

# Detailed Profiling of the Volatile Oxygenated Fraction of Mandarin Essential Oils by Using the Off-Line Combination of High-Performance Liquid Chromatography and Comprehensive Two-Dimensional Gas Chromatography-Mass Spectrometry

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**Abstract** The aim of the herein described research was the detailed qualitative analysis of the volatile oxygenated fractions of four genuine mandarin (*Citrus deliciosa* Ten.) essential oils (green, yellow, red, and Mexican), by using the off-line combination of high-performance liquid chromatography (HPLC) and comprehensive two-dimensional gas chromatography-quadrupole mass spectrometry (GC×GC-qMS). Detailed qualitative profiling was performed through a pre-separation (normal phase) HPLC process aimed at the isolation of the oxygenated compounds; the target fraction was then concentrated and subjected to cryogenically modulated GC×GC-qMS analysis. The oxygenated constituents contribute most to the essential oil aroma and are also used for the evaluation of quality and genuineness. In such a respect, a deeper analytical view on the qualitative composition of such constituents is certainly useful. In fact, many compounds are herein related to mandarin essential oils for the first time.

**Keywords** Comprehensive two-dimensional gas chromatography · Volatile oxygenated fraction · Mandarin essential oil · LC//GC · Quadrupole mass spectrometry

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## Introduction

Mandarin (*Citrus deliciosa* Ten.) essential oil is a rather complex mixture characterized by a volatile and a non-volatile fraction, with the former comprised in the 90–96 % range (Shaw 1979). The volatile fraction consists mainly of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives; characteristic compounds of mandarin oil are the sesquiterpene aldehyde  $\alpha$ -sinensal, the aromatic ester methyl *N*-methylantranilate, and the monoterpene alcohol thymol (Frizzo et al. 2004).

Mandarin essential oil is a product with high economical importance, it being used in a wide range of industrial products, such as perfumes, cosmetics, soaps, foods, and beverages. The oil cold extraction technique can vary according to the ripening stage of the fruits (begins in September when the fruits are still unripe and ends in March, considering the Northern Hemisphere). Extraction machines such as the “pelatrice,” the brown oil extractor (BOE), and the screw press (“Torchio”) are generally used. Commonly, mandarin essential oils are classified based on their color (green, yellow, and red) and present different characteristics and prices. One of the most appreciated products is obtained by using the screw press on unripe mandarins, with the final oil presenting a light yellow color. If the same fruits are treated with the BOE, then a green oil will be attained. The processing of ripe fruits will result in the generation of red/orange oils, independently from the extraction approach (Dugo et al. 2011a).

More than half a century has passed since the first GC analysis of the volatile fraction of *Citrus* essential oils was reported: Chromatograms relative to bergamot and lemon oils showed not more than four to five peaks (Liberti and Conte

1956). The economical importance of *Citrus* essential oils, in general, has always acted as a stimulant for the use of state-of-the-art chromatography instrumentation for their characterization.

Nowadays, the most commonly employed method for the qualitative analysis of the volatile fraction of *Citrus* essential oils is gas chromatography coupled with mass spectrometry (GC-MS); the latter, combined with linear retention index (LRI) information, can be considered as a useful tool for the analysis of essential oil volatiles (Marriott et al. 2001). However, the complete separation of *Citrus* oil volatiles can be an extremely complicated task if a single GC column is used. For example, Sebastiani et al. (1983) failed to separate all the constituents of a lemon oil on one of the five stationary phases subjected to evaluation covering the polarity range available at that time.

For a more detailed view on essential composition, it is advisable to perform two GC runs on different stationary phases (Sebastiani et al. 1983), to use a heart-cutting or comprehensive two-dimensional GC system (Dugo et al. 2011b; Mondello et al. 2005), or to pre-separate the oil into different chemical classes by using high-performance liquid chromatography (HPLC) and prior to the GC analysis (Chamblee et al. 1985; Munari et al. 1990; Mondello et al. 1994; Mondello et al. 1995; Tranchida et al. 2013; Zoccali et al. 2015).

The off-line combination of liquid and gas chromatography (LC//GC) has been rather widely applied: For instance, Chamblee et al. (1985) pre-separated a lime essential oil by coupling three different silica columns in series. Fractions were collected, concentrated, and analyzed directly by GC-MS, with 23 previously unreported constituents (from now onwards defined as “new”) tentatively identified by the authors. In 1990, an online liquid-gas chromatography (LC-GC) instrument was used by Munari et al. (1990) for the pre-separation of lemon essential oil in four classes, namely hydrocarbons, aldehydes, esters, and alcohols, prior to their GC characterization.

Comprehensive two-dimensional GC has also been used for the analysis of *Citrus* essential oils; for example, Mondello et al. (2005) compared GC and GC×GC for the analysis of lemon essential oil, highlighting two strong points of the multidimensional approach, specifically separation power and group-type pattern formation. However, it was also found that highly concentrated compounds (e.g., limonene) severely overloaded the modulator. In another research, Tranchida et al. (2013) used the off-line combination of LC and comprehensive two-dimensional GC (LC//GC×GC), by using quadrupole mass spectrometry (qMS) for qualitative analysis. The oxygenated fraction was isolated by using normal phase (NP) LC and then reduced in volume prior to GC×GC-qMS analysis. Highly detailed two-dimensional chromatograms were attained for bergamot and sweet orange

oils, with several compounds reported for the first time. The same LC//GC×GC-qMS approach is herein described for the analysis of green, yellow, red, and Mexican mandarin essential oils.

## Experimental

### Samples and Sample Preparation

A C<sub>7</sub>-C<sub>30</sub> *n*-alkane series was kindly provided by Sigma-Aldrich/Supelco (Bellefonte, PA, USA), for the calculation of LRI values.

Genuine cold-pressed samples of mandarin oils (green, yellow, red) were provided by Simone Gatto s.r.l. (San Pier Niceto, Messina); a Mexican mandarin cold-pressed oil was provided by Citrojugo S.A. de C.V. Tecomán (Colima, Mexico). Prior to LC analyses, the oils were diluted 1:2 (v/v) in *n*-hexane.

### LC Pre-Separation

Pre-separation of the essential oils was obtained by using an LC//GC system (Shimadzu, Kyoto, Japan), previously described (Tranchida et al. 2013; Zoccali et al. 2015).

LC conditions: a 100 × 3 mm ID × 5 μm *d<sub>p</sub>* silica column (SUPELCOSIL LC-Si, Sigma-Aldrich/Supelco) 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 % methyl tert-butyl ether (MTBE), until the end of the analysis. Injection volume 20 μL.

LC fractions: The oxygenated compounds of all essential oils were collected approximately from 7.5 to 12 min (1575 μL).

Data were acquired by using a photodiode array detector in the range of 190–390 nm, while the sampling frequency was 1.56 Hz.

Prior to GC×GC-qMS injection, the fractions were reduced to a volume of ≈100 μL (under a gentle stream of nitrogen).

### 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). The primary column, an SLB-5ms 30 m × 0.25 mm ID × 0.25 μm *d<sub>f</sub>* column (silphenylene polymer—Sigma-Aldrich/Supelco), was connected to an uncoated capillary segment (1.5 m × 0.18 mm ID, used to create a double 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<sub>f</sub>* column (Sigma-Aldrich/

Supelco), by using another union (SGE). Modulation was carried out every 5 s, by using a loop-type modulator (under license from Zoex Corporation, Houston, TX, USA). The duration of the hot pulse (400 °C) was 400 ms. GC conditions: Temperature program was 50–250 °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.

The sample was analyzed in the full-scan mode by using a mass range of 40–360 *m/z* and spectra generation frequency 33 Hz; interface and ion source temperatures were 250 and 200 °C, respectively. MS ionization mode: electron ionization. Data were collected by the GCMS Solution software (Shimadzu); bidimensional visualization was carried out by using the ChromSquare v.2.2 software (Shimadzu). The MS database employed was the FFNSC 3.0 (Shimadzu).

## Results and Discussion

The NP LC separation of the oxygenated compounds from monoterpene and sesquiterpene hydrocarbons was easily achieved, with such compounds weakly retained on the silica column. Cryogenically modulated GC×GC enabled the separation and detection of several hundreds of compounds per analysis. Compound identification was performed by using both MS database LRI and spectral information. On the basis of recent research, the following levels of identification were applied: (I) “reliably” identified compound: MS database similarity  $\geq 90\%$  and experimental LRI value within a  $\pm 5$  LRI unit window, with respect to the database value; (II) “presumably” identified compound: either MS database similarity  $\geq 90\%$  or experimental LRI value within a  $\pm 5$  LRI unit window; a presumably identified compound cannot be characterized by a similarity match  $< 80\%$  or an experimental LRI value outside a  $\pm 10$  LRI unit range; (III) “tentatively” identified compound: MS database similarity  $> 75\%$  and experimental LRI value within a  $\pm 15$  LRI unit range, compared to the database value (Tranchida et al. 2013). The experimental LRI values herein reported were calculated considering the retention time of the most intense modulated peak. The contribution of the second-dimension medium polarity stationary phase was considered negligible, due to the short length of the column (1 m).

Considering the four mandarin essential oil samples (green, yellow, red, and Mexican), 179 different compounds were identified, and to the best of the authors’ knowledge, 110 (61.5 % of the total number) of these are new ones. Such data are reported in Table 1 and visualized graphically in Fig. 1.

### Green Mandarin Oil

Altogether, 82 oxygenated compounds were identified in the green mandarin oil; of such constituents, 42, 21, and 19

analytes were reliably, presumably, and tentatively identified, respectively. The identified analytes belonged to the following chemical classes: 16 [aliphatic aldehyde (AliAld)], 5 [monoterpene aldehyde (MAld)], 2 [sesquiterpene aldehyde (SAld)], 2 [aliphatic ketone (AliK)], 6 [monoterpene ketone (MK)], 5 [aliphatic alcohol (AliA)], 17 [monoterpene alcohol (MA)], 6 [sesquiterpene alcohol (SA)], 8 [aliphatic ester (AliE)], 4 [monoterpene ester (ME)], 6 [monoterpene oxide (MO)], 1 [sesquiterpene oxide (SO)], and 4 (Other). To the best of the authors’ knowledge, 35 of these compounds have never been reported previously in mandarin oil (in general); of these, five were identified only in the sample of green mandarin oil, namely  $\gamma$ -terpinen-7-al [(MAld), identification level (IL) II], karahanenone [(MK), IL III], *trans*- $\alpha$ -hydroxy-pinocamphone [(MK), IL III], *n*-dodecanol [(AliA), IL III], and  $\omega$ -16-hydroxy-hexadec-6-enoic acid lactone [(Other), IL III].

### Yellow Mandarin Oil

Altogether, 118 oxygenated compounds were identified in the yellow mandarin oil; of such constituents, 47, 45, and 26 solutes were reliably, presumably, and tentatively identified, respectively. The identified analytes belonged to the following chemical groups: 23 (AliAld), 5 (MAld), 4 (SAld), 7 (AliK), 7 (MK), 7 (AliA), 22 (MA), 10 (SA), 12 (AliE), 9 (ME), 6 (MO), 2 (SO), and 4 (Others). To the best of the authors’ knowledge, 66 of these compounds have never been reported previously in mandarin oil; of these, 24 were identified only in the sample of yellow oil, specifically 2-ethyl-hexanal [(AliAld), IL II], 2,5,9-trimethyl-deca-4(*Z*),9-dien-1-al [(AliAld), IL I], undec-9(*Z*)-en-1-al [(AliAld), IL III], octadec-13-(*Z*)-en-1al [(AliAld), IL II],  $\alpha$ -terpinen-7-al [(MAld), IL II], ethyl butyl ketone [(AliK), IL I], heptan-2-one [(AliK), IL II], tridecan-2-one [(AliK), IL II], (*Z*)-jasmone [(AliK), IL II], umbellone [(MK), IL II], prenol [(AliA), IL II], *neo*-*iso*-dihydrocarveol [(MA), IL III], *cis*-chrysantenol [(MA), IL II], lavandulol [(MA), IL III], *trans*-pinene hydrate [(MA), IL III],  $\gamma$ -curcumen-12-ol [(SA), IL III], gleenol [(SA), IL III], germacra-4(15),5,10(14)-trien-1-a-ol [(SA), IL III], *neo*-intermedeol [(SA), IL II], *cis*-lanceol [(SA), IL II], octadec-13(*Z*)-en-1-yl acetate [(AliE), IL III], citronellyl tiglate [(ME), IL III], lavandulyl acetate [(ME), IL III], and *allo*-aromadendrene epoxide [(SO), IL II].

### Red Mandarin Oil

Altogether, 115 oxygenated compounds were identified in the red mandarin oil; of such constituents, 58, 43, and 14 solutes were reliably, presumably, and tentatively identified, respectively. The high complexity of red mandarin oil can be observed in the four expansions illustrated in Fig. 2a–d. Almost the entire two-dimensional space was exploited, highlighting

**Table 1** Compounds identified in mandarin essential oils classified according to the chemical class

No.	Class	Mandarin				
		Green	Yellow	Red	Mexican	Legend
	Aldehydes					
	Aliphatic					
10	2-Methylpentanal			II		c
2	<i>n</i> -Hexanal			II		a, b
	2-Ethylhexanal		II			c
6	<i>n</i> -Heptanal	I	I	I		a, b
5	Hept-4( <i>Z</i> )-en-1-al		I	I		c
11	<i>n</i> -Octanal	I	I	I	I	a, b
17	<i>n</i> -Nonanal	I	I	I	II	a, b
27	Non-2( <i>E</i> )-en-1-al			I		a
34	<i>n</i> -Decanal	I	I	I	II	a, b
47	Dec-2( <i>E</i> )-en-1-al	I	I	I	III	a, b
31	Dec-4( <i>Z</i> )-en-1-al		I	I		b
59	Dec-2( <i>E</i> ),4( <i>E</i> )-dien-1-al	I	I	I		a, b
	2,5,9-Trimethyl-deca-4( <i>Z</i> ),9-dien-1-al		I			c
57	<i>n</i> -Undecanal	I	I	II	I	a, b
64	Undec-2( <i>E</i> )-en-1-al	II	II	III		a, b
67	Undec-8( <i>Z</i> )-en-1-al			I		c
	Undec-9( <i>Z</i> )-en-1-al		III			c
96	Undec-10-en-1-al			III		c
71	<i>n</i> -Dodecanal	I	I	I	II	a, b
79	Dodec-2( <i>E</i> )-en-1-al	I	I	I	II	a, b
78	Dodec-2( <i>E</i> ),6( <i>Z</i> )-dien-1-al	I	I	I	II	a, b
85	Dodec-2( <i>E</i> ),4( <i>E</i> )-dien-1-al		I	I		c
83	<i>n</i> -Tridecanal	I	I	II		a
88	Tridec-2( <i>E</i> )-en-1-al	I	I	I		a
95	<i>n</i> -Tetradecanal	I	II	I	II	a, b
100	Tetradec-2( <i>E</i> )-en-1-al	I	I	I	II	a, b
91	Tetradec-7( <i>Z</i> )-en-1-al		II	I		c
109	Hexadec-9-( <i>Z</i> )-en-1-al	I		I		c
114	14-Methyl-hexadec-8( <i>Z</i> )-en-1-al			II		c
	Octadec-13-( <i>Z</i> )-en-1-al		II			c
	Monoterpene					
	Campholenal				II	c
25	Citronellal	I	I	I	II	a, b
43	Cuminaldehyde			I		c
49	Geranial	I	I	I	II	a, b
	<i>p</i> -Mentha-3-en-7-al				I	a
41	Neral	I	I	I	II	a, b
51	Perillaldehyde	I	I	I	I	a, b
	Phellandrenal				I	c
	$\alpha$ -Terpinen-7-al		II			c
	$\gamma$ -Terpinen-7-al	II				c
	Sesquiterpene					
103	2( <i>E</i> ),6( <i>Z</i> )-Farnesal		II	I		c
105	( <i>E</i> , <i>E</i> )-Farnesal		II	II		c

Table 1 (continued)

No.	Class	Mandarin				
		Green	Yellow	Red	Mexican	Legend
106	$\alpha$ -Sinensal	I	I	I	I	a, b
102	$\beta$ -Sinensal	III	I	I		c
	Ketones					
	Aliphatic					
4	Butyrene	II	II	II		c
1	Methyl butyl ketone			II		c
	Ethyl butyl ketone		I			c
	Heptan-2-one		II			c
7	5-Methyl-hepta-3-one	II	III	II		c
	6-Methyl-hepta-5-en-3-one		I			a, b
	Tridecan-2-one		II			c
77	Geranylacetone			II		c
	Chyptone				III	c
	(Z)-Jasmone		II			c
	Monoterpene					
	Camphor					
44	Carvone	I	I	I	II	a, b
	(Z)-Dihydrocarvone				II	a, b
13	Dihydrotagetone			III		c
15	Fenchone	I	II	I		c
	Karahanenone	III				c
21	Limona ketone		II	II	II	c
	<i>trans</i> - $\alpha$ -Hidroxy-Pinocamphone	III				c
	Pinocarvone	II	II			c
46	Piperitone	II	II	II		a, b
	<i>trans</i> -Ocimenone		II			a
	Umbellenone		II			c
82	<i>cis</i> -Geranylacetone			I		c
	Alcohols					
	Aliphatic					
	Prenol		II			c
8	6-Methyl-heptan-2-ol	III		III		c
	<i>n</i> -Octanol		III		II	a, b
	Non-2( <i>E</i> )-en-1-ol		III			c
	Non-2( <i>Z</i> )-en-1-ol		III			c
	Nona-2( <i>Z</i> ),6( <i>Z</i> )-dien-1-ol				III	c
48	Dec-2-en-1-ol			II		c
	<i>n</i> -Dodecanol	III				c
	Dodec-2( <i>E</i> )-en-1-ol	III				b
80	Dodec-8( <i>Z</i> )-en-1-ol		III	III		c
97	Tetradec-9( <i>Z</i> )-en-1-ol	II		III		c
107	<i>n</i> -Pentadecanol	III	II	III		c
	<i>n</i> -Hexadecanol		III			b
	Pinacol				III	c

Table 1 (continued)

No.	Class	Mandarin				
		Green	Yellow	Red	Mexican	Legend
	Aldehydes					
	Monoterpene					
	<i>cis</i> -Carveol				II	a, b
37	<i>trans</i> -Carveol		I	II	II	a, b
56	Carvacrol			II		c
35	1,6-Dihydrocarveol	II	II	II		c
33	<i>neo</i> -Dihydrocarveol	II	II	II	II	c
	<i>neo-iso</i> -Dihydrocarveol		III			c
	<i>cis</i> -Chrysantenol		II			c
40	Citronellol			I		a, b
54	<i>p</i> -Cymen-7-ol			II		b
30	<i>p</i> -Cymen-8-ol	II	I	I	II	a, b
26	<i>iso</i> -Pulegol	II	I	II		a, b
28	<i>iso-iso</i> -Pulegol	I		I		c
	Lavandulol		III			c
55	Limonen-10-ol		II	III	III	c
16	Linalool	I	I	I	I	a, b
20	<i>cis-p</i> -Menth-2-en-1-ol	I	I	I	III	a
24	<i>trans-p</i> -Menth-2-en-1-ol	II	II	II		c
23	<i>cis-p</i> -Mentha-2,8-dien-1-ol	II	I	I	II	a
19	<i>trans-p</i> -Mentha-2,8-dien-1-ol	I	I	I	I	a, b
	Neomenthol	II	II			c
	Nerol				II	a, b
98	Pelargol			III		c
52	Perilla alcohol			III		a, b
	<i>trans</i> -Pinene hydrate		III			c
	Pinocampheol				III	c
15	<i>cis</i> -Sabinene hydrate	I	I	I	II	a, b
18	<i>trans</i> -Sabinene hydrate	I	I	II	III	a, b
29	Terpinen-4-ol	I	III	I	II	a, b
32	$\alpha$ -Terpineol	I	I	I	I	a, b
53	Thymol	I	I	I	I	a, b
39	3,7-Dimethyl-octa-7-en-1-ol	I	I	I	I	c
	Sesquiterpene					
	( <i>Z</i> )- $\alpha$ - <i>trans</i> -Bergamotol	III	III			c
84	Cubebol	II	II	II		a
	10- <i>epi</i> -Cubebol				III	c
	$\gamma$ -Curcumen-12-ol		III			c
86	$\alpha$ -Elemol	I	II	II		a
	Gleenol		III			c
	Germacra-4(15),5,10(14)-trien-1-a-ol		III			c
92	<i>allo</i> -Hedycaryol		III	III		c
99	Intermedeol	II		I		c
	<i>neo</i> -Intermedeol		II			c
	<i>cis</i> -Lanceol		II			c
87	( <i>E</i> )-Nerolidol	II	I	I		a
101	( <i>Z</i> )- $\alpha$ -Santalol			II		c

Table 1 (continued)

No.	Class	Mandarin				
		Green	Yellow	Red	Mexican	Legend
89	Spathulenol	III		II		a, b
	Esters					
	Aliphatic					
9	Isopropyl tiglate		II	II		c
14	Butanoic acid-1-methyl-2-oxopropyl ester		III	III		c
3	Isobutyl propionate	III	III	II		c
	Pentyl acetate		III			c
50	Pentyl allyl butyrate			II		c
70	Decyl acetate			II		b
	Dec-7(Z)-en-1-yl acetate	III	II			c
	Dec-9-en-1-yl acetate	III	II			c
94	Dodecyl acetate			I		a, b
69	Dodec-5(Z)-en-1-yl-acetate	III	II	III		c
108	Tetradec-7(Z)-en-1-yl acetate	III	II	II		c
110	Tetradec-11(Z)-en-1-yl acetate	III	III	II		c
113	Tetradec-9(Z),11(E)-dien-1-yl acetate	II	II	II		c
	Hexadec-11(E)-en-1-yl acetate	III	III			c
	Octadec-13(Z)-en-1-yl acetate		III			c
	Monoterpene					
66	<i>cis</i> -Carvyl acetate	III	II	II		c
61	<i>trans</i> -Carvyl acetate			II		c
38	<i>trans</i> -Chisantenyl acetate		II	II		c
63	Citronellyl acetate	I	II	I		a, b
	Citronellyl formate		I			a
75	Citronellyl propionate			I		c
104	Citronellyl isobutyrate			II		c
	Citronellyl tiglate		III			c
68	Geranyl acetate	I	I	I		a, b
	<i>iso</i> -Isopulegyl acetate				III	c
	Lavandulyl acetate		III			c
45	Linalyl acetate			I		a, b
36	Linalyl formate			III		c
	Linalyl isobutyrate				III	c
73	<i>p</i> -Menth-1-en-9-yl acetate			II		c
60	Myrtenyl acetate			I		c
65	Neryl acetate	I	II	I		a, b
76	Neryl propionate			I		c
74	Perillyl acetate		II	II		c
	Sesquiterpene					
111	Farnesyl acetate			II		c
	Oxides					
	Monoterpene					
	1,8-Cineole	I	I	I	II	a, b
	6,7-Epoxyborneol				I	c



**Table 1** (continued)

No.	Class	Mandarin				
		Green	Yellow	Red	Mexican	Legend
	Aldehydes					
22	<i>cis</i> -Limonene oxide	I	I	I	I	a, b
	<i>trans</i> -Limonene oxide	II	II		II	a, b
	<i>trans</i> -Linalool oxide				III	a
	( <i>E</i> )-Myroxide	III	III		III	a
42	Ascaridole	I	I	I		c
58	Isoascaridole	I	I	I	II	c
	Sesquiterpene					
	<i>allo</i> -Aromadendrene epoxide		II			c
90	Caryophyllene oxide	II	III	II	II	a, b
	Humulene epoxide I				III	c
	Others					
12	Arbuscolone			I		c
	Benzoic acid <2-[[[4-(4-hydroxy-4-methylpentyl)-, 3-cyclohexen-1-yl]methylene]amino]-, methyl->ester	I	I			c
81	$\delta$ -Decalactone	II	II	II		c
	$\omega$ -16-Hidroxy-hexadec-6-enoic acid lactone	III				c
62	Methyl anthranilate			II		a
72	Methyl <i>N</i> -methylantranilate	I	I	I	I	a, b
93	$\delta$ -Undecalactone		II	II		c

Peak number (*No.*) refers to red mandarin sample. Refer to section 3.0 for information on the identification levels I, II, and III. Legend: compound, to the best of the authors' knowledge, identified for the first time in industrially cold-extracted oils, reported in published research since 1987 (a); compound, to the best of the authors' knowledge, identified for the first time in laboratory oils (manual pressure applied to the peel, solvent extraction, or distillation), reported in published research since 1979 (b); and compound, to the best of the authors' knowledge, identified for the first time in mandarin oils (c)

the importance of the GC $\times$ GC-qMS approach to achieve the detailed characterization of mandarin essential oil. Despite the high number of detected peaks, many remained unidentified, probably due the low intensity of the signal or the lack of the correct spectrum in the MS database.

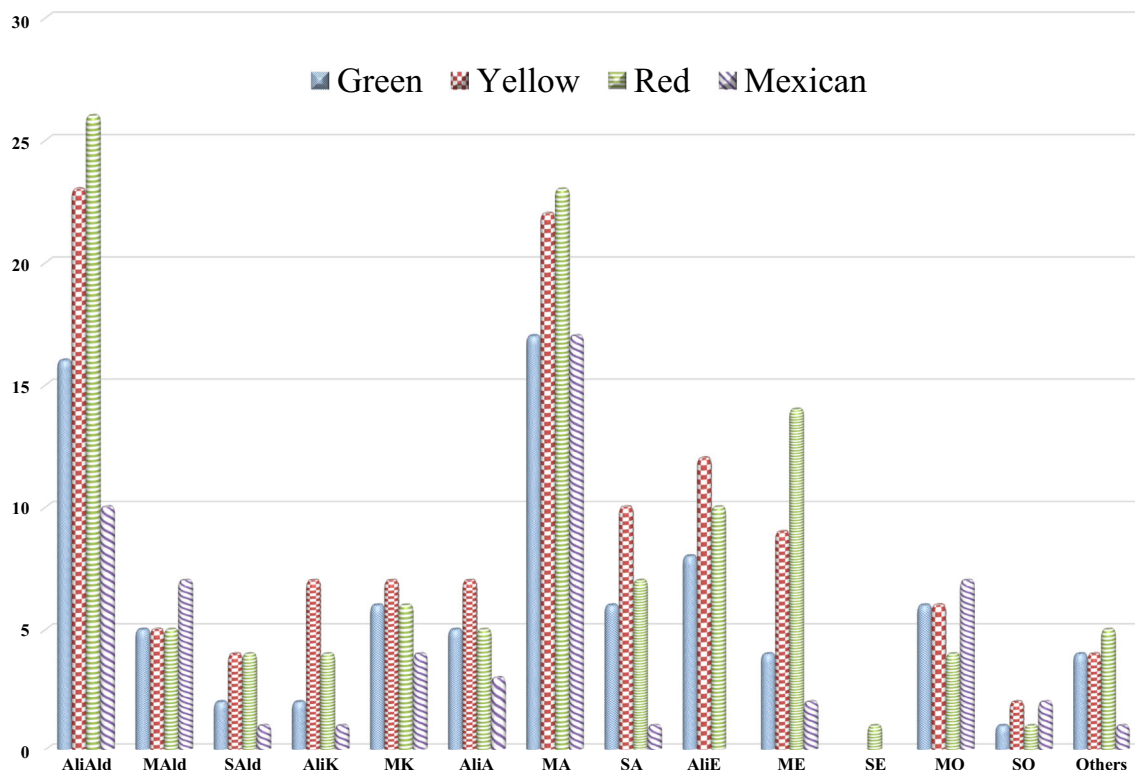
The identified analytes belonged to the following chemical groups: 26 (AliAld), 5 (MAld), 4 (SAld), 4 (AliK), 6 (MK), 5 (AliA), 23 (MA), 7 (SA), 10 (AliE), 14 (ME), 1 [sesquiterpene ester (SE)], 4 (MO), 1 (SO), and 5 (Other). To the best of the authors' knowledge, 60 of these solutes have never been described previously in mandarin oil; of these, 23 were identified only in the sample of red oil, namely 2-methyl pentanal [(AliAld), IL II], undec-8(*Z*)-en-1-al [(AliAld), IL I], undec-10-en-1-al [(AliAld), IL III], 14-methyl-hexadec-8(*Z*)-en-1-al [(AliAld), IL II], cuminaldehyde [(MAld), IL I], methyl butyl ketone [(AliK), IL II], geranyl acetone [(AliK), IL II], dihydrotagetone [(MK), IL III], *cis*-geranylacetone [(MK), IL I], dec-2-en-1-ol [(AliA), IL II], carvacrol [(MA), IL II], pelargol [(MA), IL III], (*Z*)- $\alpha$ -santalol [(SA), IL II], pentyl allyl butyrate [(AliE), IL II], *trans*-carvyl acetate [(ME), IL

II], citronellyl propionate [(ME), IL I], citronellyl isobutyrate [(ME), IL II], linalyl formate [(ME), IL III], *p*-menth-1-en-9-yl acetate [(ME), IL II], myrtenyl acetate [(ME), IL I], neryl propionate [(ME), IL I], farnesyl acetate [(SE), IL II], and arbuscolone [(Other), IL I].

### Mexican Mandarin Oil

Altogether, 56 oxygenated compounds were identified in the Mexican oil; of such constituents, 14, 28, and 14 solutes were reliably, presumably, and tentatively identified, respectively. The identified analytes belonged to the following chemical classes: 10 (AliAld), 7 (MAld), 1 (SAld), 1 (AliK), 4 (MK), 3 (AliA), 17 (MA), 1 (SA), 2 (ME), 7 (MO), 2 (SO), and 1 (Other). To the best of the authors' knowledge, 16 of these analytes have never been reported previously in mandarin oil; of these, 11 were identified only in the sample of Mexican oil, namely campholenal [(MAld), IL II], phellandrenal [(MAld), IL I], chryptone [(AliK), IL III], nona-2(*Z*),6(*Z*)-dien-1-ol [(AliA), IL III], pinacol [(AliA), IL III], pinocampheol





**Fig. 1** Graph illustrating the number of compounds (y-axis) vs. the class of compounds (x-axis), identified in the GC×GC-qMS. The columns refer, respectively, to green, yellow, red, and Mexican mandarin sample

[(MA), IL III], 10-*epi*-cubebol [(SA), IL III], *iso*-isopulegyl acetate [(ME), IL III], linalyl isobutyrate [(ME), IL III], 6,7-epoxymyrcene [(MO), IL I], and humulene epoxide I [(SO), IL III].

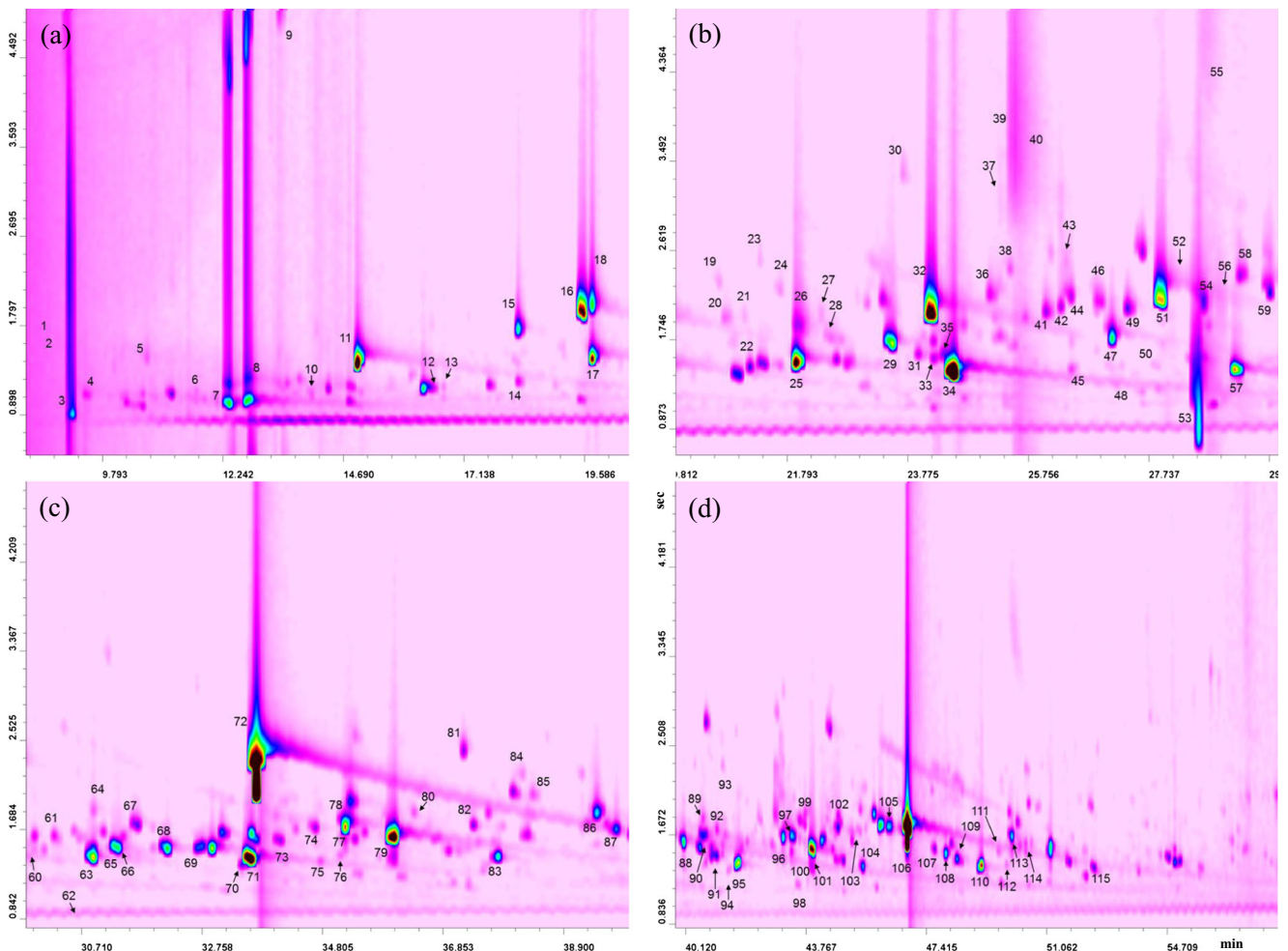
### Mandarin Oil Comparison

Considering the four types of samples subjected to LC//GC×GC-qMS analysis, 179 different compounds were identified; of these, and to the best of the authors' knowledge, 110 are reported here for the first time in mandarin oils. Such compounds belong to the following chemical classes: 12 (AliAld), 5 (MAld), 3 (SA), 9 (AliK), 8 (MK), 11 (AliA), 14 (MA), 10 (SA), 13 (AliE), 14 (ME), 1 (SE), 3 (MO), 2 (SO), and 5 (Other). A pie chart reported in Fig. 3 summarizes the aforementioned data.

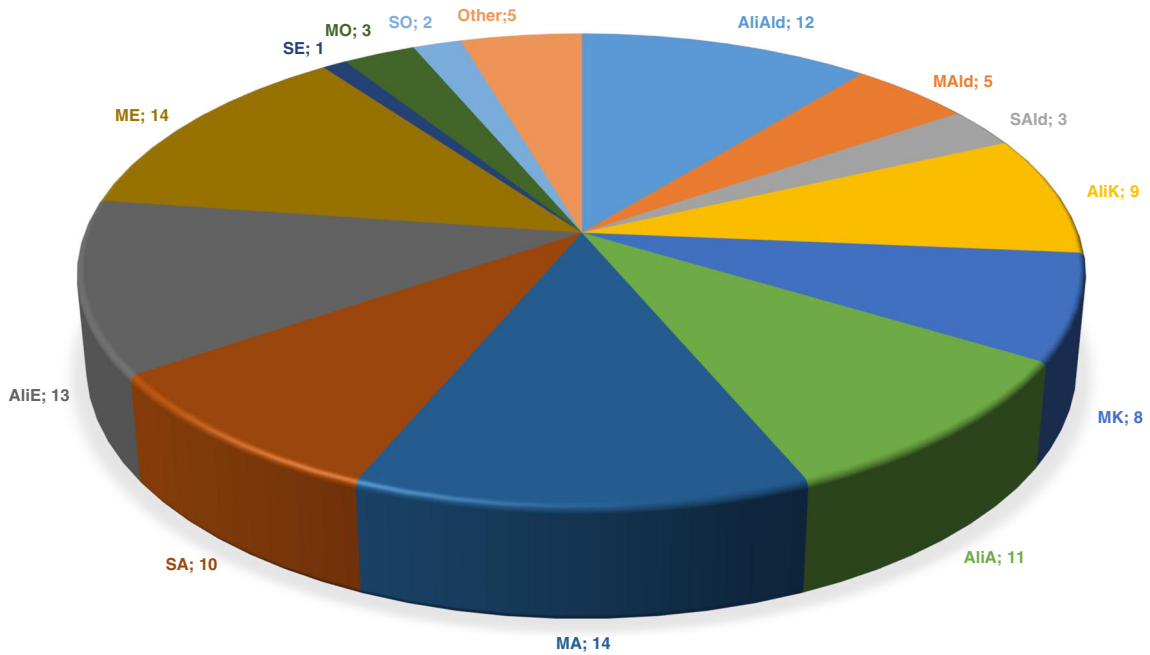
Among the investigated samples, the highest number of compounds was identified in yellow mandarin oil (118), followed by red mandarin oil (115), green mandarin oil (82), and finally the Mexican oil (56). AliAld and MA represent the families with the highest number of compounds in all samples. Moreover, and as can be seen in both Fig. 1 and Table 1, red mandarin is the essential oil with the highest number of AliAld and MA constituents (26 and 23, respectively). On the other hand, SE and SO

families were those with the lowest numbers of members. With regard to the number of detected compounds (by the GC×GC-qMS software), red mandarin oil was the richest with about 240 compounds, followed by the yellow ( $\approx 218$ ), green ( $\approx 155$ ), and Mexican ( $\approx 115$ ) oils. As can be deduced, in all cases, only approx. 50 % of the detected compounds were subjected to one of the three levels of identification, while the remaining part remained unidentified.

A higher number of oxygenated compounds are herein reported, compared to previously published work. For example, Dugo et al. (2011a) reported 142 different oxygenated compounds in genuine cold-pressed, commercial, and laboratory-extracted mandarin oils. It is noteworthy that the data reported by Dugo et al. (2011b) (in a book chapter) were derived from 55 research works covering three decades (1979–2009). Out of the 142 oxygenated compounds, 66 are herein reported (46 %). Such a percentage is certainly not a low one, especially considering the fact that the present investigation is compared with 55 others, performed across a wide time period, under both a great variety of instrumental conditions and sample sources. Furthermore, 37 more analytes were herein reported, compared to the total sum of 142 solutes previously reported.



**Fig. 2** Sequential chromatogram expansions, relative to the GC×GC-qMS analysis of oxygenated compounds in sample red mandarin. For peak identification in Fig. 2a–d, refer to Table 1



**Fig. 3** Pie chart illustrating the number and the families of the compounds reported for the first time in mandarin essential oils

## Conclusions

A detailed investigation of the oxygenated fraction of four types of mandarin essential oil has been performed. The off-line coupling of LC and of GC×GC-qMS offered high selectivity, sensitivity, and separation power, enabling the detection and identification of an unprecedented number of compounds. As already reported, the proposed method has potentially opened a new analytical door, allowing the profound analysis of essential oils, obviously belonging not only to the *Citrus* genus.

## Compliance with Ethical Standards

**Conflict of Interest** Mariosimone Zoccali declares that he has no conflict of interest. Peter Q. Tranchida declares that he has no conflict of interest. Ivana L. Bonaccorsi declares that she has no conflict of interest. Paola Dugo declares that she has no conflict of interest. Luigi Mondello declares that he has no conflict of interest. Giovanni Dugo declares that he has no conflict of interest.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Informed consent is not applicable for this study.

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