Talanta 99 (2012) 213–224

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

On line characterization of 58 phenolic compounds in *Citrus* fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry

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ARTICLE INFO

Article history:
Received 17 February 2012
Received in revised form
16 May 2012
Accepted 19 May 2012
Available online 26 May 2012

Keywords:
Phenolic compounds
Flavonoids
Mass spectrometry
Structural characterization
HPLC
Citrus
Juices

ABSTRACT

Polyphenol profile of *Citrus* juices of sweet orange, tangerine, lemon and grapefruit from Spanish cultivars was obtained by High-Performance Liquid Chromatography with Diode Array Detection coupled to Electrospray ionization and Triple Quadrupole Mass Spectrometry. Fifty eight phenolic compounds of five different classes were identified in these *Citrus* juices. Flavanone: *O*-dihexoside of naringenin; flavones: apigenin-7-*O*-rutinoside-4′-*O*-glucoside, luteolin-7-*O*-neohesperidoside-4′-*O*-glucoside, luteolin-6-*C*-glucoside, 6,8-di-*C*-acylhexosides of chrysoeriol and diosmetin, 6*C*- and 8*C*-glucoside-*O*-pentoside of apigenin, apigenin-6-*C*-hexoside-*O*-hexoside and apigenin-8-*C*-hexoside-*O*-acylrhamnoside; flavonols: 7-*O*-rutinosides of quercetin, kaempferol, isorhamnetin and tamarixetin, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-rutinoside-7-*O*-glucoside, tamarixetin-3-*O*-rutinoside-7-*O*-glucoside, isorhamnetin-3-*O*-hexoside-7-*O*-rhamnosylhexoside, 3-*O*-rhamnoside-7-*O*-rhamnosylhexoside of quercetin and isorhamnetin and kaempferol-3-*O*-rhamnosylhexoside-7-*O*-rhamnoside; hydroxycinnamic acids: *O*-hexoside of ferulic and sinapic acid; and, coumarins: *O*-hexoside and *O*-rhamnosylhexoside of scopoletin, had not previously been reported in *Citrus* juices to our knowledge. Structures have been assigned on the basis of the complementary information obtained from retention time, UV–visible spectra, scan mode MS spectra, and fragmentation patterns in MS^2 spectra obtained using different collision energies. A structure diagnosis scheme is provided for the identification of different phenolic compounds.

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1. Introduction

The positive effects of *Citrus* fruit consumption on human health were of common knowledge centuries before researchers begun to unravel the complexity of such food matrices. Over the past decades, a large number of studies have been carried out with the aim of identifying the bioactive components present in different parts of *Citrus* fruits, in an attempt to gain a deeper understanding of the correlation between diet, health benefits and reduced risk of diseases.

Nowadays, an amount of data has been collected on the biomedical properties of many relevant nutraceuticals [1]. In this context, several epidemiological studies have associated the consumption of phenolic compounds, and more specifically flavonoids, with lower risks of different types of cancer [2] and cardiovascular diseases [3], and have shown that they posses antioxidant, anti-inflammatory and anti-ageing activity [4]. *Citrus* fruits are the main winter fruits consumed in the Mediterranean diet, so they are the main source of dietary flavonoids, especially flavanone and flavones with flavonols present in lower concentration [5] although polymethoxylated flavones have been also found in large amounts in the peel of some *Citrus* [6]. Flavonoids found in different parts of *Citrus* fruits usually do not occur normally as aglycones [7,8] but rather as glycosides [9].

Apart from their beneficial properties in food, which have conferred on them a relevant role as nutraceuticals [10], polyphenols are chemotaxonomic markers due to their specifity and ubiquity, and they have proven to be chemical markers for food authentication demanded by food producers, consumers and regulatory bodies [11–13]. Characteristic phenolic compounds

Abbreviations: Nar, Naringenin; Eri, Eriodictyol; Isk, isosakuranetin; Hes, hesperetin; Heri, homoeriodictyol; Lut, luteolin; Dio, diosmetin; Chrys, chriysoeriol; Api, apigenin; Kaem, kaempferol; Que, quercetin; Iso, isorhamnetin; Tam, tamarixetin; Fer, ferulic acid; Snp, sinapic acid; Sco, scopoletin; rha, rhamnoside; hex, hexoside; pent, pentoside; glc, glucoside; rut, rutinoside; nhes, neohesperidoside; Acylgly, acylglycoside; FVNN, flavanone; FVN, flavone; FVL, flavonol; DFVL, Dihydroxyflavonol; HCA, hydroxycinnamic acid; CM, coumarin

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http://dx.doi.org/10.1016/j.talanta.2012.05.042

have been successfully used for the determination of adulteration of *Citrus* juices [14–16] and *Citrus* jam [17] with cheaper fruits.

For the investigation of structure–activity relationships and food quality control of natural polyphenolic compounds, it is also important to have access to rapid and reliable methods for the analysis and identification of these natural phenolic compounds in all their many forms. Among the methods used for the determination of phenolic compounds, the most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to diode array detection (DAD) and mass spectrometry (MS) with atmospheric pressure ionization techniques, i.e., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APcI). With the use of tandem MS technologies (MS/MS) in combination with collision-induced dissociation (CID), MS/MS spectra of a range of flavonoid structures have been investigated and compared, obtaining fragmentation rules and fragmentation patterns that enable discrimination and identification of a wide range of phenolic compounds [18–20].

In the present paper, a comprehensive characterization of phenolic compounds in *Citrus* juices (sweet orange, tangerine, lemon and grapefruit) from Spanish cultivars by HPLC-DAD-ESI-CID-MS/MS is reported. The structural information provided by online technical HPLC-DAD-ESI-CID-MS/MS scan and product ion scan mode led to identify and characterize successfully 58 phenolic compounds in *Citrus* fruit juices using the mechanisms and fragmentation patterns established in the previous study with phenolic compounds standards [19]. Although some of the phenolic compounds have been previously described in literature, 25 phenolic compounds have been detected for the first time in *Citrus* in this work.

2. Experimental

2.1. Reagents, solvents and standard phenolics

Methanol and dimethyl sulfoxide (Romil, Chemical Ltd, Heidelberg, Germany) were of HPLC grade. Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Glacial acetic acid, ascorbic acid and sodium fluoride provided by Merck (Darmstadt, Germany) were of analytical quality. All solvents used were previously filtered through 0.45 μm nylon membranes (Lida, Kenosha, WI, USA).

Phenolics standards were supplied as follows: eriodictyol-7-*O*-rutinoside, eriodictyol-7-*O*-neohesperidoside, naringenin-7-*O*-rutinoside, hesperetin-7-*O*-rutinoside, hesperetin-7-*O*-neohesperidoside, isosakuranetin-7-*O*-rutinoside, hesperetin, homoeriodictyol, ferulic acid, sinapic acid, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucofuranoside, quercetin-3-*O*-glucopyranoside, quercetin-3-*O*-rhamnoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, kaempferol-7-*O*-neohesperidoside, kaempferol-3-*O*-robinoside-7-*O*-rhamnoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin, tamarixetin, myricetin, scopoletin, luteolin-7-*O*-glucoside, luteolin-6-*C*-glucoside, luteolin-8-*C*-glucoside, luteolin-3′,7-di-*O*-glucoside, luteolin-4′-*O*-glucoside, diosmetin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, apigenin-6-*C*-glucoside, apigenin-8-*C*-glucoside, apigenin-7-*O*-neohesperidoside, apigenin-7-*O*-rutenoside, diosmetin, chrysoeriol and sinensetin from Extrasynthèse (Genay, France); while naringenin, 5′-caffeoylquinic acid, caffeic acid, *p*-coumaric acid and quercetin-3-*O*-rutinoside were provided by Sigma-Aldrich Chemie (Steinheim, Germany); apigenin-8-*C*-glucoside-4′-*O*-rhamnoside, kaempferol-3-*O*-(*p*-coumaroyl)glucoside, tangeretin and nobiletin by Chromadex (Santa Ana, CA, USA); and naringenin-7-*O*-neohesperidoside, quercetin dehydrated and apigenin by Fluka Chemie (Steinheim, Germany).

All stock standard solutions (in concentrations ranging from 250 to 2500 μg/mL, depending on each phenolic compound) were prepared in methanol, except for hesperetin-7-*O*-rutinoside, hesperetin, homoeriodictyol, chrysoeriol and isorhamnetin that was dissolved with water–dimethyl sulfoxide (80:20, v/v), and all were stored at 4 °C in darkness.

2.2. Fruit samples

Fruits of four different *Citrus* species: sweet orange (*Citrus sinensis*) (nine cultivars), tangerine (*Citrus reticulate* and *Citrus unshiu*) (seven cultivars), lemon (*Citrus lemon)* (four cultivars) and grapefruit (*Citrus paradise)* (five cultivars), produced in Spain during the years 2003–2005 were purchased from a local market at maturity.

2.3. Citrus juice preparation

Three batches of fruit (1 kg) were constituted for each fruit cultivar and harvest. Each batch was peeled separating the flavedo and the albedo from the pulp and squeezed using a home juicer. The collected juice after measuring its volume, was mixed with 50 mL of an aqueous solution containing ascorbic acid 0.2 g/mL and sodium fluoride 0.2 g/mL, in order to inactive polyphenoloxidases and prevent phenolic degradation [21], and centrifuged at 6000 r.p.m. for 15 min at 4 °C. Aliquots of 1 mL were sampled, stored at −20 °C and lyophilized later. The freeze-dried material was stored at room temperature in a desiccator in darkness until analysis.

2.4. Analytical procedure

2.4.1. Solvent extraction of freeze-dried samples and RP-HPLC

Extraction was performed following a previously optimized procedure [22]. The HPLC system was a Waters (Milford, USA) Alliance 2695 coupled to a Waters 2996 DAD. A reversed-phase Phenomenex (Torrance, USA) Luna C18(2) column (150 × 4.6 mm i.d. and particle size 3 μm) with a Waters Nova-Pack C18 guard column (10 × 3.9 mm i.d., 4 μm) was used. A gradient program was employed [22].

2.4.2. Mass spectrometry

Mass spectra were obtained on a Micromass (Milford, MA, USA) Quattro micro-triple quadrupole mass spectrometer coupled to the exit of the diode array detector and equipped with a Z-spray ESI source. A flow of 70 μL/min from the DAD eluent was directed to the ESI interface using a flow-splitter. Nitrogen was used as desolvation gas, at 300 °C and a flow rate of 450 L/h, and no cone gas was used. A potential of 3.2 kV was used on the capillary for positive ion mode and 2.6 kV for negative ion mode. The source block temperature was held at 120 °C.

Two independent runs, one for the MS^1 full scan mode and another for MS^2 product ion scan mode were carried out at 1 scans/s and inter-scan delay of 0.1 s. MS^1 full scan spectra, within the *m*/*z* range 50–1000, were performed in the positive mode at different cone voltages (15, 30 and 45 V) and in the negative mode at −30 V. MS^2 product ion spectra in positive mode were recorded using argon as collision gas at 1.5×10^{-3} mbar and under different collision energies in the range 5–40 eV and optimized cone voltages. The optimum cone voltages were those which produced the maximum intensity for protonated molecular ion $[M+H]^+$ and protonated aglycone ion $[Y_0]^+$ in the previous MS^1 experiments.

The nomenclature adopted to denote the fragment ions for glycoconjugates was proposed by Domon and Costello [23] (Fig. 1). The flavonoid aglycone fragment ions have been designed according to the nomenclature proposed by Ma et al. [24] (Fig. 2).

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