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Analysis of the sesquiterpene fraction of *citrus* essential oils by using the off-line combination of high performance liquid chromatography and gas chromatography-based methods: a comparative study†

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Abstract: The present investigation is based on the off-line combination of high-performance liquid chromatography (HPLC), with different high-resolution gas chromatography (GC) methods (LC//GC), for the analysis of the sesquiterpene hydrocarbon (SH) fraction of various *Citrus* essential oils. Attention was directed to such constituents because the SH profile is often highly specific and can vary considerably between different *Citrus* oil-types. The (normal phase) HPLC step was exploited for the separation of the hydrocarbons, from the oxygenated compounds, hence generating a simplified sub-sample. After, the hydrocarbon fraction was concentrated and subjected to analysis, using conventional GC-quadrupole mass spectrometry (qMS) and GC-flame ionization detection (FID), comprehensive two-dimensional GC-qMS and comprehensive two-dimensional GC-FID, and cold-injection GC-qMS. The latter approach was exploited to avoid and evaluate solute rearrangements occurring under hot-injection conditions. The data derived from each approach were compared, to evaluate each analytical approach. Apart from the comparative scopes, an overall highly-detailed view (both qualitative and quantitative) was attained on the SH fraction of each essential oil, with many constituents reported here for the first time. Copyright © 2015 John Wiley & Sons, Ltd.

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Keywords: comprehensive two-dimensional gas chromatography; sesquiterpene hydrocarbons; *Citrus* essential oil; quadrupole mass spectrometry; LC//GC

Introduction

The content of sesquiterpene hydrocarbon (SH) compounds, in the volatile fraction of industrially cold-pressed *Citrus* essential oils, can vary from about 0.1% in some sweet and bitter orange oils, to nearly 9% in Key lime oils. Even if some SH constituents are found in almost all *Citrus* oils, the SH profile is often highly specific and can vary considerably between different oil-types. A great deal of such data is in good agreement, even though contradictions do exist, especially in relation to minor constituents. For instance, the presence of a specific SH compound is often given by a single research group.[1,2] Such an occurrence can be related, in great part, to the use of different analytical approaches.

The great majority of the analytical results, in the field of *Citrus* oil volatiles, has been attained through gas chromatography–mass spectrometry (GC-MS), mainly using single quadrupole mass analyzers, and commercially-available MS databases.[2] Although such an approach does often provide satisfactory results, inconveniences can often arise, due to different causes. One, for example, is related to the reliability of MS identification. The latter can be enhanced if spectral searches are accompanied by analyte retention information, such as linear retention indices (LRIs).[3,4] In fact, sesquiterpene hydrocarbons are characterized by similar structures and so often a series of different matches, with good similarity values, can be attained for the same analyte. A further problem is related to overlapping at the GC column outlet of: (I) two or more hydrocarbons, and/or (II) hydrocarbon and oxygenated compounds. Such co-elutions, which are caused by insufficient peak capacity and/or a lack of selectivity, have been discussed recently by Dugo *et al.*[2] In such cases, the reliable identification and quantification of minor SH constituents becomes an excessive challenge, especially for those oils in which the overall SH fraction is

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present in low amounts. Finally, possible rearrangements can occur in hot GC injectors (e.g., germacrenes) which can cause the generation of incorrect analytical data.[5]

Considering the aforementioned difficulties, then the following GC-MS features are desirable: (I) an MS database equipped with LRI data, (II) a GC method characterized by high peak capacity, selectivity and sensitivity, (III) use of a cold GC injector [e.g., cold on column or programmed-temperature vaporizer (PTV)].

In relation to features I and II, an off-line method proposed by Tranchida *et al.*, is herein considered.[6] Specifically, the hydrocarbon and oxygenated compounds contained in sweet orange and bergamot oils, were separated using normal-phase (NP) high performance liquid chromatography (HPLC). Each HPLC fraction was collected, reduced in volume, and then subjected to the most powerful GC-based technique today-available, cryogenically-modulated comprehensive two-dimensional gas chromatography-quadrupole mass spectrometry (LC//GC×GC-qMS). The volatiles were identified through MS database matching, using LRI information as a filter. The concentrated HPLC fractions gave origin to chromatograms characterized by many well-separated peaks, due to three fundamental GC×GC characteristics, namely the enhanced peak capacity, selectivity and sensitivity. In general, the analytical advantages of GC×GC have often been exploited for the analysis of essential oils.[7] It is also noteworthy that the off-line combination of HPLC and 1D GC is not new to the field of *Citrus* essential oils.[1,2,8]

In the present research, the approach employed by Tranchida *et al.* was applied to the study of the SH fraction of several industrially cold-pressed Citrus essential oils. Furthermore, the sesquiterpene fraction of the same samples were subjected to GC×GC-qMS and GC×GC-FID, conventional GC-FID and GC-qMS, to attain qualitative and % data. Finally, cold-injection PTV GC-qMS conditions were also applied to all the samples, to evaluate possible rearrangements that can occur using a hot injector. The information derived gave a highly-detailed view on the SH fraction of each essential oil.

Experimental

Samples and sample preparation

A C_7-C_{30} *n*-alkane series was kindly provided by Sigma-Aldrich/Supelco (Bellefonte, PA, USA), for the calculation of LRI values. A C_9 *n*-alkane (IS) hexane solution (10 mg L^{-1}) was used for internal standardization purposes.

Genuine cold-pressed samples of lemon (two), bergamot (two), sweet orange (two), clementine, bitter orange (two), Italian mandarin (green, yellow, red), pink grapefruit oils were provided by Simone Gatto s.r.l. (San Pier Niceto, Messina, Italy); a clementine oil was from Citrofood s.r.l. (Pace del Mela, Messina, Italy), while lime (Key A, Key B and Persian) and mandarin Mexican oils were provided by Citrojugo S.A. de C.V. Tecomán (Colima, Mexico). Prior to LC analyses the oils were diluted 1:2 (*v/v*) in hexane.

LC pre-separation

All the essential oils were pre-separated using an LC//GC system (Shimadzu, Kyoto, Japan), previously described.[6]

LC conditions: a 100 × 3 mm ID × 5 μm d_p silica column (SUPELCOSIL LC-Si, Sigma-Aldrich/Supelco, Bellefonte, PA, USA) was operated under the following gradient conditions (flow: 0.35 mL/min): 0–4.5 min (100% hexane); from 4.5 to 6.0 min 100% MTBE (until the end of the analysis). Injection volume: 20 μL.

LC fractions: hydrocarbons were collected from approximately 1.5 to 3 min, in relation to the detector response. Data were acquired by using a photodiode array detector in the range 190–390 nm, while the sampling frequency was 1.5625 Hz.

Prior to GC injection, the fractions were reduced to a volume of 0.1 mL (under a gentle stream of nitrogen). The boiling points of sesquiterpene hydrocarbons are high enough to avoid the necessity of precautions to prevent losses.

GC×GC-qMS analysis

All GC×GC-qMS applications were carried out on a system, consisting of a GC2010 gas chromatograph, and a QP2010 Ultra quadrupole mass spectrometer (Shimadzu, Kyoto, Japan).

The primary column, an SLB-5ms 30 m × 0.25 mm ID × 0.25 μm d_f column [silphenylene polymer equivalent to 5% diphenyl polysiloxane (Sigma-Aldrich/Supelco, Bellefonte, PA, USA)], was connected to an uncoated capillary segment (1.5 m × 0.18 mm ID, used to create a delay loop), by using an SGE SilTite mini-union (SGE, Ringwood, Victoria, Australia). The uncoated capillary was then connected to a segment of Supelcowax-10 (100% polyethylene glycol) 1.0 m × 0.10 mm ID × 0.10 μm d_f column (Sigma-Aldrich/Supelco), by using another union (SGE, Ringwood, Victoria, Australia). Modulation was carried out every 5 sec, by using a loop-type modulator (under license from Zoex Corporation, Houston, TX, USA). The duration of the hot pulse (400°C) was 400 msec.

GC oven temperature programme: 50°C to 250°C at 3°C/min. Carrier gas, helium, was supplied at an initial pressure of 173.5 kPa (constant linear velocity). Injection temperature: 250°C.

Injection mode and volume: split (1:20), 1.0 μL.

MS parameters: the sample was analyzed in the full scan mode using a mass range of 40–360 *m/z*; spectra generation frequency: 33 Hz; interface and ion source temperatures were 250°C and 200°C, respectively. MS ionization mode: electron ionization.

Data were collected by the GCMS Solution software (Shimadzu, Kyoto, Japan); bidimensional visualization was carried out by using the ChromSquare *v*.2.0 software (Shimadzu, Kyoto, Japan), while the MS databases employed were the FFNSC 2.0 (Shimadzu, Kyoto, Japan) and 'The atlas of spectral data of sesquiterpene hydrocarbons' (EB-Verlag, Hamburg, Germany).

GC×GC-FID analysis

All GC×GC-FID applications were carried out on a system, consisting of a GC2010 gas chromatograph. The columns, delay loop and unions were the same as in section '**GC×GC-qMS analysis**'. Modulation conditions were the same as in section '**GC×GC-qMS analysis**'.

GC oven temperature programme: same as in section '**GC×GC-qMS analysis**'. Carrier gas, He, was supplied at an initial pressure of 213 kPa (constant linear velocity). Injection conditions were the same as in section '**GC×GC-qMS analysis**'.

FID parameters: temperature 280°C, sampling frequency 50 Hz.

Data were collected by the GC Solution software (Shimadzu, Kyoto, Japan); bidimensional visualization was carried out by using the ChromSquare *v*.2.0 software.

GC-qMS analysis

All GC-qMS applications were carried out on a system, consisting of a GC2010 gas chromatograph, and a QP2010 Ultra quadrupole mass spectrometer.

Column: SLB-5ms 30 m × 0.25 mm ID × 0.25 μm d_f. GC oven temperature programme: 50°C to 250°C at 3°C/min. Carrier gas, He, was supplied at an initial pressure of 26.7 kPa (constant linear velocity). Injection temperature: 250°C. Injection mode and volume: split (1:20), 1.0 μL.

MS parameters: the sample was analyzed in the full scan mode using a mass range of 40–360 *m/z*; spectra generation frequency: 2 Hz; interface

and ion source temperatures were 250°C and 200°C, respectively. MS ionization mode: electron ionization.

Data were collected by the GCMS Solution software. The MS databases employed were the FFNSC 2.0 and 'The atlas of spectral data of sesquiterpene hydrocarbons'.

GC-FID analysis

All GC-FID applications were carried out on a GC-2010 Plus system (Shimadzu, Kyoto, Japan).

Column: same as in section 2.5. GC oven temperature program: same as in section '**GC-qMS analysis** '.

Carrier gas, He, was supplied at an initial pressure of 99.5 kPa (constant linear velocity). Injection conditions were the same as in section '**GC-qMS analysis**'. FID parameters: temperatures 280°C, sampling frequency 12.5 Hz. Data were collected by the GC Solution software.

Cold-injection PTV GC-qMS analysis

All cold-injection PTV GC-qMS applications were carried out on a system, consisting of a GC2010 gas chromatograph, and a QP2010 Ultra quadrupole mass spectrometer, equipped with an Optic 4 injector (ATAS GL International, Eindhoven, The Netherlands).

Column: same as in section '**GC-qMS analysis**'. GC oven temperature programme: 35°C (2 min) to 100°C (75 min) at 20°C/min. Carrier gas, He, was supplied at an initial pressure of 43.6 kPa (constant linear velocity). The Optic 4 injector was temperature-programmed as follows: from 35°C (2 min) to 100°C at 2°C/sec. Injection conditions were the same as in section 2.5.

MS parameters: the same as in section '**GC-qMS analysis**'. Data were collected by the GCMS Solution software.

Main GC operational conditions are summarized in Table 1.

Results and discussion

An HPLC-PDA sweet orange oil extracted chromatogram (210 nm), highlighting the response of the hydrocarbon fraction, is shown in Figure 1. The sesquiterpene hydrocarbons were weakly retained on the silica column and eluted just after the dead volume. It is noteworthy that, in general, SH retention times were very stable.

For peak assignment the following levels of identification were considered: (I) 'reliably' identified compound: MS database similarity equal to, or above 90%, and experimental LRI value within a ± 5 LRI unit window, with respect to the database value, (II) 'presumably' identified compound: either MS database similarity ≥ 90%, or experimental LRI value within a ± 5 LRI unit window; a 'presumably' identified compound cannot be characterized by a similarity match < 80%, or an experimental LRI value outside a ± 10 LRI unit range; (III) 'tentatively' identified compound: MS database similarity above 75% and experimental LRI value within a ± 15 LRI unit range, compared to the database value.[6] With regards to the stability of LRI values, it has been demonstrated that when using polysiloxane-based phases these can undergo considerable variations when using different temperature programmes; on the contrary, LRI values have been found to be very stable when using the same temperature conditions.[9] The temperature programme applied in the present study is equal to that used to construct the MS database, and so any database match falling outside the ± 15 LRI unit range was not considered. Finally, the influence of the short 'wax' second dimension on the calculated LRI values of the sesquiterpene hydrocarbons was presumed to be negligible.

Table 2 reports the % composition of the SH fraction (g/100 g essential oil), of the nine *Citrus* oils, isolated by using an HPLC step, and determined through GC-FID and GC×GC-FID analysis (identification was performed through GC-qMS and GC×GC-qMS analyses), with the support of cold-injection PTV GC-qMS data. FID response factors for sesquiterpene hydrocarbons are equal, or very similar, and so such information is commonly used to generate % information.[1,2] Database LRI values are also reported in the table. In principle, even if GC×GC with dual simultaneous MS/FID detection could have been used, this would have caused a loss in sensitivity.

A heat map constructed by using the data shown in Table 2 is illustrated in Figure S1 (supplementary material). With regard to GC-FID and GC×GC-FID analysis, the total number of single

Table 1. Experimental conditions for the one- and two-dimensional GC applications. Abbreviations: D1/D2 = first/second dimension; ALV = average linear velocity; acq. = acquisition. In all applications, the first or the only column was an SLB-5ms 30 m × 0.25 mm ID one; the second GC dimension was always a Supelcowax-10 1 m × 0.10 mm ID column

Method	Inlet pressure (kPa)	ALV (cm/s)	Loop ALV (cm/s)	D2 ALV (cm/s)	Detection (mass range, acq. frequency)
GC×GC-qMS	173.5	20 (D1)	44	224	40-360 *m/z*-33 Hz
GC×GC-FID	213	20 (D1)	44	194	50 Hz
GC-qMS	26.7	30			40-360 *m/z*-2 Hz
GC-FID	99.5	30			12.5 Hz
PTV GC-qMS	43.6	35			40-360 *m/z*-2 Hz

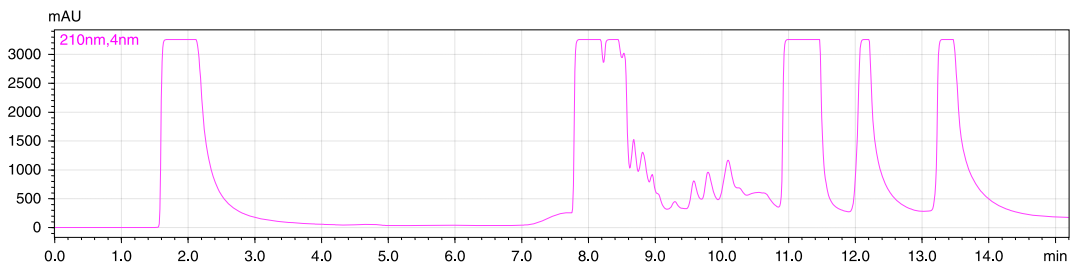

Figure 1. Sweet orange essential oil HPLC-PDA chromatogram (extracted at 210 nm)

Table 2. Percentage composition (g/100 g essential oil) of the SH fraction (defined by using an internal standard), isolated by using an HPLC step, and determined through GC-FID and GC×GC-FID analysis (identified through GC-qMS and GC×GC-qMS analyses), with the support of cold-injection PTV GC-quadMS. Refer to section 3.0 for information on the identification levels I, II and III (when two values are reported in parenthesis the first is related to the GC-qMS result). Database LRI values. Percentage composition ranges with no standardization (both experimental and derived from the literature) are reported at the bottom

	Database LRI	Lemon 1	Lemon 2	Bergamot 1	Bergamot 2	Sweet Orange 1	Sweet Orange 2	Clementine 1	Clementine 2	Bitter Orange 1	Bitter Orange 2
δ-Elemene	1335	-	-	0.4 (I,I)	0.6	-	-	-	-	-	-
α-Cubebene	1349	traces[a] (I)	traces	traces[c,e] (II,I)	-	0.2 (I,I)	0.2	0.3 (I,I)	0.3	0.1 (I,II)	0.1
Cyclosativene	1367	-	-	-	-	0.4[e] (II,I)	0.4	0.5[d] (II,I)	0.5	-	-
α-Ylangene	1371	-	-	-	-	-	-	-	-	traces [d] (I,II)	traces
α-Copaene	1375	0.1[a] (I)	0.1	traces[e] (I,I)	traces	6.2 (II,I)	7.6	12.0 (I,I)	12.8	0.3 (I,I)	0.3
β-Bourbonene	1382	-	-	-	-	-	-	-	-	0.2[d] (I,I)	0.2
β-Elemene	1390	0.3[a] (I)	0.3	traces[d] (I,I)	0.1	0.4 (I,I)	0.4	0.3 (I,I)	0.1	0.2 (I,I)	0.2
β-Cubebene	1392	-	-	-	-	5.6 (I,I)	6.7	10.6 (I,II)	12.2	0.1 (I,I)	0.1
Sativene	1394	-	-	-	-	0.4[e] (III,III)	0.4	0.5[d] (II,II)	0.5	-	-
α-Funebrene	1403	-	-	traces [d] (I,I)	traces	0.3[e] (I,I)	0.3	0.4[d] (I,I)	0.8	traces [d] (I,II)	traces
Sibirene	1405	-	-	-	-	-	-	-	-	-	-
β-Longipinene	1405	-	-	-	-	0.1[a,e] (III)	0.1	-	-	-	-
α-Gurjunene	1406	-	-	-	-	-	-	0.1[d] (I,I)	0.1	-	-
Longifolene	1412	-	-	-	-	-	-	0.1[d] (III,III)	0.1	-	-
β-Maaliene	1415	-	-	-	-	0.1[a,e] (I)	0.1	-	-	0.1[d] (I,II)	0.1
cis-α-Bergamotene	1416	2.0 (I,I)	1.2	1.1 (I,I)	1.7	-	0.1[a,c] (I)	0.1[a] (I)	0.1	-	-
α-Santalene	1418	0.6[a] (I)	0.6	0.4[a] (II)	0.6	-	-	-	-	-	-
tr-Caryophyllene	1424	14.0 (I,I)	15.7	26.6 (I,I)	26.4	5.5 (I,I)	6.7	4.1 (I,I)	3.9	31.1 (I,I)	29.7
γ-Elemene	1432	-	-	-	-	0.1[a] (I)	0.1	-	-	-	-
tr-α-Bergamotene	1432	24.7 (I,I)	26.5	23.0 (I,I)	17.8	-	1.0[c] (II,I)	0.5 (I,I)	0.8	0.2[b] (I)	0.2
β-Copaene	1433	-	-	-	-	5.1 (II,I)	6.9	6.6 (I,I)	7.8	0.5[a,d] (I)	0.5
α-Guaiene	1438	-	-	-	-	0.3[e] (I,I)	0.3	0.4[d] (I,I)	0.6	traces [d] (III,I)	traces
Aromadendrene	1438	1.4 (II,II)	1.6	0.7 (II,II)	1.3	-	-	-	-	0.3[a] (I)	0.3
cis-β-Farnesene	1439	0.2[b] (III)	0.2	0.2[b] (III)	0.2	-	-	0.1[b] (III)	0.1	-	-
Guaia-6,9-diene	1444	-	-	-	-	-	-	-	-	-	-
epi-β-Santalene	1446	-	-	traces [b](II)	traces	-	-	-	-	-	-
Isogermacrene D	1447	-	-	-	-	0.1[a,e] (II)	0.1	-	-	0.1[b,d] (I)	0.1
α-Himachalene	1449	1.9[b,d] (III)	0.1	-	-	-	-	-	-	-	-
tr-β-Farnesene	1452	1.9 (I,I)	1.5	3.9 (I,I)	3.5	4.2 (I,I)	4.6	7.8 (I,I)	6.0	2.9 (I,I)	3.3
Spirolepechinene	1452	-	-	-	-	0.1[b,d] (I)	0.1	-	-	-	-
α-Humulene	1454	0.8 (II,I)	1.4	1.9 (III,I)	2.3	1.1 (II,I)	1.5	2.4 (I,I)	2.4	3.2 (I,I)	2.8
Sesquisabinene	1455	0.1[a,d] (I)	0.3	0.5[a,e] (I)	0.7	0.6[e] (I,I)	1.4	1.7[d] (I,I)	1.9	0.3[d] (I,II)	0.3
alloAromadendrene	1458	-	-	-	-	0.1[a] (II)	0.1	-	-	-	-
β-Santalene	1459	1.4 (II,I)	0.7	0.7 (III,I)	1.1	-	-	-	-	-	-
9-epi-*tr*-Caryophyllene	1464	traces[a,d] (II)	traces	0.1[a,e] (I)	0.1	0.1[a,e] (II)	0.1	0.1[d] (I,I)	0.3	0.2[d] (I,II)	0.2
Selina-4,11-diene	1476	-	-	-	-	-	-	-	-	-	-

(Continues)

Table 2. *(Continued)*

	Database LRI	Lemon 1	Lemon 2	Bergamot 1	Bergamot 2	Sweet Orange 1	Sweet Orange 2	Clementine 1	Clementine 2	Bitter Orange 1	Bitter Orange 2
γ-Gurjunene	1476	-	-	-	-	0.4 (II,II)	0.4		0.7[c] (I,II)	0.2[a,d] (II)	0.2
γ-Muurolene	1478	-	-	-	-	1.2 (I,I)	1.2	0.6 (I,I)	1.3	0.1 (I,II)	0.1
β-Chamigrene	1479	-	-	-	-	0.1[a,e] (II)	0.1	-	-	-	-
Germacrene D	1480	-	-	3.4 (II,I)	3.2	4.5 (II,I)	6.4	9.4 (I,I)	9.4	41.6 (II,I)	43.2
α-Neocallitropsene	1480	0.1[a] (II)	0.1	0.1[a,e] (II)	0.1	-	-	-	-	-	-
α-Curcumene	1480	-	-	-	-	-	-	-	-	-	-
γ-Curcumene	1482	0.5[d] (I,I)	0.3	0.3 (I,I)	0.3	-	-	-	-	-	-
tr-β-Bergamotene	1483	1.6 (I,I)	1.2	0.5 (III,I)	1.5	-	-	-	-	-	-
Aristolochene	1490	0.1[a,d] (II)	traces	-	-	2.0[e] (II,I)	1.2	-	-	-	-
Eremophilene	1491	-	-	-	-	-	-	0.2[d] (II,II)	0.2	-	-
Valencene	1492	1.3 (II,I)	0.7	traces [a] (II)	traces	33.7 (II,I)	25.3	0.9 (I,I)	0.9	0.1 (I,I)	0.1
β-Selinene	1492	-	-	-	-	-	-	0.2[a] (I)	0.2	-	-
α-Zingiberene	1496	-	-	-	-	-	-	-	-	-	-
Bicyclogermacrene	1497	2.6 (I,I)	4.4	1.0 (II,I)	1.2	3.3[b] (I)	3.7	2.4 (I,I)	3.6	4.0 (I,I)	4.0
α-Muurolene	1497	-	-	-	-	1.3[b] (I)	0.9	1.7[b] (I)	1.9	-	-
α-Selinene	1501	-	-	-	-	2.7[b,d] (I)	2.5	-	-	-	-
ε-Amorphene	1502	-	-	-	-	-	-	-	-	0.4[d] (I,I)	0.4
cis-α-Bisabolene	1503	3.9 (I,I)	2.5	2.7 (I,I)	2.3	-	-	-	-	-	-
Isodaucene	1504	-	-	-	-	-	-	-	-	-	-
tr, tr-α-Farnesene	1504	traces[b,c] (II)	-	0.3 (II,I)	0.3	3.0 (II,I)	4.3	12.9 (I,I)	9.5	0.3[b] (I)	0.3
α-Bulnesene	1505	-	-	-	-	0.6[e] (I,I)	0.6	0.5[a,d] (I)	0.5	-	-
δ-Amorphene	1506	-	-	-	-	-	-	-	-	-	-
β-Bisabolene	1508	37.2 (I,I)	38.6	30.9 (I,I)	30.5	0.6 (I,I)	1.2	2.6 (I,I)	1.4	0.1 (I,I)	0.5
cis-γ-Bisabolene	1511	2.0 (I,I)	1.6	0.5 (I,I)	1.1	-	-	0.1[a] (I)	0.1	-	-
Germacrene A	1511	-	-	0.1	0.1	5.3	4.8	3.0	2.6	1.9	2.1
γ-Cadinene	1512	-	-	-	-	0.2[a] (II)	0.2	0.2 (I,II)	0.2	0.1 (I,II)	0.1
δ-Cadinene	1518	0.1[a] (I)	0.1	0.1 (I,I)	0.3	6.8 (I,I)	8.1	12.7 (I,I)	13.7	1.3 (I,I)	0.9
Germacrene C	-	-	-	0.4	0.4	0.4[d]	0.4	0.1[d]	0.1	8.0	7.6
7-epi-α-Selinene	1518	0.2[a,d] (III)	0.1	-	-	2.0 (III,I)	1.2	-	-	-	-
β-Sesquiphellandrene	1523	0.2[a] (I)	0.2	0.2 (I,I)	0.2	1.2[b] (I)	0.8	2.6 (I,I)	2	0.3 (I,I)	0.3
tr-γ-Bisabolene	1528	0.3 (I,II)	0.1	0.1 (I,I)	0.1	-	-	-	-	-	-
γ-Cuprenene	1530	-	-	-	-	-	-	-	-	-	-
tr-Cadina-1,4-diene	1536	-	-	traces [d] (I,II)	traces	traces [a,e] (I)	traces	0.1[d] (I,II)	0.1	traces [d] (I,II)	traces
α-Cadinene	1538	-	-	-	-	-	-	-	-	-	-
Selina-4(15),7(11)-diene	1540	-	-	-	-	traces [a,e] (II)	traces	0.1[a,d] (II)	0.1	0.1[a,d] (II)	0.1
tr-α-Bisabolene	1540	0.7 (I,I)	0.5	0.4 (I,I)	0.8	-	0.1[a,c,e] (I)	traces [a,c,d] (II)	-	-	-
α-Calacorene	1544	-	-	-	-	-	-	traces [a,c,d] (II)	-	-	-
Germacrene B	1557	-	-	0.3 (I,I)	0.3	0.3 (II,II)	0.4	0.5 (II,I)	0.5	1.9 (I,I)	2.1

(Continues)

Table 2. *(Continued)*

	Database LRI	Lemon 1	Lemon 2	Bergamot 1	Bergamot 2	Sweet Orange 1	Sweet Orange 2	Clementine 1	Clementine 2	Bitter Orange 1	Bitter Orange 2
Experimental absolute content (g/100 g) of SH	-	1.18	1.14	1.22	1.02	0.20	0.15	0.14	0.13	0.15	0.17
% values of SH (without standardization)	-	1.32	1.28	1.44	1.38	0.21	0.17	0.17	0.17	0.18	0.20
Literature % content of SH (without standardization)	-	0.8-1.6		0.7-2.0		0.1-0.4		0.2		0.1-0.4	

		Mandarin				Lime		
	Mexican	Green	Yellow	Red	Grapefruit	Key A	Key B	Persian
δ-Elemene	-	-	-	-	-	0.5 (I,I)	0.5 (I,I)	0.2 (I,I)
α-Cubebene	0.2 (I,I)	traces (I,I)	0.1 (I,I)	0.1 (I,I)	0.2 (I,I)	traces (II,II)	0.1 (II,II)	traces [b] (II,II)
Cyclosativene	traces [a,d] (III)	traces [a,d] (III)	traces [a,d] (III)	traces [a,d] (III)	0.1[d] (I,II)	-	-	-
α-Ylangene	-	-	-	-	-	-	-	-
α-Copaene	6.7 (I,I)	2.1 (II,I)	2.2 (II,I)	2.7 (II,I)	10.7 (I,I)	traces (I,II)	traces (I,II)	traces [d] (II,I)
β-Bourbonene	-	-	-	-	0.1[b,d] (II)	traces [b,d] (II)	traces [b,d] (I)	-
β-Elemene	0.2 (II,II)	0.1 (I,I)	0.1 (I,I)	0.1 (I,I)	-	0.8 (I,II)	0.9 (I,II)	0.4 (I,I)
β-Cubebene	6.4 (I,I)	1.9 (I,I)	2.0 (I,I)	2.5 (I,II)	10.4 (I,I)	-	-	-
Sativene	-	-	-	-	-	-	-	-
α-Funebrene	-	-	-	-	0.1[a,d] (II)	-	-	-
Sibirene	-	-	-	-	0.1[a,d] (II)	-	-	-
β-Longipinene	-	-	-	-	-	-	-	-
α-Gurjunene	-	traces [a,d] (III)	traces [a,d] (III)	traces [a,d] (III)	traces [a,d] (II)	-	-	-
Longifolene	-	-	-	-	-	-	-	-
β-Maaliene	0.1[a,d] (II)	-	-	-	0.1[a,d] (I)	-	-	-
cis-*α*-Bergamotene	-	-	-	traces [a] (II)	-	1.0 (I,II)	0.9 (I,I)	1.6 (I,I)
α-Santalene	-	-	-	-	-	0.3[a] (I)	0.2[a] (I)	0.6[a] (I)
tr-Caryophyllene	20.9 (I,I)	27.8 (I,I)	27.8 (II,I)	23.3 (I,I)	39.3 (I,I)	11.6 (I,I)	13.7 (I,I)	12.1 (I,I)
γ-Elemene	-	-	-	-	-	-	-	-
tr-*α*-Bergamotene	-	-	-	0.1 (II,II)	-	14.0 (I,I)	13.4 (I,I)	23.4 (I,I)
β-Copaene	0.1[b,d] (II)	traces [b,d] (I)	traces [b,d] (I)	traces [b,d] (I)	0.3 (I,I)	-	-	-
α-Guaiene	0.3 (I,I)	0.1 (I,I)	0.1 (II,I)	0.1 (I,I)	0.3[d] (I,I)	0.6[b,d] (II)	0.7[b,d] (II)	1.1[b,d] (II)
Aromadendrene	0.1[a,d] (I)	0.1[a,d] (I)	0.1[a,d] (I)	0.1[a,d] (II)	0.2[a,d] (II)	-	-	-
cis-*β*-Farnesene	-	-	-	-	-	0.1[b] (III)	0.1[b] (III)	0.2[b] (III)
Guaia-6,9-diene	-	-	-	-	-	0.1[b,d] (I)	0.1[b,d] (I)	traces [b,d] (I)
epi-*β*-Santalene	-	-	-	-	-	0.1[b] (II)	0.1[b] (II)	0.1[b] (II)
Isogermacrene D	-	-	-	-	-	-	-	-
α-Himachalene	0.2[b,d] (II)	0.1[d] (II,II)	0.1[a,d] (II)	0.1[d] (II,II)	-	1.5[a,d] (III)	1.8[a,d] (II)	0.2[a,d] (I)
tr-*β*-Farnesene	0.1[a] (I)	0.2[a] (I)	0.2 (I,II)	0.1[a] (I)	0.8 (I,I)	1.4 (I,I)	1.3 (I,I)	2.2 (I,I)
Spirolepechinene	-	-	-	-	-	-	-	-

(Continues)

Table 2. (Continued)

	Mandarin					Lime		
	Mexican	Green	Yellow	Red	Grapefruit	Key A	Key B	Persian
α-Humulene	2.4 (I,I)	2.4 (II,I)	2.4 (II,I)	2.1 (II,I)	5.2 (II,I)	1.5 (II,I)	1.7 (II,I)	1.1 (II,I)
Sesquisabinene	-	-	-	-	0.3[a,d] (I)	0.4[a] (II)	0.3[a] (I)	0.2[a,d] (I)
alloAromadendrene	-	-	-	0.1[a,d] (II)	-	-	-	-
β-Santalene	-	-	-	-	-	0.6[b] (I)	0.6[b] (I)	1.0 (II,I)
9-epi-*tr*-Caryophyllene	0.1[d] (II,II)	traces [d] (III,II)	traces [d] (III,I)	0.1[a,d] (III)	0.1[a,d] (I)	-	-	-
Selina-4,11-diene	0.3[d] (I,I)	0.2[d] (I,I)	0.2[d] (I,I)	0.3[d] (I,I)	-	0.1[b,d] (I)	traces [b,d] (I)	traces [b,d] (I)
γ-Gurjunene	0.2[d] (I,I)	0.1[a,d] (II)	0.2[a,d] (III)	0.2[a,d] (III)	0.2[a,d] (II)	-	-	-
γ-Muurolene	0.7[a,d] (I)	-	-	-	0.3 (I,I)	-	-	-
β-Chamigrene	-	-	-	-	-	0.5[a,d] (II)	1.0[a,d] (II)	0.2[a,d] (II)
Germacrene D	1.1 (II,I)	1.8 (II,I)	1.8 (II,I)	2.3 (II,I)	9.2 (II,I)	3.9 (II,II)	3.8 (II,I)	1.2 (II,I)
α-Neocallitropsene	-	1.0[a,d] (II)	-	-	-	-	-	-
α-Curcumene	-	-	-	-	-	-	-	traces [a,d] (II)
γ-Curcumene	-	-	-	-	-	0.3 (I,I)	0.2 (I,I)	0.5 (I,I)
tr-β-Bergamotene	-	-	-	-	-	1.0 (II,II)	1.0 (II,II)	1.5 (I,I)
Aristolochene	-	-	-		-	-	-	-
Eremophilene	1.4[a,d] (I)	-	-	-	-	-	-	-
Valencene	1.8[b] (I)	0.9 (I,I)	0.9 (I,I)	1.1 (I,I)	-	0.9[a] (I)	0.5[a] (I)	0.3[a,d] (I)
β-Selinene	0.4[a] (I)	0.3 (II,I)	0.3 (I,I)	0.4 (II,I)	0.2[d] (I,I)	0.5 (I,II)	0.4 (I,II)	0.3 (I,I)
α-Zingiberene	-	-	-	0.3[a,d] (II)	-	-	-	-
Bicyclogermacrene	-	-	-	-	3.4 (I,I)	-	-	-
α-Muurolene	0.9[b,d] (II)	-	-	-	1.0[b] (II)	-	-	-
α-Selinene	16.0 (I,II)	10.2[b] (I)	10.2[b] (I)	11.9[b] (I)	-	0.9[b] (I)	0.9[b] (I)	0.5[b] (I)
ε-Amorphene	-	-	-	-	0.6[a,d] (II)	-	-	-
cis-α-Bisabolene	-	-	-	-	-	2.0 (I,I)	1.8 (I,I)	3.1 (I,I)
Isodaucene	-	-	1.8[a,d] (II)	0.7[a,d] (II)	-	-	-	-
tr, tr-α-Farnesene	31.2 (I,I)	46.8 (I,I)	45.2 (I,I)	45.9 (I,I)	-	14.6 (I,I)	15.8 (I,I)	4.8 (I,I)
α-Bulnesene	0.5[d] (I,II)	0.2[d] (II,II)	0.3[d] (II,II)	0.5[d] (II,II)	0.5[d] (I,I)	-	-	-
δ-Amorphene	-	-	-	-	0.2[a,d] (III)	-	-	-
β-Bisabolene	-	0.3 (II,II)	0.2 (II,II)	0.8 (I,I)	0.1[a] (II)	22.9 (I,I)	21.9 (I,I)	36.5 (I,I)
cis-γ-Bisabolene	-	-	-	-	-	0.3 (I,I)	0.4 (I,I)	0.5 (I,I)
Germacrene A	0.8	1	1	1.1	3.1[b] (II)	3.5	3.4	1.7
γ-Cadinene	-	-	-	-	traces (II,II)	-	-	-
δ-Cadinene	7.0 (I,I)	2.4 (II,I)	2.5 (II,I)	3.2 (II,I)	12.9 (I,I)	-	-	-
Germacrene C	-	-	-	-	-	4.9	4.2	1.3
7-epi-α-Selinene	-	-	-	-	-	0.1[b] (I)	0.1[b] (I)	0.1[b] (I)
β-Sesquiphellandrene	-	-	-	-	0.1[b,d] (II)	traces [b] (II)	traces [b] (II)	0.1[b] (I)
tr-γ-Bisabolene	-	-	0.1[a,d] (II)	0.1[a,d] (III)	-	0.3 (II,II)	0.2 (I,II)	0.2 (I,I)
γ-Cuprenene	-	0.1[a,d] (II)	0.1[a,d] (II)	-	-	-	-	traces [a,d] (II)
tr-Cadina-1,4-diene	0.1[a] (I)	-	-	-	0.1[d] (I,II)	-	-	-

(Continues)

Table 2. *(Continued)*

	Mandarin					Lime		
	Mexican	Green	Yellow	Red	Grapefruit	Key A	Key B	Persian
α-Cadinene	-	-	-	-	traces [a,d] (I)	-	-	-
Selina-4(15),7(11)-diene	-	-	-	-	-	-	-	-
tr-α-Bisabolene	-	-	-	-	-	0.5 (I,I)	0.4 (I,I)	0.7 (I,I)
α-Calacorene	-	-	-	-	-	-	-	-
Germacrene B	-	-	-	-	-	8.9 (I,I)	8.6 (I,I)	2.4[d] (I,I)
Experimental absolute content (g/100 g) of SH	0.26	0.30	0.27	0.27	0.73	6.20	5.78	3.59
% values of SH (without standardization)	0.30	0.35	0.31	0.31	0.80	8.52	7.86	4.50
Literature % content of SH (without standardization)		0.1-0.4			0.5-1.0	5.4-9.0	5.4-7.5	3.6-5.0

a) compounds identified only through LC//GC×GC-MS analysis
b) compounds identified only through per LC//GC-MS analysis
c) compound identified in only one of the samples
d) compound, to the best of the authors' knowledge, identified for the first time in industrially cold-extracted oils
e) compound, to the best of the authors' knowledge, identified only in ref. 6.

identified solutes by both approaches was considered, and then % values were extrapolated. The use of the PTV GC approach enabled the evaluation of rearrangement processes that occur during hot-injection analyses, and will be discussed later.

The last three lines of the table report information relative to the experimental absolute content of SH expressed as g/100 g of essential oil, SH% ranges (without standardization) reported in the literature, and the experimental % value (without standardization) found.[1,2,10,11]

Absolute SH quantification was carried out by using the internal standard method (C_9 alkane at a concentration of 10 mg L^{-1}), through hot-injection GC-FID analysis of each essential oil. FID response factors were the same as for C_9 alkane, namely 1. As expected, and as can be seen in Table 2, there are differences between SH% values with and without standardization.

It is noteworthy that, in many cases, the % composition of specific constituents, in the SH fraction, appears to be similar between different oils [e.g., *tr*-caryophyllene in bergamot and mandarin oils]; however, it must also be emphasized that the % composition of the same SH compounds, in the essential oil, can differ greatly considering the total SH % values (e.g., β-bisabolene in lemon and Persian lime oils).

In general, five SH compounds (α-cubebene, α-copaene, *tr*-caryophyllene, *tr*-β-farnesene, α-humulene) were present (in different percentages) in all the oils subjected to analysis. Other six constituents were contained in all the oils apart from one (indicated in parenthesis): germacrene A and germacrene D (lemon), β-bisabolene (Mexican mandarin), β-elemene, *(tr, tr)*-α-farnesene and valencene (grapefruit). Some specific minor compounds were found only in a single oil (the identification level is also reported), specifically: spirolepechinene (I) and β-longipinene (III) in sweet orange, α-calacorene (II) and longifolene (III) in clementine, α-ylangene (I) in bitter orange, α-zingiberene (II) in red mandarin, α-cadinene (I), δ-amorphene (III) and siberene (II) in grapefruit, and α-curcumene (II) in Persian lime.

As will be seen, many SH compounds for a specific *Citrus* essential oil are here reported for the first time (to the best of the authors' knowledge).

Lime oils

Prior to the description of the lime oil results, it is noteworthy that it was the only type of essential oil in which more compounds were identified through GC–qMS, compared to GC×GC-qMS.

Altogether, 35 SH (plus Germacrene A and C, through PTV injection) constituents were identified in Key A lime (six for the first time). 10 analytes were identified only through GC-qMS, five solutes only through GC×GC-qMS, while 20 SH compounds were identified by using both approaches (Table 2).

It is noteworthy that the elemenes (δ, β, γ) were derived (γ-elemene totally, and δ- and β- in part) from the Cope rearrangement of the germacrenes in the hot injector (250°C).[5] A certain amount of germacrene B remained untransformed, along with germacrene D, due to a high thermal stability.

The extent of hot-injector degradation was evaluated through a cold-injection PTV GC-qMS experiment (see section 'Cold-injection PTV GC-qMS analysis'). Germacrenes A and C were detected, germacrene B was present in a higher concentration, while the amount of germacrene D remained constant. γ-Elemene was not detected, while δ- and β-elemenes were detected at lower concentrations. On the basis of the PTV GC-qMS results, the (hot-injector)

GC×GC-FID and GC-FID information was modified accordingly, in relation to the % amounts of germacrenes and elemenes (the same procedure was performed for all the essential oils).

With regard to Key B lime oil, 35 SH (plus Germacrene A and C, through PTV injection) compounds were identified (six for the first time). 10 solutes were identified only through GC-qMS, five solutes only through GC×GC-qMS, while 20 SH compounds were identified by using both approaches (Table 2).

In Persian lime, 36 SH constituents were identified (11 for the first time). Eight solutes were identified only through GC-qMS, seven solutes only through GC×GC-qMS, while 21 SH compounds were identified by using both approaches (Table 2). Also in this case, germacrenes A and C were detected through cold-injection.

The absolute amounts of SH constituents contained in the two Key lime oils was approx. 6.2 g/100 g (type A) and 5.8 g/100 g (type B), while it amounted to 3.6 g/100 g in Persian lime.

The total amount of SH constituents contained in the two Key lime oils, obtained through FID analysis but without using the IS, were 8.5% (type A) and 7.9% (type B), while it amounted to 4.5% in Persian lime. The % values reported in the literature for industrially-produced cold-extracted oils range from 5.4 to 9.0% for type A, from 5.4 to 7.5% for type B, and from 3.6 to 5.0% for Persian lime.[1,2]

The same components were identified in the two Key lime oils, with the main constituents (each accounting for 0.72–1.42 % of the essential oil, in absolute concentrations) being β-bisabolene, *tr, tr*-α-farnesene, *tr*-α-bergamotene, and *tr*-caryophyllene. Such compounds represented 63 and 65% of the entire SH fraction, in types A and B, respectively. Furthermore, the following constituents were present in rather high amounts (each accounting for 0.08–0.55% of the essential oil, in absolute concentrations): α-humulene, germacrene A, B, C and D, *tr*-β-farnesene, *cis*-α-bisabolene, and α-himachalene (this compound was tentatively identified). All the remaining compounds were present in quantities lower than 0.08%.

In Persian lime oil, the main constituents (0.43–1.31% of the essential oil, in absolute concentrations) were β-bisabolene, *tr*-α-bergamotene, and *tr*-caryophyllene, amounting approximately to 70% of the entire SH fraction. Moreover, the following constituents were present in amounts between 0.08 and 0.17%: *tr, tr*-α-farnesene, *tr*-β-farnesene, *cis*-α-bisabolene and germacrene B. All the remaining compounds were present in quantities less than 0.08%.

As can be observed in Table 2, there were no qualitative differences between the two Key oils, but only slight % differences due to the higher SH content in the type A oil. With regard to the Persian lime oil, there were slight qualitative differences compared to the Key oils, in relation to a series of minor compounds (all presumably identified): β-bourbonene was contained only in the Key oils, while α-curcumene and γ-cuprenene were present only in the Persian oil. A series of conclusions can be made by observing the relative compositions of the SH fractions, in the different lime oils: the bergamotenes (*cis*-α-, *tr*-α-, *tr*-β-), the bisabolenes (β- and *cis*-α-), the santalenes (α- and β-), and the β-farnesenes (*cis* and *tr*) are contained in percentages 1.5–2 times higher in Persian oil, while the germacrenes (A, B, C and D), the selinenes (α- and β-), valencene, and *tr, tr*-farnesene, are present in percentages 2–3 times lower.

Lemon oil

Altogether, 30 SH constituents were identified (six for the first time) in lemon oil. Three solutes were identified only through GC-qMS, 11 solutes through GC×GC-qMS, while 16 SH compounds were identified by using both approaches (Table 2).

If the same analyte was identified at two different levels in the two samples, then the highest level was considered (the same is valid for all the other cases where two samples of the same oil were analyzed).

The amount of SH constituents contained in the two lemon oils was 1.18 g and 1.14 g/100 g. The SH percentage in the two samples, determined without using the IS, corresponded to 1.32% and 1.28% of the oil volatile fraction. The % values reported in the literature, for cold-pressed industrial oils, are in the 0.8–1.6% range, with the highest values reported for Spanish oils.[1,2] It is noteworthy that the two samples were produced in January 2013, and that the lemon oil SH fraction undergoes variations during the production season, reaching a minimum at the beginning (October/January) and a maximum in April/May. However, the proportions between the various compounds remain practically constant.[12] The main SH compounds in the two lemon oils were β-bisabolene, *tr*-α-bergamotene and *tr*-caryophyllene, which formed 0.44% (in both samples), 0.29% (sample 1)/0.30% (sample 2), and 0.17% (sample 1)/0.18% (sample 2) of the essential oil, respectively, and together approx. 76% (sample 1) and 81% (sample 2) of the SH content, respectively. Additionally, bicyclogermacrene, *cis*-α-bisabolene, and *cis*-γ-bisabolene were present in amounts exceeding 0.01% in both essential oils, while all the other compounds were present at levels $\leq$ 0.01% (Table 2). In the lemon samples the only germacrene identified was bicyclogermacrene, even when using the cold-injector method.

Bergamot oil

Altogether, 33 SH constituents were identified [three for the first time and five only once (in the previous research by Tranchida *et al.*[6])] in bergamot oil. Two solutes were identified only through GC-qMS, five solutes only through GC×GC-qMS, while 26 SH compounds were identified by using both approaches (Table 2). Germacrene A and C were determined through the cold-injection; using the latter approach, the amount of germacrene D and B remained constant, γ-elemene was not detected, while δ- and β-elemene were detected at lower concentrations.

The amount of SH constituents contained in the two bergamot oils was 1.22 g/100 g and 1.02 g/100 g.

The % of SH analytes found in the two samples, determined without using the IS, amounted to 1.44% and 1.38% of the oil volatile fraction. The values reported in the literature for industrially-produced cold-extracted oils range from 0.7 to 2.0%.[1,2,10] The high variability of such a concentration range is due to the fact that bergamot oil composition can vary greatly in relation to the harvesting period and geographical location.[1,2,10]

As aforementioned, the main SH compounds present in both bergamot samples were β-bisabolene, *tr*-α-bergamotene and *tr*-caryophyllene, which formed 0.38% (sample 1)/0.31 (sample 2), 0.28% (sample 1)/0.18% (sample 2), and 0.32% (sample 1)/0.27% (sample 2) of the essential oil, respectively, and together approximately 81% (sample 1) and 75% (sample 2) of the SH content. Additionally, *tr*-β-farnesene [0.05% (sample 1)/0.04% (sample 2)], germacrene D [0.04% (sample 1)/0.03% (sample 2)], α-humulene (0.02% in both samples), *cis*-α-bisabolene [0.03% (sample 1)/0.02% (sample 2)], and *cis*-α-bergamotene [0.01% (sample 1)/0.02% (sample 2)], were present in relatively high percentages; all other constituents were contained at levels $\leq$ 0.01% (Table 2).

Comparison between lemon, bergamot and lime oils

The SH profiles, in lemon and bergamot oils, are very similar. The qualitative differences are related to the presence, in one of the two oils, of minor constituents; in particular, the absence of germacrene A and D in lemon oil must be highlighted, because these compounds have been reported before in this oil-type.[1,2,11] The main % differences are related to the different proportions of the three main compounds, and to the different percentages of bicyclogermacrene, *tr*-β-farnesene, α-humulene and *cis*-γ-bisabolene. There are many similarities between the SH fractions of lemon, bergamot and lime oils: for example, the main three compounds, along with comparable percentages of three bergamotenes, five bisabolenes, α- and β-santalene, α-copaene and muurolenes; however, there are also major differences: the SH fraction in Key and Persian oils is characterized by significant percentages of *tr, tr*-α-farnesene, selinenes (α- and β-) and germacrene B, compounds which are absent or present in traces in lemon and bergamot oils; on the other hand, bicyclogermacrene is present only in lemon and bergamot oils. Germacrene D is contained in comparable percentages in lime and bergamot oils. Other slight qualitative differences are related to minor constituents present in lemon and bergamot oils, and not in lime oil, and vice versa.

Sweet orange oil

Altogether, 42 SH constituents were identified [three for the first time and 15 only once (in the previous research by Tranchida *et al.*[6])] in sweet orange oil. Five solutes were identified only through GC-qMS, 12 solutes only through GC×GC-qMS, while 25 SH compounds were identified by using both approaches (Table 1). Germacrene A and C were determined through the cold-injection; using the latter approach, the amount of germacrene D and B remained constant, δ-elemene was not detected, while β- and γ-elemene were detected at a lower percentage.

The amount of SH constituents contained in samples 1 and 2 was 0.20 g/100 g and 0.15 g/100 g, respectively.

The % of SH analytes, determined without using the IS, amounted to 0.21% and 0.17%, in samples 1 and 2, respectively. Values derived from the literature, for industrially-produced cold-extracted oils, range from 0.1 to 0.4%, with highest values found in an Israelian oil.[1,2]

The main compound was valencene, which formed 0.07% (sample 1) and 0.04% (sample 2) of the essential oil, and approximately 34% (sample 1) and 25% (sample 2) of the SH content. Other compounds present in relatively high percentages (about 0.01% each) were: δ-cadinene, *tr*-caryophyllene, α- and β- copaene, β-cubebene, *tr*-β-farnesene, germacrene D and germacrene A, which together with valencene formed about 77% of the SH fraction in both samples. All other SH solutes were contained in lower amounts (Table 2).

Clementine oil

Altogether, 39 SH constituents were identified (15 for the first time) in clementine oil. Two solutes were identified only through GC-qMS, seven solutes only through GC×GC-qMS, while 30 SH compounds were identified by using both approaches (Table 2). Germacrene A and C were determined through cold-injection; using the latter approach, the amount of germacrene D and B remained constant, δ- and γ-elemene were not detected, while β-elemene was detected at a lower percentage.

The amount of SH constituents contained in the two clementine oils was 0.14 g/100 g (sample 1) and 0.13 g/100 g (sample 2).

The % of SH analytes, determined without using the IS, amounted to 0.17%, in both samples. Data reported in the literature, for industrially-produced clementine oils, are limited; however, it has been reported that the total amount of SH constituents corresponds to about 0.2%, and between 0.1–0.6% in cold-extracted lab oils.[2]

The most abundant compounds, in agreement with data reported in the literature were: δ-cadinene, α-copaene, β-cubebene, *tr, tr*-α-farnesene, and germacrene D. These compounds represent each about 0.01–0.02% of both clementine oils; moreover, the five constituents form 57% of the SH fraction. Additionally, β-copaene and *tr*-β-farnesene accounted each for approx. 0.01% of the oils, while the remaining compounds were present at lower levels (Table 2).

Comparison between sweet orange and clementine oils

Among the SH compounds identified in sweet orange and clementine oils, 34 are common to both oils; 31 compounds are present in comparable relative quantities, with percentages slightly higher in clementine oil. Valencene, which represents approx. 29% (average of the two samples) of the SH fraction in sweet orange oil, is present at a level lower than 1% in clementine oil; moreover, *tr, tr*-α-farnesene is contained at a level of 3.7% (average of the two samples) in sweet orange, while it is three times higher in clementine. The qualitative differences between the two oils are related to 16 minor SH compounds, present in one or the other oil, all of which determined with great difficulty through the GC-qMS analysis of the essential oil. Among such minor-amount constituents, the most abundant were α-selinene, 7-epi-α-selinene, and aristolochene, in sweet orange oil (2.6%, 1.6%, and 1.6%, respectively, expressed as an average value of the two samples). In both oils, santalenes and curcumenes are absent, such as in bitter orange, mandarin, and grapefruit oils, while α- and γ-muurolene are present, such as in grapefruit and Mexican mandarin oils.

Bitter orange oil

Altogether, 32 SH constituents were identified (13 for the first time) in bitter orange oil. Three solutes were identified only through GC-qMS, four solutes only through GC×GC-qMS, while 25 SH compounds were identified by using both approaches (Table 2). Germacrene A and C were determined through the cold-injection; using the latter approach, the amount of germacrene B was higher, that of germacrene D remained constant, while δ- and γ-elemene were not detected, and β-elemene was detected at a lower concentration.

The amount of SH constituents contained in the two bitter orange oils was 0.15 g/100 g (sample 1) and 0.17 g/100 g (sample 2).

The % of SH analytes, determined without using the IS, amounted to 0.18% and 0.20%, in sample 1 and 2, respectively. Values derived from the literature, for industrially-produced cold-extracted oils, range from 0.1 to 0.4%.[1,2] An exception to such values was reported for a Cuban oil, for which *tr*-α-bergamotene and β-bisabolene were reported as the only constituents, at a total level of 0.8%.[2]

The most abundant compounds, in agreement with data reported in the literature, were germacrene D (0.06% and 0.07% in sample 1 and 2, respectively) and *tr*-caryophyllene (0.05% in both samples), representing approx. 73% of the SH fraction in both

oils.[1,2] Bicyclogermacrene, *tr*-β-farnesene and α-humulene were present in an amount each correspondent to approx. 0.005% of the essential oil; all other identified compounds were present in lower quantities.

The composition of the SH fraction of bitter orange oil is rather different from the other analyzed oils, with only a few common characteristics: for example, bergamotenes and bisabolenes are absent or present in traces, as in mandarin and grapefruit oils; compared to sweet orange, clementine, mandarin and grapefruit oils, α- and β-cubebene are present, with a much lower percentage of the β- isomer; furthermore, considering the same oils, α- and β-copaene are present, though in much lower percentages; compared to sweet orange, clementine, and grapefruit oils, *tr*-cadine-1,4-diene, γ- and δ-cadinene are present, though δ-cadinene is contained in much lower percentages; the percentage of *tr*-β-farnesene is similar to that found in lemon, bergamot, sweet orange, Key and Persian lime oils, while the content of *tr, tr*-farnesene is similar to that of lemon and bergamot oils; the percentage contents of caryophyllenes are high as observed in lemon, bergamot, mandarin, grapefruit, Key and Persian lime oils. Finally, curcumenes and santalenes are absent, as observed in sweet orange and clementine oils, while traces of isogermacrene D are present, as in sweet orange oil.

Mandarin oil

Altogether, 27 SH constituents were identified in Mexican mandarin oil (12 for the first time). Four solutes were identified only through GC-qMS, eight solutes only through GC×GC-qMS, while 15 SH compounds were identified by using both approaches (Table 2). Germacrene A was identified and quantified through the cold-injection experiment, while β-elemene was detected at a lower concentration.

In Italian green mandarin oil, 26 SH constituents were identified (11 for the first time). Two solutes were identified only through GC-qMS, seven solutes only through GC×GC-qMS, while 17 SH compounds were identified by using both approaches (Table 2). Germacrene A was determined through the cold-injection experiment, while β-elemene was detected at a lower percentage.

In Italian yellow mandarin oil, 27 SH constituents were identified (12 for the first time). Two solutes were identified only through GC-qMS, eight solutes only through GC×GC-qMS, while 17 SH compounds were identified by using both approaches (Table 2). Germacrene A was determined through the cold-injection experiment, while β-elemene was detected at a lower percentage.

In Italian red mandarin oil, 30 SH constituents were identified (13 for the first time). Two solutes were identified only through GC-qMS, 11 solutes only through GC×GC-qMS, while 17 SH compounds were identified by using both approaches (Table 2). Germacrene A was determined through the cold-injection experiment, while β-elemene was detected at a lower percentage.

The amount of SH constituents contained in the Mexican mandarin oil was 0.26 g/100 g lower than that determined in the three Italian mandarin oils. The % of SH analytes, determined without using the IS, amounted to 0.30%.

Among the Italian oils, there were slight differences between green mandarin (0.30 g/100 g), and the yellow and red samples (0.27 g/100 g). The % of SH analytes, determined without using the IS, amounted to 0.35% in the green mandarin oil, and to 0.31% in both yellow and red mandarin oils. Such differences have been reported with the highest level of SH compounds occurring at the beginning of the season, in green mandarin oils.[1,2]

The content of SH constituents, reported for industrial cold-extracted mandarin oils, is between 0.1 and 0.4%, with an exception represented by FMC-extracted Cuban oils.[1,2]

In the four samples subjected to analysis, 38 compounds were identified; β-maaliene, eremophilene, α-γ-muurolene and *tr*-cadina-1,4-diene were identified only in the Mexican sample; the following compounds were found in at least one of the Italian samples, but not in the Mexican one: α-gurjunene, *cis*- and *tr*-α-bergamotene, allo-aromadendrene, γ-cuprenene, α-neocallitropsene, α-zingiberene, isodaucene, β-bisabolene, *tr*-γ-bisabolene.

The most abundant compounds, in agreement with data reported in the literature, were *tr, tr*-α-farnesene, *tr*-caryophyllene, α-selinene, which accounted for 0.08, 0.05 and 0.04% of the Mexican mandarin oil, respectively, and 0.12–0.14%, 0.06-0.08% and 0.03% in the Italian oils, respectively.[1,2] Such constituents, in the Mexican oil, represent 68% of the SH fraction, and 81–85% in the Italian oils. α-Copaene, β-cubebene, δ-cadinene, and α-humulene were present in amounts between 0.005% and 0.02% in all four oils, along with germacrene D only in the Italian mandarin oils. The first three of such compounds, in the Mexican mandarin oil, are present in double or triple percentages compared to the Italian oils (Table 2). All other identified compounds were present in percentages lower than 0.005%.

The SH fraction in mandarin oils is characterized by the highest percentages, compared to the other oils, of *tr, tr*-α-farnesene, and α-selinene, and the lowest of *tr*-β-farnesene. The limited similarities with the other oils are related to the percentages of valencene, compared to clementine, lemon and Key A, and that of germacrene D, compared to Persian lime. Moreover, some common compositional features were found between the Mexican oil and sweet orange oils (presence of α-and γ-muurolene, and percentages of α-and β-cubebene and of α-copaene).

Grapefruit oil

Altogether, 33 SH constituents were identified in grapefruit oil (18 for the first time). Four solutes were identified only through GC-qMS, 12 solutes only through GC×GC-qMS, while 17 SH compounds were identified by using both approaches (Table 2). This was the only case in which germacrene A was identified through the hot-injector method, and obviously was detected at a higher concentration through the cold-injection approach; finally, and using the latter approach, β-elemene was not detected.

The amount of SH constituents contained in the grapefruit oil was 0.73 g/100 g. The % of SH analytes, determined without using the IS, amounted to 0.80%.

The content of SH constituents, reported for industrial cold-extracted grapefruit oils, is between 0.5 and 1.0%, even though levels up to 3% have been reported.[1,2] With regard to industrially-produced red and yellow grapefruit oils, no substantial differences are reported in the literature in relation to the SH fraction.[1,2]

The most abundant compound was *tr*-caryophyllene, which accounted for 0.29% of the essential oil and for 39.3% of the SH fraction. Moreover, δ-cadinene (0.1%), α-copaene (0.08%), β-cubebene (0.08%), germacrene D (0.07%), α-humulene (0.04%) and bicyclogermacrene (0.02%) were present in significant amounts, and together with *tr*-caryophyllene, accounted for 91% of the SH fraction. All other identified compounds were present in percentages lower than 0.01% (Table 2), in agreement with data reported in the literature for industrial oils.[1,2]

The SH fraction in grapefruit oils was characterized by the highest percentages, compared to all the other oils, of *tr*-caryophyllene, and in the absence of valencene; moreover, it was the only oil with traces of α-cadinene. Similarities with the other oils were found in: the presence of α- and β-cubebene and of α- and β-copaene, as in sweet orange, clementine, bitter orange, and mandarin oils, with the percentages of the cubebenes nearly identical to that in clementine oil, and in the amounts of copaenes similar to that present in Mexican mandarin; a content of γ- and δ-cadinene, and *tr*-cadine-1,4-diene, comparable to that of sweet orange and clementine oils.

Conclusions

A detailed investigation of the SH fraction of a variety of commercially-important *Citrus* oils, has been performed. Altogether, 375 compounds were determined through LC//GC×GC-qMS/FID, 332 through the LC//GC-qMS/FID method, while 275 compounds were determined using both techniques. Consequently, in 100 cases it was GC×GC that proved to be more useful than GC; the same was valid for GC, over GC×GC, for 57 analytes.

With regard to the LC//GC×GC-qMS/FID approach, it was the enhanced sensitivity of cryogenically-modulated GC×GC, more than the high resolution, which enabled the identification of a higher number of compounds; in fact, there was limited separation in the second dimension due to the same polarity of the SH analytes. It must be added that modulation, in some cases, did destroy the resolution of closely-eluting first-dimension compounds, hindering the identification of a series of unknowns (assigned through LC//GC-qMS/FID).

The advantages of an LC pre-fractionation step, prior to a GC-based analysis, must also be emphasized in such an application type, because it enables the generation of less-complex, and chemically-homogeneous sub-samples. Finally, the use of cold-injection PTV GC-qMS was useful in providing a truer of the composition of the samples.

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