Fast HPLC for the Analysis of Oxygen Heterocyclic Compounds of Citrus Essential Oils†

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A fast HPLC method for the determination of the oxygen heterocyclic compounds of citrus essential oils was developed. Five different oils were analyzed under identical conditions, by reversed-phase HPLC with photodiode array detector, for a direct comparison of the composition of their oxygen heterocyclic fraction. Analysis time was 7 min. The oils analyzed were lemon, bergamot, mandarin, sweet orange, and bitter orange. The method developed is good for rapid screening or fingerprinting of these essential oils; a slightly slower method is recommended for higher resolution and better quantitative results.

Keywords: *Citrus oils; coumarins; psoralens; polymethoxylated flavones; fast HPLC*

INTRODUCTION

Analysis time is becoming an important issue today, and fast HPLC methods are needed. The analysis time is affected by several variables: retention factor, flow rate, and column length. If shorter columns are used, with smaller particle size, it is possible to achieve good efficiency in a shorter analysis time. Fast HPLC columns can be 3 cm long, with conventional internal diameter (4.6 mm) and 3 μm particle size. This columns provide several advantages such as shorter retention time and lower backpressure, and therefore higher flow rates can be applied, providing efficiency that is comparable with standard dimensions columns.

The quality control of essential oils is necessary to ensure the genuineness of the product, the shelf life, and the storage conditions. Adulteration of citrus oils can be easily performed by adding less valuable oils, fractions, or synthetic components. Among citrus oils, sweet orange oil and/or its fractions are the most common adulterant for all the others, so that an analytical methodology for a fast detection of small amounts of sweet orange in other oils is necessary. Moreover, if distilled fractions or single components are used for adulterating citrus oils, it becomes necessary to adjust the oil absorbance in the UV range where oxygen heterocyclic components adsorb (McHale et al., 1988). In fact, this analytical control is routinely carried out for citrus oil genuineness control. To mask the adulteration performed, which would decrease the adsorbance value of the reconstituted oil, extraneous components are used that will absorb in the same range as the nonvolatile natural components of the oil. This kind of adulteration can be easily revealed by HPLC analysis. Different contamination could also be due to nonhomogeneity of the fruit processed or to insufficient cleanliness of the production line between the extractions from different fruits.

Quality control methods reported in the literature mostly pertain to the analysis of the volatile components by GC, because this is the most important fraction for flavor evaluation. However, nonvolatile oxygen heterocyclic compounds can be more helpful for determining the oil purity, because this fraction is simpler in composition. Furthermore, the components present in some citrus essential oils may be undesirable. For example, bergapten, naturally present in bergamot oil, is photosensitive (Shi et al., 1990), and therefore its concentration in lotions, perfumes, or other skin products must be below certain values. It is therefore useful to be able to determine the concentration of bergapten in the bergamot oils used as fragrances in these products.

The literature reports that mostly normal-phase HPLC methods are used for the determination of the nonvolatile components of citrus essential oils (Dugo et al., 1997; McHale, 1989; Bianchini et al., 1980; Shu et

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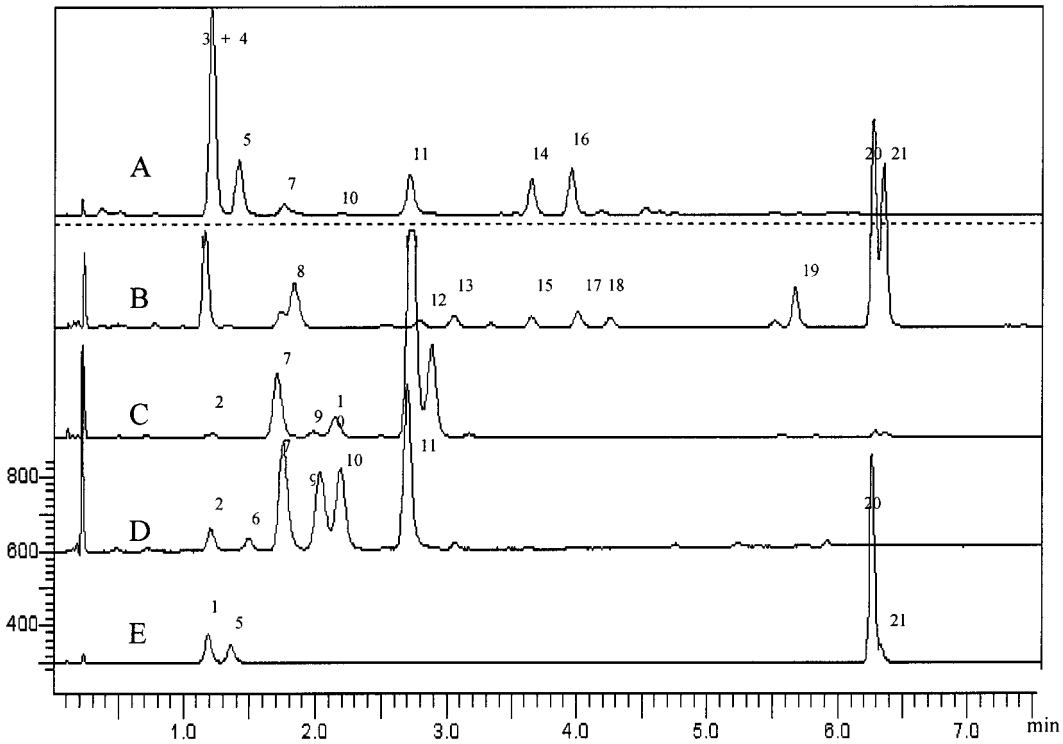

Figure 1. HPLC chromatograms obtained under identical conditions for five citrus essential oils: (A) bitter orange, (B) lemon, (C) mandarin, (D) sweet orange, and (E) bergamot. P-E C18, 3 cm × 4.6 mm, 3 μm column. Gradient conditions in text.

al., 1975). Reversed-phase HPLC is now becoming more common, and methods have been reported for coumarins, psoralens, and polymethoxylated flavones (PMFs) (Bianchini et al., 1981; Rouseff et al., 1979; Zeigler et al., 1995). The analysis time, so far reported, is usually long (often 20−45 min), and no single method is reported for the analysis of the oxygen heterocyclic fractions of the most common citrus oils.

In this article, one easy and rapid HPLC method, using short 3 cm columns with conventional internal diameter (4.6 mm) and 3 μm particle size and a method development software package (P-E Turbo Method Development), for the determination of oxygen heterocyclic compounds in five citrus oils is described.

MATERIALS AND METHODS

The five citrus essential oils analyzed were obtained from various producers in Sicily during the 1996 production season. The samples were genuine cold-pressed lemon, bergamot, red mandarin, sweet blond orange, and bitter orange essential oils. The samples were analyzed using the P-E (Perkin-Elmer Corp., Norwalk, CT) series 200 HPLC system, with the P-E model 235C photodiode array (PDA) detector and the P-E series 200 quaternary pump. The experimental conditions were as follow: column, P-E C_{18}, 3 cm × 4.6 mm i.d., 3 μm; solvent A, acetonitrile; solvent B, water; gradient elution program, initial 30% A/70% B for 0.5 min, gradient in 4 min to 80% A/20% B, hold for 2.5 min; flow rate, 1.5 mL/min; detection, 315 nm; bandwidth, 5 nm. The spectra were acquired in auto mode to determine peak purity; sample, 1:100 dilutions in acetonitrile were prepared for each essential oil, sample volume injected, 20 μL.

The analytical HPLC system was equipped with the P-E Turbochrom Professional Workstation, including the spectral application program Turboscan and the Turbo Method Development software. The latter was helpful for the rapid determination of the optimal eluent composition. The system searched in an automated program for the best separation, reducing greatly the method development time. This method was completely developed and tested within 3 days.

Table 1. Tentative Peak Identification in Lemon, Bergamot, Bitter Orange, Mandarin, and Sweet Orange Oils (see Figure 1)

peak	compound
1	citropten
2	sinensetin
3	meranzin
4	isomeranzin
5	bergapten
6	3,3′,4′,5,6,7-hexamethoxy flavone
7	nobiletin
8	oxypeucedanin
9	tetra-*O*-methylscutellarein
10	3,3′,4′,5,6,7,8,-heptamethoxy flavone
11	tangeretin
12	imperatorin
13	phellopterin
14	osthol
15	isoimperatorin
16	epoxybergamottin
17	5-isopent-2′-enyloxy-7-methoxy coumarin
18	5-isopent-2′-enyloxy-8-(2′,3′-epoxyisopentyloxy) psoralen
19	8-geranyloxy coumarin
20	bergamottin
21	5-geranyloxy 7-methoxy coumarin

RESULTS AND DISCUSSION

Figure 1 shows the HPLC chromatogram of the five oils analyzed, and Table 1 reports peak identification. Identification was tentatively done by comparison of UV spectra and the literature data (Dugo et al., 1997; Ziegler et al., 1995). More information (preferably LC/MS) is needed to confirm the identified components (Sendra et al., 1988; Kefford et al., 1970; Murray et al., 1982). As can be seen in Figure 1, the separation achieved for the five oils on the 30 mm × 4.6 mm C_{18} column under identical chromatographic conditions was

Table 2. Reproducibility of Five HPLC Analyses of Bitter Orange Oil

		retention time		area	
peak	compound	*X*	% RSD	*X*	% RSD
3 + 4	meranzin + isomeranzin	1.27	1.24	1 060 003	0.33
5	bergapten	1.50	1.35	274 538	0.51
7	nobiletin	1.84	1.91	86 737	0.54
10	3,3′,4′,5,6,7,8-hepta-methoxyflavone	2.29	1.95	9 070	14.18
11	tangeretin	2.85	1.39	236 746	0.22
14	osthol	3.84	0.83	193 977	0.32
16	epoxybergamottin	4.17	0.87	257 741	0.7

satisfactory to characterize each essential oil, rendering possible an immediate comparison of the oils.

Bitter orange oil contains three classes of components (coumarins, psoralens, and PMFs) and was used to prove reproducibility of the method. Table 2 reports the average values for retention time and peak areas, relative to five injections of the bitter orange oil and the resulting relative percent standard deviation (% RSD) to evaluate the method reproducibility. The precision of retention times is very good, typically 1−2% RSD. Peak areas are very reproducible, less than 1% RSD for all peaks except peak 10, which is poor as a result of the lack of precision for the integration of such small peak.

Sweet orange and mandarin essential oils have very similar composition and differ from the other oils containing only PMFs. They can be differentiated from each other by comparing two values: Sweet orange contains the 3,3′,4′,5,6,7-hexamethoxy flavone, which is not present in mandarin oil (Dugo et al., 1997), and the ratio nobiletin/tetra-*O*-methylscutellarein greatly differs for these oils. This ratio is approximately 2/1 for sweet orange oil and about 14/1 for mandarin oil.

Lemon and bergamot oils have three components in common but the two oils can be easily differentiated from the ratio 5-geranyloxy-7-methoxy coumarin/bergamottin, which is about 0.1 for bergamot and 1.0 for lemon oil. Lemon and bergamot oils can be easily differentiated from sweet orange and mandarin oils because they contain only coumarins and psoralens, whereas orange and mandarin oils contain only PMFs. The components typical for sweet orange and mandarin oils are easily detectable in lemon and bergamot oils. Therefore, by determining the presence of polymethoxylated flavones in lemon essential oil from the chromatographic profile or by checking peak purity and maximum absorbance, of the coeluting components, it is possible to determine sweet orange and mandarin oil contamination in lemon and bergamot oils. Lemon and bergamot oils can also be differentiated from bitter orange, because they contain bergamottin and 5-geranyloxy-7-methoxy coumarin. Bitter orange oil is different from all the other analyzed oils for the presence of two characteristic components, meranzin and isomeranzin.

The proposed method is simple and rapid; it allows the differentiation of the citrus essential oils analyzed, permitting detection of possible cross contamination.

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