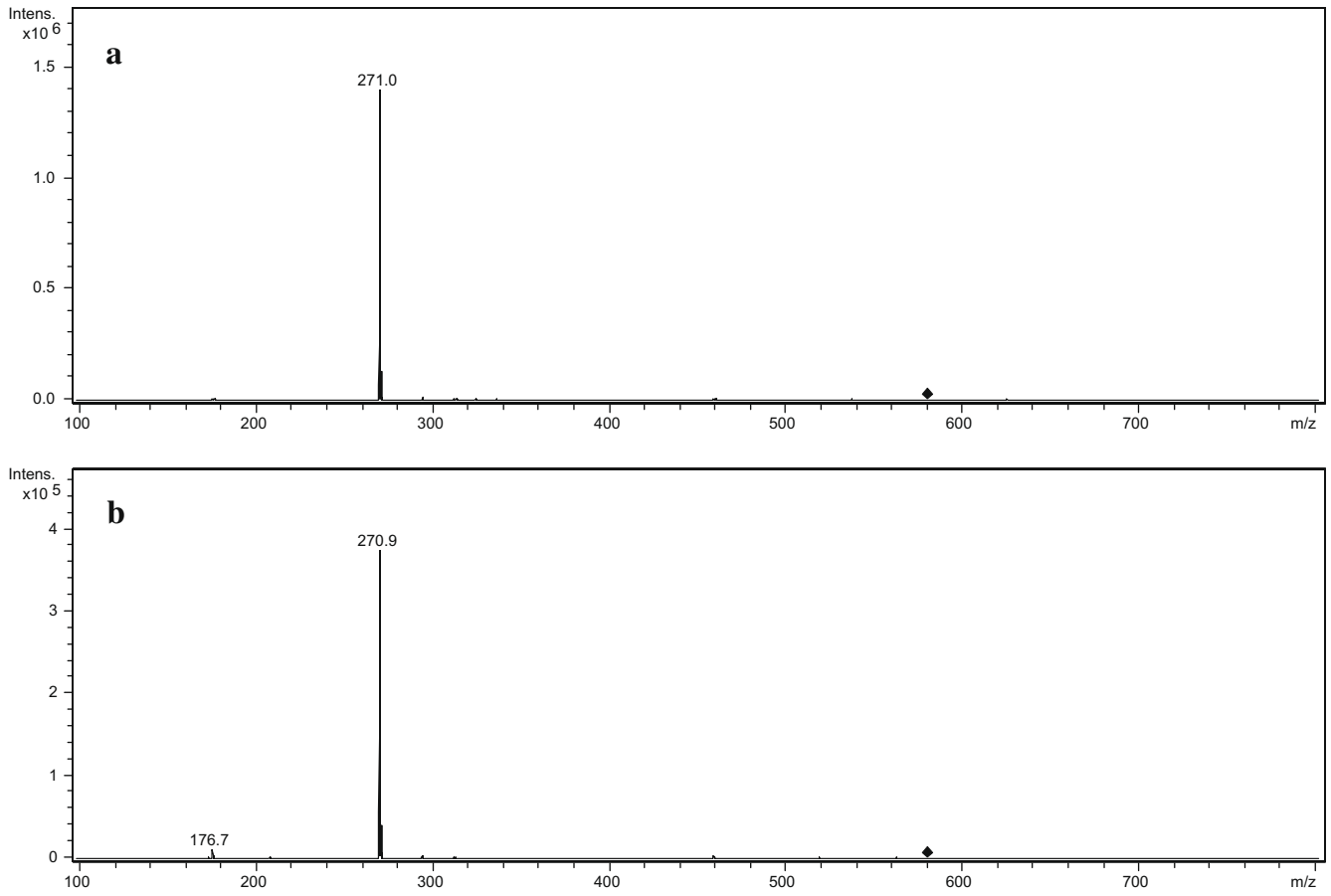

Fig. 3A. (a) MS/MS narirutin (*m*/*z* 579.2) standard, (b) MS/MS narirutin (*m*/*z* 579.2) in sweet orange peel sample.

capillary was fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid established electrical contact between the capillary effluent and water for the electrospray needle. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonics.

2.3. Extraction procedures

Five extraction procedures were prepared in order to choose the best conditions for the extraction of naringin, neohesperidin, narirutin and hesperidin from the orange peel samples. Basically, the extraction procedures are very similar but some modifications have been carried out. The conditions of each extraction procedure were as follows.

2.3.1. Extraction A

0.2 g of the dried sample were weighted and extracted with 10 ml of methanol, the solution was shaken on vortex for 5 min and then centrifuged at 4500 rpm for 10 min. The solution was filtered through 0.2 μm filter and collected in a round bottom flask. The concentrated methanol was evaporated by rotary pump at 40 °C, and the sample re-dissolved using 2 ml of MeOH:DMSO (50:50, v/v). Finally the extract was kept in the freezer until the analysis. The samples were diluted 1:1 in water before analysis.

2.3.2. Extraction B

The same as extraction procedure A but the solution was shaken with magnetic stirrer for 2 h.

2.3.3. Extraction C

The same as extraction procedure A but the dry residue was resolved in 2 ml of MeOH:H_2O (50:50, v/v).

2.3.4. Extraction D

0.2 g of the sample were weighted and extracted with 10 ml of MeOH:DMSO (50:50, v/v) The solution was shaken at a room temperature for 2 h and then centrifuged at 4500 rpm for 10 min. The solution was filtered through 0.2 μm filter. Finally the samples were kept in the freezer until analysis. The samples were diluted 1:1 in water before analysis.

2.3.5. Extraction E

The same as extraction procedure D but the solution was shaken on vortex for 5 min.

2.4. CE–ESI–IT–MS procedure

In order to develop the CE–ESI–IT–MS method, to obtain the best selectivity, sensitivity and resolution, the extract C previously described was used.

CE separation was carried out on a fused-silica capillary of 50 μm i.d. with a total length of 100 cm (corresponding to the MS detection length).

Before first use, the bare capillaries were conditioned by rinsing with 0.5 M sodium hydroxide for 20 min, followed by a 10 min rinse with water. Capillary conditioning was done by flushing for 2 min sodium hydroxide, 4 min with water, and then for 10 min

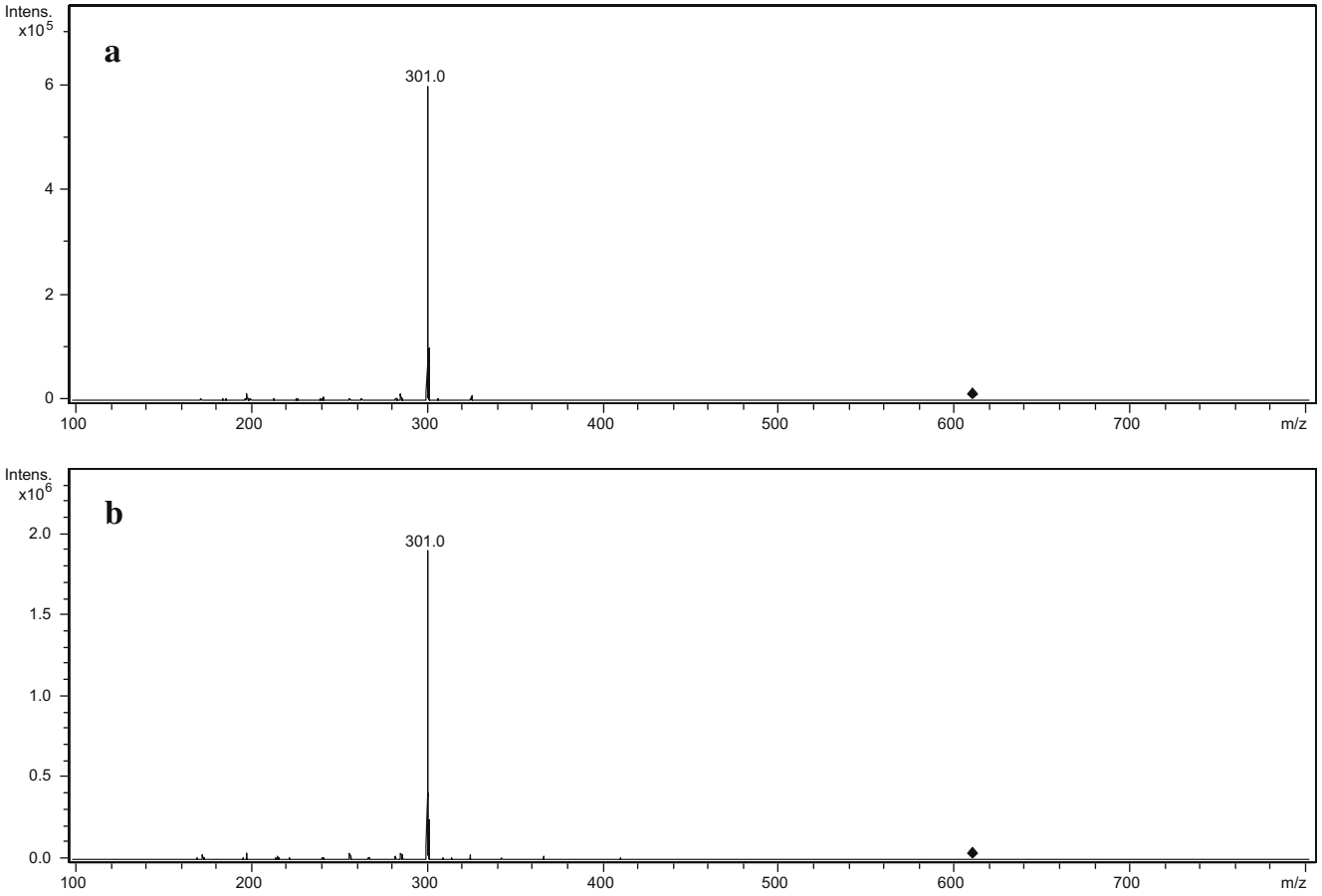

Fig. 3B. (a) MS/MS hesperidin (*m*/*z* 609.3) standard, (b)MS/MS hesperidin (*m*/*z* 609.3) in sweet orange peel sample.

Table 1
Analytical parameters of the proposed method.

Analyte	RSD	LOD (mg/l)	LOQ (mg/l)	Calibration range (mg/l)	Calibration equations	R^2
Naringin	2.35	0.99	3.30	5–50	$y = 505738x + 2E + 06$	0.9858
Neohesperidin	2.62	0.23	0.72	5–50	$y = 640452x + 1E + 06$	0.9886
Narirutin	2.71	0.38	1.58	25–80	$y = 532136x - 9E + 06$	0.9974
Hesperidin	3.50	1.15	3.85	25–80	$y = 152140x - 0.821550$	0.9996

with the separation buffer. During all the capillary conditioning was used a pressure of 20 ψ (1 ψ = 6895 Pa). At the end of the day the capillary was rinsed for 10 min water and 5 min flush air. The CE conditions used in the method were a buffer solution of 200 mM boric acid adjusted with ammonium hydroxide at pH 9.5. Samples were injected hydrodynamically in the anodic end in low pressure mode (0.5 ψ) for 5 s. Electrophoretic separations were performed at 25 kV which caused a current intensity of 40 μA.

The optimum ESI–IT–MS parameters were a sheath liquid isopropanol/water 60:40 with 0.1% (v/v) TEA delivered at a flow rate of 0.28 ml/h, a drying gas flow rate of 5 l/min at 300 °C, compound stability 25% and a nebulizer gas pressure of 6 ψ was supplied for ESI formation.

The mass spectrometer was run in the negative ion mode and the capillary voltage was set at 4000 V. The ion trap scanned at 100–800 *m*/*z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 5.00 ms, the target count at 20,000 and the trap drive level at 100%.

3. Results and discussion

3.1. Selection of extraction procedure

The CE–ESI–IT–MS method was applied to the analysis of main polyphenols in bitter and sweet orange peel extracts (see Section 2.3). Under the optimised CE–ESI–IT–MS conditions described above it is possible to analyse main compounds in the different types of extraction procedures and to carry out a comparative study of the extraction capacity. The compounds with *m*/*z* 579.2, from sweet and bitter orange peels, were extracted using the procedures A–C; the compounds with *m*/*z* 609.2, from sweet and bitter orange peels, were extracted using the procedures C–E. Therefore, the extraction procedure C has been selected due to presence of the target compounds in the extract.

3.2. Identification of main polyphenols by MS/MS analysis

The peaks of the main phenolic compounds in orange peel were easily identified by comparing both migration time and MS/MS

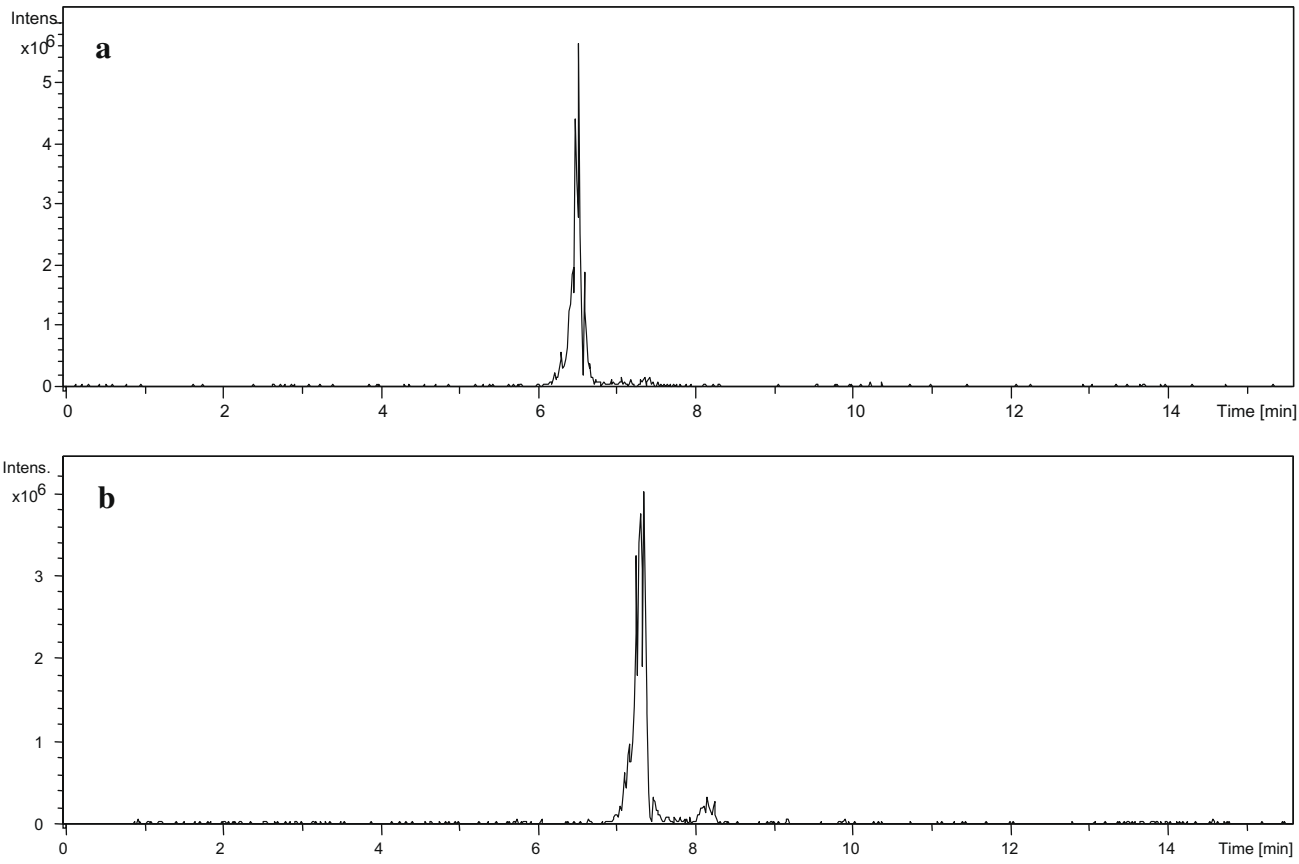

Fig. 4A. Extracted ion electropherograms of: (a) naringin and (b) neohesperidin in bitter orange peel sample.

data obtained from bitter and sweet orange peel samples with standards. MS/MS can be used to obtain fragment ions of structural relevance for identifying target compounds in a highly complex matrix. As these compounds had the same (m/z): naringin and narirutin (m/z 579.2), neoheredin and hesperedin (m/z 609.2), MS/MS experiments of both kinds of samples comparing with the MS/MS of standards were useful in order to identify these compounds. Figs. 2A and 2B show the MS/MS spectra of naringin and neohesperidin standards and in the bitter orange peel sample. Besides, Figs. 3A and 3B show the MS/MS spectra of narirutin and hesperidin standards and in the sweet orange peel sample. Thus, using the MS/MS spectra it is possible to prove that the compounds under the current study correspond with the assignment proposed.

3.3. Analytical parameters of the method proposed

We carried out a study to check the repeatability of the proposed method, as well as to establish the calibration curves to quantify naringin and neohesperidin in bitter orange peel and narirutin and hesperidin in sweet orange peel.

3.4. Repeatability study

Repeatability of the CE–ESI–IT–MS analysis was studied by performing series of separations using the optimised method on the extracts in the same day (intraday precision, $n = 5$) and on three consecutive days (interday precision, $n = 15$). The relative standard deviations (RSDs) of analysis time and peak area were determined. The intraday repeatability of the analysis time (expressed as RSD) was 0.22%, whilst the interday repeatability was 0.89%. The intraday repeatability of the peak area (expressed as RSD) was 6.5%, whilst the interday repeatability was 6.9% adequate for the aim of this work.

3.5. Calibration curves

In order to quantify the amount of each compound in the bitter orange peel, naringin and neohesperidin, a calibration curve was prepared with the standards between the ranges from 5 to 50 mg/l including five replicated of each point. In the same way, in order to quantify the amount of the sweet orange peel compounds, hesperidin and narirutin, a calibration curve was prepared with the standards between the ranges from 25 to 80 mg/l including five replicated of each point. All calibration curves showed good linearity in the studied range of concentration. Regression coefficients were higher than 0.985 for narigin and neohesperidin and higher than 0.997 for narirutin and hesperidin. All the features of the proposed method are summarised in Table 1.

3.6. Quantification of the main polyphenols in bitter and sweet orange samples

The proposed method was applied to the quantification of naringin, neohesperidin, narirutin and hesperidin in bitter and sweet orange peel real samples. In Figs. 4A and 4B the extracted ion electropherogram for each target compound of bitter and sweet orange peel are shown. The studied compounds were diluted in order to fix them in the calibration range. Finally, the results expressed in mg analyte/g of dry weight peel ($n = 5$; value = $X \pm SD$) were 5.1 ± 0.2 and 7.9 ± 0.7 mg/g of naringin and neohesperidin in bitter orange peel and 26.9 ± 2.1 and 35.2 ± 3.6 mg/g of narirutin and hesperidin in sweet orange peel, respectively.

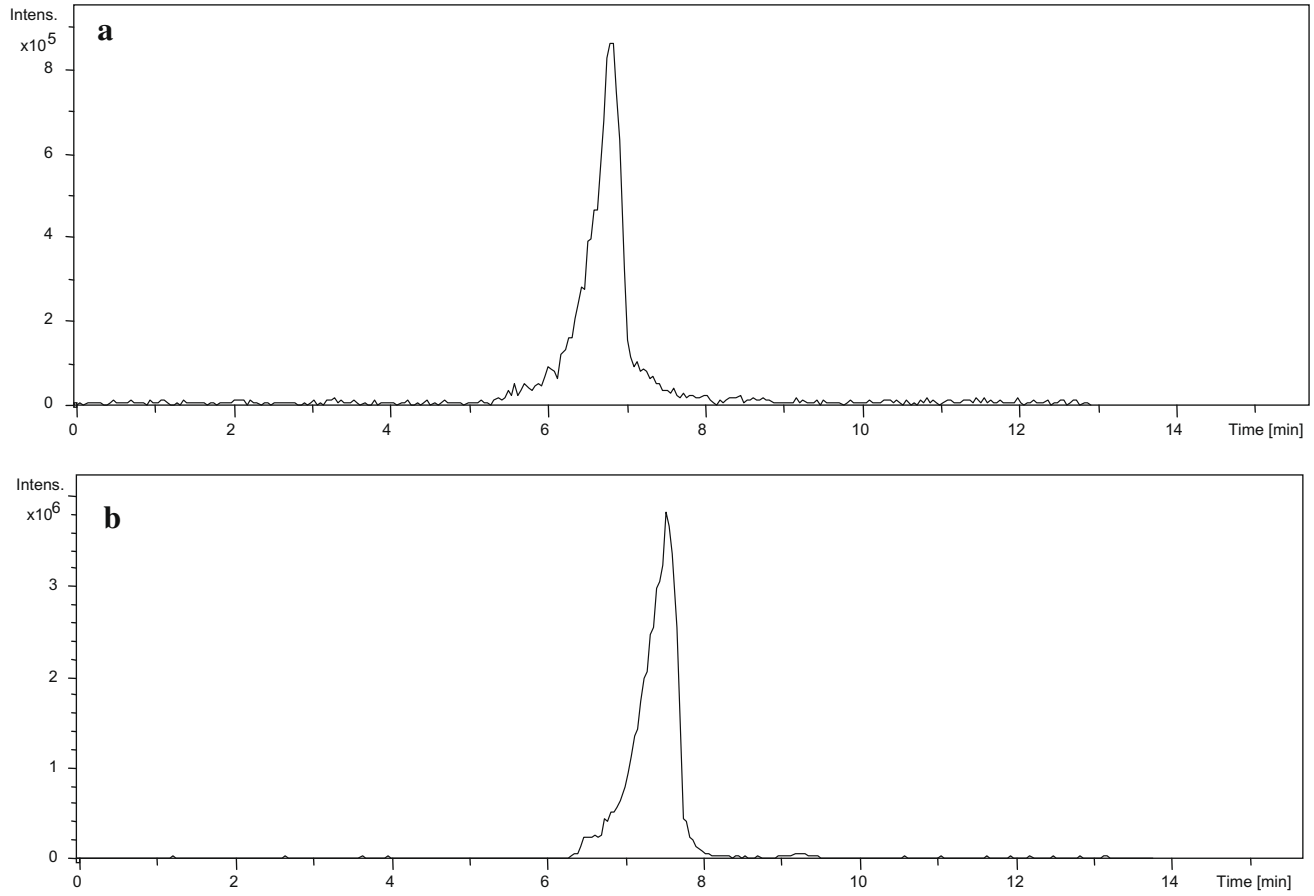

Fig. 4B. Extracted ion electropherograms of: (a) narirutin and (b) hesperidin in sweet orange peel sample.

4. Conclusions

The food and agricultural processing industries generate substantial quantities of phenolics-rich by-products, which could be valuable natural sources of antioxidants. In oranges, the peel represents roughly half of the fruit mass. The highest concentrations of flavonoids in citrus fruit occur in peel. In this work we propose the characterisation, using MS/MS experiments, and quantification of the distinctive phenolic compounds (naringin, neohesperidin, narirutin and hesperidin) from the peel of sweet and bitter oranges. The CE–ESI–IT–MS allowed to differentiate naringin from narirutin and hesperidin from neohesperidin and it showed to be suitable for the analysis of this type of natural compounds.

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