A Simple and Rapid HPLC-PDA MS Method for the Profiling of *Citrus* Peels and Traditional Italian Liquors*

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Key words
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- flavanones
- flavones
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- protoalkaloids
- HPLC-PDA MS

Abstract

A chromatographic method for the qualitative and quantitative characterization of peels and preparations based on different species of *Citrus* was developed in order to obtain a complete profile of the constituents, including flavonoids and protoalkaloids. Commercial peels of sweet orange, lemon, mandarin, and grapefruit were analyzed. Seventeen constituents including flavanones, flavones, polymethoxyflavones, and protoalkaloids were identified by HPLC-PDA, HPLC-MS, and HPLC-MS/MS using a comparison of retention times and UV-Vis and MS spectra with reference standards and literature data. The total amount of flavanones [neoeriocitrin (**5**), naringin (**8**) and hesperidin (**9**)] and polymethoxflavones [sinensetin (**12**), nobiletin (**14**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**15**), and tangeretin (**16**)] was determined and expressed as naringin (**8**) or hesperidin (**9**), and sinensetin (**12**), respectively. The protoalkaloid synephrine was detected in all samples, except in grapefruit, but its content was lower than the limit of quantification. Qualitative and quantitative chemical profiles of three different Italian aromatic liquors ("Limoncello", "Arancello", and "Mandarinetto"), prepared according to traditional recipes, were also analyzed.

Supporting information available online at http://www.thieme-connect.de/products

Introduction

The genus *Citrus* (Rutaceae) represents the largest sector of the world's fruit production, with more than 100 million tons produced each season, among which the major commercially important orange fruit accounted for almost 45 million tons. Around 34% of these products are used for juice production, yielding large amounts of peels (roughly half of the fruit mass), which represent a source of molasses, pectin, and essential oil. The waste material is usually dried and sold as cattle feed, mixed with dried pulps [1]. *Citrus* peels have recently been considered potential sources of polyphenols, in particular polymethoxyflavonoids and flavanones (mainly glycosides), due to their potent antioxidant, anti-inflammatory, antithrombogenic, antiatherogenic, and hypolipidemic/hypoglycemic effects [2–11]. Additionally, polymethoxyflavonoids have antiproliferative activity [12–16].

Other characteristic constituents of *Citrus* peels are phenolic acids and protoalkaloids, principally represented by *p*-synephrine and *p*-octopamine, which are weak adrenergic agonists, active on both α- and β-adrenoceptors, but orders of magnitude less active than norepinephrine [17]. Protoalkaloids have been involved in the last decade in safety issues. Several reports of adverse effects due to the use of bitter orange as a replacement for ephedra in weight-loss dietary supplements have recently emerged, speculating that synephrine and/or octopamine can increase cardiac output and blood pressure [18]. Consequently, EFSA (European Food Safety Authority) has set a maximum limit for the daily intake of 6.7 mg of synephrine in food supplements [19]. Accordingly, developed analytical methods are mainly focused on the identification of the constituents of bitter orange (*Citrus aurantium* L.).

At the same time, in many regions of the world, other *Citrus* peels are used for relieving stomach upset, inflammatory syndromes, and infections

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[13]. In Italy, *Citrus* peel alcoholic preparations originally developed by Italian monks during the thirteenth and fourteenth centuries as a medicinal drink to treat and/or prevent illnesses have recently evolved into more palatable liquors as aperitifs and after-dinner digestives. However, most of them are merely flavored products made with pure essential oils or commercial essences rather than an infusion of *Citrus* peels according to the original recipe. The quality control of aromatic herbal liquors is mainly based on the physicochemical properties, from the alcoholic grade, pH, etc., till the assessment of the volatile fraction chemical composition [20–22].

In the current European Pharmacopoeia, there are several monographs on *Citrus* peels and their preparations (i.e., fluid extract, tincture, syrup), but the quality is generally related to the volatile oil content [23–25]. Only the monograph "Mandarin epicarp and mesocarp" includes a quality control based on flavonoids, in particular on the content of a flavanone glycoside (minimum 3.5 per cent of hesperidin) [26].

Up to now, several analytical methods have been developed for the quantification of phenethylamine alkaloids (e.g., synephrine, octopamine) in *Citrus* peels and fruits, extracts thereof, and food supplements; most of them use chromatographic (HPLC, GC) and electrophoretic (CE) determinations [27]. Methods have also been developed for the simultaneous characterization of protoalkaloids and organic acids [28], flavanones and protoalkaloids [29], flavanones, hydroxycinnamic acids, and protoalkaloids [30], both polymethoxyflavonoids and flavanones [31], or hydroxycinnamates and polymethoxylated flavones [7].

In this study, we developed a simple and rapid HPLC-PDA and HPLC-MS/MS method suitable for simultaneous qualitative and quantitative analysis of flavones, flavanones, polymethoxyflavones, and protoalkaloids in *Citrus* peels and to obtain a full polyphenol profile of traditional Italian liquors.

Results and Discussion

Several commercial *Citrus* peels (sweet orange, lemon, grapefruit, and mandarin) and homemade traditional liquors obtained by the infusion of lemon, orange, and mandarin peels were analyzed. Different extraction methods such as maceration and Soxhlet extraction were explored, and three extraction solvents, 100% methanol, 70%, and 80% ethanol, were compared for extraction efficiency of the targeted phytochemical constituents. The extraction protocols were finally adopted based on the number and intensity of the protoalkaloid and flavonoid peaks that were detected in the HPLC-MS trace. Accordingly, hydroalcoholic solutions were selected for sweet orange, lemon, and grapefruit, while methanol was chosen for mandarin. The extracts were analyzed by HPLC-PDA, HPLC-MS, and HPLC-MS/MS for the qualitative evaluation of the constituents, while the quantification was made by HPLC-PDA. The chromatographic conditions were selected to obtain chromatograms with optimal resolution of the adjacent peaks. As the stationary phase, two columns, Zorbax® Eclipse XDB-C18 (4.6 × 150 mm, 5 µm) and Vydac® C_{18} (4.6 × 250 mm, 5 µm), were evaluated, and the first one that showed the best performance was selected. Formic acid was used as a mobile phase modifier since it significantly reduced the peak tailing of the polyphenols and alkaloids in our experiments. Moreover, gradient elution was used to achieve a better separation of the constituents. Under the optimum gradient conditions reported in the Material and Methods section, a baseline separation of all target peaks was achieved. The PDA wavelengths were set at 240, 280, 330, and 350 nm. MS data of flavones, flavanones, and polymetoxyflavones were recorded in the negative and positive ionization modes while the alkaloids were analyzed only in the positive ion mode. Selected ion monitoring (SIM) was employed to identify the target components. Individual monitored ions are summarized in **Table 1**. Flavanones and polymethoxyflavones were quantified by HPLC-PDA using an external standard method (see Material and Methods). Limit of detection (LOD) and limit of quantification (LOQ) were 2.46 ng and 12.30 ng, respectively, for synephrine, 1.00 ng and 7.67 ng for naringin, 1.32 ng 9.86 ng for hesperidin, and 1.78 ng 10.70 ng for sinensetin.

The results of the qualitative analysis of sweet orange peels are reported in **Table 1**. Ten compounds were identified and their structures are shown in **Fig. 1**. **Fig. 2** presents the TIC chromatograms in positive and negative ion modes. Vicenin-2 (**2**; MW 594; R*t:* 13.0 min) gave a $[M - H]^-$ ion at *m/z* 593.4. For further confirmation, this ion was subjected to MS/MS investigation to produce secondary fragments {*m/z* 575 $[(M - H)\text{-}18]^-$, 503 $[(M - H)\text{-}90]^-$, 473 $[(M - H)\text{-}120)]^-$, 383 $[(M - H)\text{-}210)]^-$, 353 $[(M - H)\text{-}240)]^-$}, which were in agreement with those previously reported in the literature [32,33]. The compound with R*t* 26.3 min (**7**; *m/z* 579.4) is a structurally, not fully identified isomer of naringin (**8**), probably differing from the latter by the nature or location of the sugar residue. The MS/MS analysis of **7** produced the same fragmentation pattern as **8** {*m/z* 579 $[M - H]^-$, 459 $[(M - H)\text{-}120)]^-$, 313 $[(M - H)\text{-}266)]^-$, 271 $[(M - H)\text{-}308)]^-$} [34]. However, in contrast to naringin (**8**) and its strong fragment ion at *m/z* 459, the most abundant fragment was observed at *m/z* 271.0 $[(M - H)\text{-}308]^-$, resulting from the loss of the disaccharide unit. The peaks at 27.5 and 32.0 min were identified as hesperidin (**9**) and sinensetin (**12**), respectively, in comparison with standards. The compound at R*t* 29.3 min (*m/z* 593.4 $[M - H]^-$) was attributed to poncirin (**10**) from its UV and mass spectra. In fact, even if vicenin-2 (**2**) also present in sweet orange has the same molecular weight, both compounds could be reliably distinguished by comparison with literature data [31] and from different elution times and MS/MS data. Poncirin (**10**) gave essentially a fragment ion with *m/z* 285.2, resulting from the cleavage of the glycosidic bond and loss of neohesperidose (*m/z* 308), leaving the aglycone isosakuranetin {*m/z* 285 $[(M - H)\text{-}308)]^-$}. This ion is not observed in the case of vicenin-2 (**2**) because, in this case, the characteristic fragmentation is due to a ring opening on the sugar moiety. The peak at R*t* 30.0 min is due to three coeluting substances with *m/z* 726.6 $[M - H]^-$, 696.6 $[M - H]^-$, and 711.2 $[M - H]^-$. Among these, only the peak at *m/z* 726.6 could be identified. It was assigned to citrusin III (**11**), a cyclic peptide consisting of seven aminoacids (Gly-Ser-Pro-Leu-Leu-Pro-Tyr). The peak at R*t* 32.4 min (**13**) is an unidentified structural isomer of nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) (**14**), having the same UV spectrum and fragment ions in the positive ionization mode {*m/z* 403.3 $[M + H]^+$; *m/z*: 388.4 $[(M + H)\text{-}14]^+$; 373.3 $[(M + H)\text{-}30]^+$; 355.4 $[(M + H)\text{-}48]^+$; 313.5 $[(M + H)\text{-}90]^+$; 151.2 $[(M + H)\text{-}151]^+$}. Nobiletin (**14**) was identified at 33.8 min. Finally, the peak at R*t* 33.2 min corresponded to 3,5,6,7,8,3′,4′-heptamethoxyflavone (**15**) [33], while that at R*t* 33.6 min was found to be tangeretin (**16**) [33].

In the lemon peel, 11 compounds were identified (**Table 1**). **Fig. 3** shows the positive and negative TIC chromatograms. The peaks at R*t* 13.0 (**2**), 26.3 (**7**), 27.5 (**9**), 32.9 (**14**), 33.3 (**15**), and 33.7 (**14**) min correspond to compounds already identified

Table 1 Constituents of extracts identified by HPLC-DADPDA-MS and HPLC-MS/MS analyses.

Compound	Citrus plant	Rt (min)	Positive ion mode	Negative ion mode	MS/MS fragmentation	UV maximum (nm)	Identification
1	Lemon	11.9		609.4 $[M-H]^-$		270, 349	Luteolin 6,8-di-C-glucoside (Lucenin-2)
2	Sweet orange Lemon Mandarin	13.0		593.4 $[M-H]^-$	593.2 $[M-H]^-$, 575.2 $[(M-H)-18]^-$, 503.1 $[(M-H)-90]^-$,473.1 $[(M-H)-120)]^-$, 383.1 $[(M-H)-210)]^-$, 353.1 $[(M-H)-240)]^-$	271, 335	Apigenin 6,8-di-C-glucoside (Vicenin-2)
3	Lemon	13.7		623.4 $[M-H]^-$	623.5 $[M-H]^-$, 605.4 $[(M-H)-18)]^-$, 533.4 $[(M-H)-90)]^-$, 503.3 $[(M-H)-120)]^-$, 413.4 $[(M-H)-210)]^-$, 383.3 $[(M-H)-240)]^-$	271, 348	Diosmetin 6,8-di-C-glucoside (Lucenin 2,4′-dimethylether)
4	Lemon	14.4		623.4 $[M-H]^-$	623.4 $[M-H]^-$, 605.4 $[(M-H)-18)]^-$, 533.4 $[(M-H)-90)]^-$, 503.3 $[(M-H)-120)]^-$, 413.3 $[(M-H)-210)]^-$, 383.3, $[(M-H)-240)]^-$	271, 347	Chrysoeriol 6,8-di-C-glucoside (Stellarin-2)
5	Lemon	22.1		595.3 $[M-H]^-$		283, 325	Eriodictyol 7-O-neohesperidoside (Neoeriocitrin)
6	Lemon	24.5		593.4 $[M-H]^-$		260, 292, 350	Luteolin 7-O-rutinoside
7	Sweet orange Lemon Grapefruit Mandarin	26.3		579.4 $[M-H]^-$	579.3 $[M-H]^-$, 459.2 $[(M-H)-120)]^-$, 313.1 $[(M-H)-266)]^-$, 271.0 $[(M-H)-308)]^-$	214, 226, 284, 334	Naringin isomer
8	Grapefruit Mandarin	27.3	579.4 $[M-H]^-$	579.3 $[M-H]^-$, 459.2 $[(M-H)-120)]^-$, 313.1 $[(M-H)-266)]^-$, 271.0 $[(M-H)-308)]^-$		214, 226, 284, 334	Naringenin 7-O-neohesperidoside (Naringin)
9	Sweet orange Lemon Mandarin	27.5	611.1 $[M+H]^+$	609.4 $[M-H]^-$		228, 284, 334	Hesperetin 7-O-rutinoside (Hesperidin)
10	Sweet orange Mandarin Grapefruit	29.3		593.4 $[M-H]^-$	285 $[(M-H)-308)]^-$	282, 330	Isosakuranetin 7-O-neohesperidoside (Poncirin)
11	Sweet orange	30.0	728.6 $[M+H]^+$	726.6 $[M-H]^-$			Citrusin III
12	Sweet orange Mandarin	32.0	373.4 $[M+H]^+$			214,239, 269, 331	5,6,7,3′,4′-Pentamethoxyflavone (Sinensetin)
13	Sweet orange	32.4	403.3 $[M+H]^+$			270, 330	Nobiletin isomer
14	Sweet orange Lemon Mandarin	32.8	403.3 $[M+H]^+$		388.4 $[(M+H)-14]^+$, 373.3 $[(M+H)-30]^+$, 355.4 $[(M+H)-48]^+$, 313.5 $[(M+H)-90]^+$, 151.2 $[(M+H)-151]^+$,	248, 270, 330	5,6,7,8,3′,4′-Hexamethoxyflavone (Nobiletin)
15	Sweet orange Lemon Mandarin	33.2	433.4 $[M+H]^+$		418.4 $[8M+H)-15]^+$, 403.3 $[(M+H)-30]^+$, 165.2 $[(M+H)-268]^+$,	254, 343	3,5,6,7,8,3′,4′-Heptamethoxyflavone
16	Sweet orange Lemon Mandarin	33.6	373.4 $[M+H]^+$		358.3 $[(M+H)-15]^+$, 343.3 $[(M+H)-30]^+$, 325.3 $[(M+H)-48]^+$, 283.3 $[(M+H)-90]^+$, 151.3 $[(M+H)-222]^+$	271, 336	5,6,7,8,4′-Pentamethoxyflavone (Tangeretin)

in the sweet orange peels. Lucenin-2 (**1**) (R*t:* 11.9 min) was identified by MS/MS analysis. Lucenin-2 generated a quasi-molecular ion at *m/z* 609.4 [(M – H)$^-$]. Fragments were detected at *m/z* 591 [(M – H) – 18]$^-$, 519 [(M – H)-90]$^-$, 489 [(M – H)-120]$^-$, 399 [(M – H)-210]$^-$, and 369 [(M – H)]$^-$240]$^-$ [33]. The identity of lucenin-2 4′-methylether (**3**) (R*t*: 13.7 min) was confirmed by the characteristic MS and MS/MS data and the retention time [32, 33]. The peak at R*t* 14.4 min corresponded to stellarin-2 (chrysoeriol 6,8-di-C-glucoside) (**4**) and was confirmed by UV and MS data [34]. The substance at R*t*: 22.1 min was identified as neoeriocitrin (**5**). In the MS/MS spectrum, three ions were present at *m/z* 595.3 [M – H]$^-$, 459 [(M – H)-136]$^-$, and 287.2 [(M – H)-308]$^-$. The main fragment at *m/z* 287.2 corresponded to the aglycone eryodictyol, resulting from the loss of the disaccharide neohesperidose. Literature data [33] report that neoeriocitrin (**5**) (a neohesperidoside) is able to form the fragment *m/z* 459 [(M – H)-136]$^-$ that is not observed for the isomer eriotricin (a rutinoside), which presents a same UV spectrum. The loss of a fragment of *m/z* 136 may correspond to a retro-cyclization that involves the aglycone.

In the grapefruit peel, three peaks have been identified (**Table 1**). The TIC chromatographic traces in the positive and negative modes are shown in **Fig. 4**. At R*t* 26.3 min, the naringin isomer **7** was identified, while naringin (**8**) was detected at R*t* 27.3 min, and poncirin (**10**) at R*t*: 29.3 min.

In the mandarin peel, nine flavonoids were identified and are reported in **Table 1**. All compounds were also found in previous samples and their structures have been identified as described above. **Fig. 5** shows the positive and negative TIC chomatograms.

Detection of the alkaloid synephrine in the four samples was performed by HPLC-MS and HPLC-MS/MS. The comparison of the TIC in the positive ion mode of all *Citrus* species is shown in **Fig. 6**. Synephrine was identified by comparison of its retention time and mass spectra with those of the reference standard ([M + H]$^+$ *m/z* 168). Its content was, however, below the LOQ in the HPLC-PDA analysis (detection at 280 nm). Synephrine was detected in mandarin, sweet orange, and lemon commercial peels, but not in grapefruit, which is consistent with a previous report [35].

Flavonoids were quantified in peels and liquors. Hesperidin (**9**) was used as a reference standard for the quantification of all flavanones in the sweet orange extract, while naringin (**8**) was employed for lemon, grapefruit, and mandarin samples. Polymethoxyflavones in all samples were quantified and expressed as sinensetin (**12**). The data are reported in **Table 2**.

Sweet orange peels are the richest matrix in polymethoxyflavones, while the highest content of flavanones was found in grapefruit peels. The percentage of flavanones in the lemon and mandarin samples are similar, but mandarin contains more polymethoxyflavones than lemon peels.

HPLC-PDA analyses were also performed to obtain qualitative and quantitative polyphenol profiles of the liquors. A high content of flavanones, mainly glycosides, was found in the investigated preparations. Thus, in 40 mL (typical glass) of Arancello, there were 15.8 mg of flavanones expressed as hesperidin, while 22.0 mg were found in the same volume of Mandarinello, and 39.6 mg in Limoncello, the richest liquor. In all preparations, a few milligrams (1–3 mg for a 40-mL glass) of polymethoxyflavones were found. No protoalkaloids were detected in the tested samples.

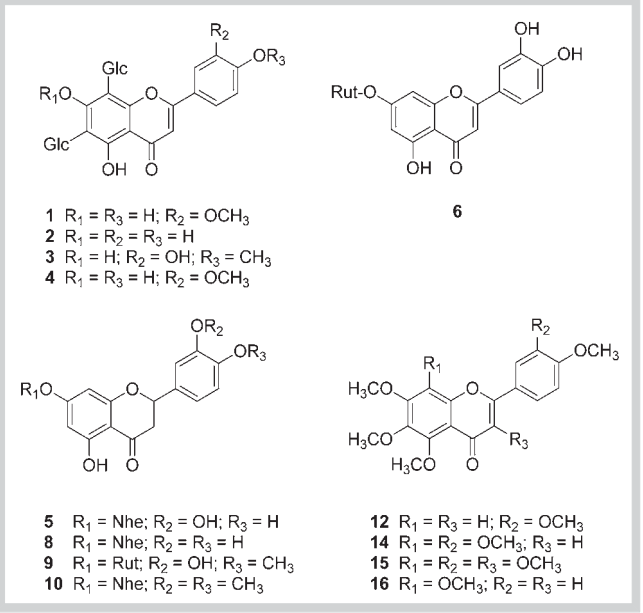

Fig. 1 Structures of identified flavonoids. Glc: glucose; Rut: rutinose; Nhe: neohesperidose.

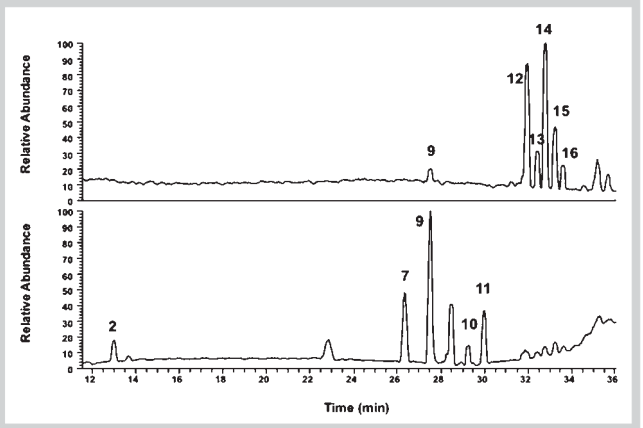

Fig. 2 Total ion current (12–36 min) in positive (top) and negative (bottom) ionization modes of a sweet orange sample.

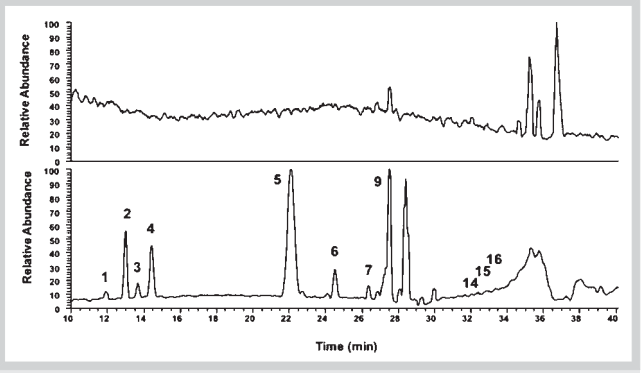

Fig. 3 Total ion current (10–40 min) in positive (top) and negative (bottom) ionization modes of a lemon sample.

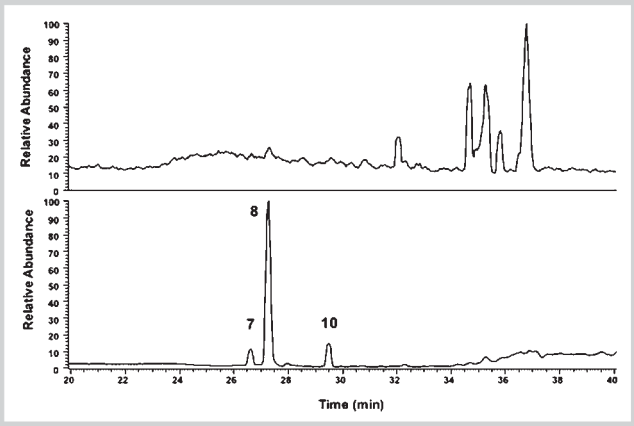

Fig. 4 Total ion current (10–40 min) in positive (top) and negative (bottom) ionization modes of a grapefruit sample.

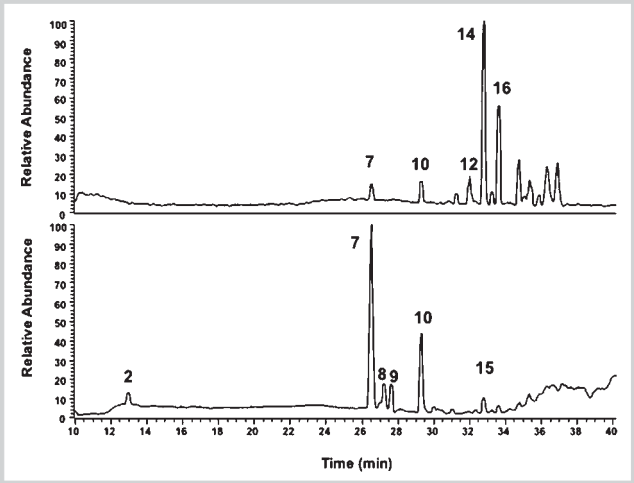

Fig. 5 Total ion current (10–40 min) in positive (top) and negative (bottom) ionization modes of a mandarin sample.

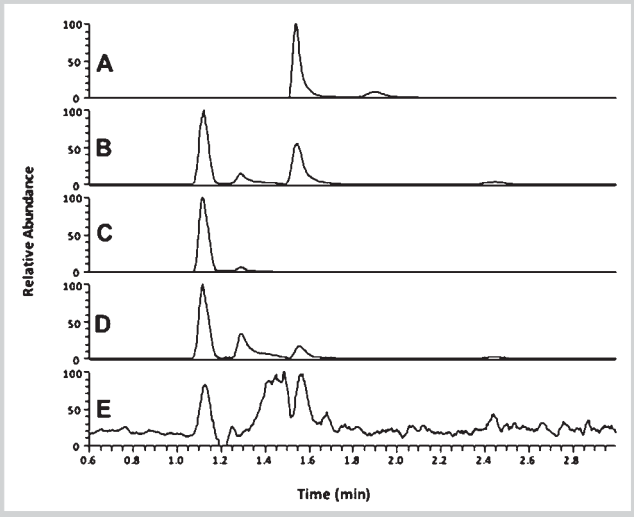

Fig. 6 Total ion current (positive ionization mode) of synephrine standard (**A**), mandarin (**B**), grapefruit (**C**), sweet orange (**D**), and lemon (**E**) samples.

Table 2 Content of flavanones and polymethoxyflavones in *Citrus* peels.

Sample	% of Flavanones/ herbal drug (expressed as naringin)	% of Polymethoxyflavones/ herbal drug (expressed as sinensetin)
Sweet orange	0.79% (expressed as hesperidin)	1.30%
Lemon	4.92%	0.05%
Grapefruit	14.00%	0.62%
Mandarin	3.53%	0.60%

In conclusion, the profiles of flavonoids and other constituents such as synephrine were analyzed in the peels of four species of *Citrus, Citrus sinensis* (L.) Osbeck, *Citrus limon* (L.) Burm. fil., *Citrus paradisi* Macfad., and *Citrus reticulata* Blanco. The main constituents were *O*-glycosylated flavanones [mainly represented by neoeriocitrin (**5**), naringin (**8**) and hesperidin (**9**)], *O*-glycosylated and *C*-glycosylated flavones [lucenin-2 (**1**), vicenin-2 (**2**), lucenin-2 4′-methyl ether (**3**), stellarin-2 (**4**), and luteolin7-O-rutinoside (**6**)], and polymethoxyflavones [sinensetin (**12**), nobiletin (**14**), 3,5,6,7,8,3′,4′-heptametoxyflavone (**15**), and tangeretin (**16**)]. Protoalkaloids such as synephrine were not detected or were detected under the limit of quantification.

The developed method is applicable to the quality control of *Citrus* peels and their preparations, including extracts, food supplements, and traditional liquors, in order to support their quality and safety. Some of these liquors are protected by the European regulation forbidding the addition of nature-identical flavoring substances. Consequently, in addition to GC analysis, determination of the profile of the nonvolatile constituents could represent a simple, easy way to perform analysis to guarantee the quality of these traditional liquors.

Materials and Methods

Chemicals

Anaytical grade solvents for extraction and HPLC grade solvents (acetonitrile, formic acid, DMSO and MeOH) were purchased from Sigma-Aldrich. EtOH (96%) was from Riedel-de-Haën Rdh Soherchemikalien GmbH & Co. Ultrapure deionized water (18 MX) was obtained through a Milli-Q system (Millipore).

Plant materials

The peels of *C. sinensis* (sweet orange, lot n. CD 16781), *C. limon* (lemon, lot n. R7146Z), *C. paradisi* (grapefruit, lot n. R7144Z), and *C. reticulata* (mandarin, lot n. R7145Z) were kindly offered by Amway GmbH.

Citrus liquors were prepared using organic fruits from Tuscany, namely 12 lemon fruits (approx. 1 kg) for Limoncello and 20 mandarin fruits (approx. 1.6 kg) for Mandarinello, or from Sicily, namely 10 orange fruits (approx. 2 kg) for Arancello.

Reference compounds

Naringin (naringenin 7-O-neohesperidoside, **8**), hesperidin (hesperetin 7-O-rutinoside, **9**), sinensetin (3′,4′,5,6,7-pentamethoxyflavone, **12**), and synephrine were purchased from Extrasynthèse and used as standards for qualitative and quantitative analyses. Their purity was higher than 98%.

Extraction and sample preparation

The methanol extract of the mandarin peels was prepared by maceration of 2 g of herbal drug with 100 mL of MeOH 100% (thrice). Hydroalcoholic extracts of lemon and grapefruit peels were prepared by maceration of 2 g of herbal drugs with 100 mL of EtOH/H_2O 70 : 30 (thrice), whereas hydroalcoholic extracts of sweet orange peels were obtained by maceration of 2 g of peels with 100 mL of EtOH/H_2O 80 : 20 (thrice). After 3 days, the extracts were filtered and evaporated to dryness. Extraction yields were 26.2% for sweet orange peels, 49.2% for lemon peels, 45.9% for grapefruit peels, and 23.1% for mandarin peels. The dry extracts were solubilized in MeOH/DMSO 4 : 1, ultrasonicated for 5 min, and centrifuged for 4 min at 14 000 rpm prior to injection into HPLC.

Liquors were prepared in our laboratory, according to traditional recipes. Fruits were accurately peeled out and the skins, consisting of the flavedo parts, were put into 1 L of EtOH (96% vol.) and left to draw for 2 weeks. After this time, lemon, orange, and mandarin peels were taken out of the alcohol, and a syrup made with 1 L of water plus 800 g of sugar was added to the ethanolic extract. The liquor obtained was let to rest for a couple of days before being analyzed.

HPLC-PDA and HPLC-MS/MS analyses

For qualitative analysis, MS and MS/MS experiments were conducted using an HPLC Surveyor coupled to an LTQ equipped with an ESI interface (Thermo Electron). Mass spectrometry and electrospray operating parameters were optimized for negative and positive polarities. The following final settings were used: Sheath gas flow rate (arb): 30, Aux gas flow rate (arb): 9, Sweep gas rate (arb): 5, Capillary temp. (°C): 280.00, Capillary voltage (V): 11.00, Tube lens (V): 60.00, Normalized collision energy: 25, 20. MS spectra were acquired from *m*/*z* 240 to 100 in negative and positive ion modes.

For quantitative analysis, an HP 1100 L instrument with a diode array detector controlled by an HP 9000 workstation (Agilent Technologies) was used. Data were processed with HP ChemStation software (Agilent).

Separations were performed at 27 °C on a Zorbax Eclipse XDB-C18, (150 mm × 4.6 mm, 5 µm, Agilent). The mobile phase consisted of H_2O at pH 3.2 adjusted by formic acid (solvent A), methanol (solvent B), and acetonitrile (solvent C). The flow rate was 1 mL/min and the total run time was 45 min. The following gradient profile was used: 0–10 min, 0% B, 0–15% C; 10–20 min, 0–10% B, 15–10% C; 20–25 min 10% B, 10–20% C; 25–35 min, 10–15% B, 20–75% C; 35–40 min, 15–10% B, 75–80% C; 40–45 min, 10–0% B 80–0% C, with an equilibration time of 10 min. The sample-injected volume was 10 µL. UV spectra were recorded between 200 and 600 nm. Chromatographic profiles were recorded at 240, 280, 330, and 350 nm. The identification of the constituents was performed by comparing the retention time and the UV, MS, and MS/MS spectra of the peaks in the samples with those of authentic reference samples or literature data.

Quantification of flavonoids

Calibration curves: Standard solutions were freshly prepared by serial dilutions of stock solutions in MeOH/DMSO 4 : 1 to obtain the following concentrations: 2.28, 3.43,11.43, 13.71, and 22.85 µg/mL for hesperidin (**9**), 3.15, 1.89, 1.26, 0.88, and 0.25 for sinensetin (**12**), and 14.1, 8.46, 5.64, 3.95, and 1.12 µg/mL for naringin (**8**).

Limit of detection and limit of quantification: Serial dilutions of standard solutions were used for determinating the limits of detection (LOD; S/N ≥ 3) and limits of quantification (LOQ; S/N ≥ 10). Synephrine, naringin (**8**), hesperidin (**9**), and sinensetin (**12**) were accurately weighed and dissolved in MeOH.

Quantitative determination of constituents: The external standard method was applied. Quantification was performed using regression curves. Measurements were performed at 280 nm for flavanones and 350 nm for polymethoxyflavones. Individual peaks of flavanones and polymethoxyflavones were quantified in the HPLC-PDA trace as hesperidin (**9**) (sweet orange) or naringin (**8**), and sinensetin (**12**), respectively. The amounts were then added to obtain the total contents of flavanones and polymethoxyflavones. Results are expressed as the mean of three separate experiments.

Supporting information

HPLC-UV traces (UV 350 nm) for the peel extracts of sweet orange, lemon, grapefruit, and mandarin are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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