

## Characterization of Mandarin (*Citrus deliciosa* Ten.) Essential Oil. Determination of Volatiles, Non-Volatiles, Physico-Chemical Indices and Enantiomeric Ratios

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An investigation of 27 samples of mandarin essential oils (*Citrus deliciosa* Tenore), industrially produced in Sicily during the 2007-2008 season, was performed to determine the composition of the volatile fraction by GC/FID and GC/MS-LRI, the enantiomeric distribution of some monoterpene hydrocarbons and linalol by Es-GC, the non-volatile oxygen heterocyclic components by RP-HPLC/PDA and the physico-chemical indices (relative density, refractive index, optical rotation, residue on evaporation, and UV spectroscopic CD value). This study up-dates the information available in the literature on Sicilian mandarin (*C. deliciosa* Ten.) essential oils, and provides information on the composition and quality parameters for the evaluation of this product.

**Keywords:** *Citrus deliciosa* Ten., essential oil, Es-GC, GC, GC/MS, LRI, RP-HPLC, quantitative analysis.

The essential oil obtained from mandarin is a valuable product employed in the cosmetic and food industries due to its characteristic and appreciated organoleptic properties. The botanical identification of mandarin is often unequivocal in the literature. Webber [1] classified the most important mandarins in four taxonomic groups: King, Satsuma, Mandarin, and Tangerine. Tanaka [2] recognized at least 36 species in five taxonomic groups. Swingle [3a,3b] included nearly all mandarins in a single species, *C. reticulata* Blanco. Hodgson [4a] revised the taxonomic systems of Tanaka and Swingle and suggested that the economically important mandarins could be clustered into four species: *C. unshiu* ('Satsuma'), *C. reticulata* Blanco ('Ponkan', 'Dancy', 'Clementine'), *C. deliciosa* Tenore (Willow-leaf or Mediterranean), and *C. nobilis* Loureiro ('King'). A more recent study [4b] on molecular markers has generally suggested that mandarin cultivars challenge the separation of the group into several species, as proposed by Tanaka [2], although their close relationship can represent a single species composed of many taxonomic groups, as previously proposed by Swingle [3a,3b].

The complexity of the mandarin group from the botanical point of view, and the absence of the specification of the cultivar studied by different authors render the characterization of mandarin essential oil quite complex. Although many papers indicate *C. reticulata* Blanco, Sicilian production is limited to *C. deliciosa* Tenore [5a].

The volatile fraction of cold pressed mandarin essential oils ranges between 96% and 98% of the whole oil. Its composition is characterized by numerous components, mainly terpenes and their oxygenated derivatives. A review of the composition of the volatile fraction of cold pressed mandarin essential oil was published by Dugo *et. al.* (2002) [5b] in a book on Citrus edited by Dugo and Di Giacomo in 2002. That data was based on a review of the literature up to 1999. Since then, several papers have been published on the volatile fraction of mandarin essential oils [6a-6p].

Many of the volatile components of mandarin essential oils are optically active, and the determination of the enantiomeric distribution can represent a useful tool for the characterization of this

product. A review of the chiral components of the oil was published by Mondello *et al.* (2002) [7a]. Since then, few articles have been published on the chiral separation of enantiomeric components of mandarin essential oil [6e,6f,7b,7c].

Approximately 2-4% of the oil is composed of non-volatile compounds, and among these polymethoxyflavones (PMF) are usually determined for the characterization of cold pressed mandarin essential oil. This class of components is also important for their numerous biochemical and physiological effects [7d]. In fact, PMFs, which are exclusive to the *Citrus* genus [7d], are directly associated with the inhibition of enzymes involved in inflammation, prevent cancer initiation and proliferation, and reduce the occurrence of cardiovascular disease by limiting the hepatic production of cholesterol. A review of the composition of the oxygen heterocyclic components of citrus essential oils was published by Dugo and McHale [8] also in 2002. Since then, the literature lacks information on the non-volatile fraction of mandarin essential oil. The only data available are a study of the characterization of Murkott tangerine [6d] and a recent qualitative study from our laboratory [9]. None of the recent literature, however reports a complete view of the volatile, non-volatile and chiral components and physico-chemical indices determined in a large set of samples of industrial mandarin oil.

The present study gives an update with an accurate characterization of 27 samples of Sicilian mandarin oils (*C. deliciosa* Ten. cv. Tardivo di Ciaculli) industrially cold pressed. The samples were preliminarily investigated by determination of specific gravity, refractive index, optical rotation and UV absorbance (CD value). The investigation was then completed by the qualitative and quantitative analysis of the volatiles by GC/MS-LRI and GC/FID, of the non-volatile oxygen heterocyclic fraction by reversed phase liquid chromatography (RP-HPLC), and the determination of the enantiomeric ratio (ER) of several monoterpene hydrocarbons and linalol by enantioselective gas chromatography (Es-GC/FID).

Preliminary evaluation of the samples of mandarin oils is given by the measurement of physico-chemical properties. These are not confirmatory of the genuineness of the essential oil, but contribute to its characterization, and can provide a first indication of either adulteration or alteration of the product. The

**Table 1:** Physico-chemical indices ranges of 27 mandarin oils.

|                        | Range         | ISO 3517:2002 [10a]                  |
|------------------------|---------------|--------------------------------------|
| Relative density       | 0.845-0.851   | 0.848-0.855                          |
| Refractive index       | 1.4751-1.4753 | 1.474-1.478                          |
| Optical rotation       | +69°          | (+) $64^{\circ}$ - ( $+75^{\circ}$ ) |
| Residue on evaporation | 2.3-3.0%      | 1.6 - 4.0%                           |
| CD value               | 0.45-1.23     | *                                    |

\* not listed in the ISO 4735-2002(E) regulation [10b] relative to CD value determination.

physicochemical indices of the samples analyzed are reported in Table 1 as variability ranges and compared with the reference values given by ISO regulations. The results appear in good agreement with those obtained from previous unpublished studies on Sicilian mandarin essential oil carried out in our laboratory. The low minimum for the CD value occurred in those samples with methyl-*N*-methylantranilate at its lower values. However, although CD is considered a non-separative tool for the quantitative determination of oxygen heterocyclic compounds of cold pressed citrus essential oils, in mandarin essential oil this value is also affected by the presence of methyl-*N*-methylantranilate, which absorbs in the same UV region. For these samples the amount of polymethoxyflavones was, in fact, within the ranges reported in the literature.

The composition of the volatile fraction, determined by GC-FID with LRI is summarized in Table 2. The averages and ranges of variation are reported for the 27 samples of mandarin oils analyzed. In the same table, the linear retention indices (LRI) of the components identified are indicated and the correspondent LRI reported in the literature by two different databases [10c,11]. The main components determined in the samples here analyzed were limonene (70.5%), followed by  $\gamma$ -terpinene (19.5%),  $\alpha$ - and  $\beta$ -pinene (2.4% and 1.7%, respectively) and myrcene (1.7%). None of the other components exceeded 1%. The samples of mandarin essential oil analyzed in this study are also characterized by the presence of methyl *N*-methylantranilate (0.4%) and  $\alpha$ -sinensal (0.3%).

A comparison with recently published data showed good agreement with some of the results for the volatile composition of mandarin oil [6e,6f,6n,6p]. The results published by Lota *et al.* for 15 species of mandarin oil include 10 samples of different cvs of *C. deliciosa* [6k]. Among these, the chemical compositions of the volatile fraction of the four samples of the cvs. Willow-leaf, de Chios, Avana Apireno and Tardivo di Ciaculli were very similar to those determined in the present study, while the compositions of the other samples differed greatly.

**Table 2:** Ranges and average composition (% of peak areas) of the volatile components determined in 27 samples of mandarin oil analyzed by GC-FID, Similarity Index (S.I.) determined by GC-MS, and experimental and literature LRI. tr: trace amount.

| Comps                                    | Identification (GC/MS-LRI) |          |               |                 | Quantitative analysis |       |              |
|------------------------------------------|----------------------------|----------|---------------|-----------------|-----------------------|-------|--------------|
|                                          | S.I.                       | LRI Exp. | LRI FFNSC[11] | LRI Adams [10c] | Min                   | Max   | Average      |
| $\alpha$ -Thujene                        | 98%                        | 925      | 927           | 924             | 0.83                  | 1.01  | <b>0.89</b>  |
| $\alpha$ -Pinene                         | 98%                        | 933      | 933           | 932             | 2.26                  | 2.75  | <b>2.43</b>  |
| Camphene                                 | 98%                        | 949      | 953           | 946             | 0.01                  | 0.02  | <b>0.02</b>  |
| Sabinene                                 | 98%                        | 972      | 972           | 969             | 0.24                  | 0.27  | <b>0.25</b>  |
| $\beta$ -Pinene                          | 98%                        | 978      | 978           | 974             | 1.57                  | 1.82  | <b>1.67</b>  |
| Myrcene                                  | 97%                        | 988      | 991           | 988             | 1.65                  | 1.78  | <b>1.70</b>  |
| Octanal                                  | 95%                        | 1003     | 1006          | 998             | 0.11                  | 0.15  | <b>0.14</b>  |
| $\alpha$ -Phellandrene                   | 95%                        | 1007     | 1007          | 1002            | 0.06                  | 0.08  | <b>0.07</b>  |
| $\alpha$ -Terpinene                      | 95%                        | 1017     | 1018          | 1014            | 0.29                  | 0.44  | <b>0.40</b>  |
| <i>p</i> -Cymene                         | 98%                        | 1025     | 1025          | 1020            | 0.24                  | 0.36  | <b>0.30</b>  |
| Limonene+( <i>Z</i> )- $\beta$ -Ocimene  | 93%                        | 1032     | 1030          | 1024            | 68.64                 | 72.06 | <b>70.47</b> |
| ( <i>E</i> )- $\beta$ -Ocimene           | 97%                        | 1045     | 1046          | 1044            | 0.01                  | 0.02  | <b>0.02</b>  |
| $\gamma$ -Terpinene                      | 98%                        | 1059     | 1058          | 1054            | 18.20                 | 20.72 | <b>19.48</b> |
| Octanol                                  | 98%                        | 1077     | 1076          | 1063            | 0.02                  | 0.05  | <b>0.03</b>  |
| Terpinolene                              | 98%                        | 1086     | 1086          | 1086            | 0.67                  | 0.86  | <b>0.78</b>  |
| Linalool+ <i>trans</i> -Sabinene hydrate | 98%                        | 1099     | 1101          | 1095            | 0.10                  | 0.21  | <b>0.16</b>  |
| Nonanal                                  | 98%                        | 1107     | 1107          | 1100            | 0.02                  | 0.03  | <b>0.03</b>  |
| <i>cis</i> -Limonene oxide               | 97%                        | 1134     | 1134          | 1132            | tr                    | tr    | <b>tr</b>    |
| <i>trans</i> -Limonene oxide             | 98%                        | 1138     | 1138          | 1137            | -                     | tr    | <b>tr</b>    |
| Camphor                                  | 94%                        | 1144     | 1149          | 1141            | tr                    | 0.01  | <b>0.01</b>  |
| Citronellal                              | 98%                        | 1152     | 1152          | 1148            | 0.02                  | 0.03  | <b>0.03</b>  |
| Terpinen-4-ol                            | 98%                        | 1182     | 1180          | 1174            | 0.02                  | 0.05  | <b>0.03</b>  |
| $\alpha$ -Terpineol                      | 98%                        | 1196     | 1195          | 1186            | 0.07                  | 0.21  | <b>0.15</b>  |
| Decanal                                  | 97%                        | 1207     | 1209          | 1201            | 0.04                  | 0.08  | <b>0.06</b>  |
| Citronellol                              | 94%                        | 1230     | 1232          | 1223            | -                     | 0.01  | <b>tr</b>    |
| <i>cis</i> -Carveol                      | 98%                        | 1232     | 1232          | 1226            | -                     | 0.01  | <b>tr</b>    |
| Thymol methyl ether                      | 94%                        | 1233     | 1239          | 1232            | 0.01                  | 0.02  | <b>0.01</b>  |
| Neral                                    | 97%                        | 1238     | 1238          | 1235            | -                     | 0.04  | <b>0.01</b>  |
| Carvone                                  | 95%                        | 1241     | 1241          | 1239            | -                     | 0.01  | <b>tr</b>    |
| Geraniol                                 | 96%                        | 1256     | 1255          | 1249            | -                     | tr    | <b>tr</b>    |
| Geranial                                 | 96%                        | 1270     | 1268          | 1264            | tr                    | 0.02  | <b>0.01</b>  |
| Perillaldehyde                           | 94%                        | 1279     | 1278          | 1269            | 0.02                  | 0.03  | <b>0.03</b>  |
| Thymol                                   | 94%                        | 1295     | 1293          | 1289            | 0.02                  | 0.06  | <b>0.04</b>  |
| Undecanal                                | 94%                        | 1310     | 1309          | 1305            | tr                    | 0.03  | <b>0.01</b>  |
| ( <i>E,E</i> )-2,4-Decadienal            | 94%                        | 1323     | 1322          | 1315            | -                     | tr    | <b>tr</b>    |
| $\alpha$ -Terpinyl acetate               | 94%                        | 1350     | 1349          | 1346            | -                     | tr    | <b>tr</b>    |
| Citronellyl acetate                      | 94%                        | 1352     | 1350          | 1350            | -                     | 0.01  | <b>tr</b>    |
| Neryl acetate                            | 96%                        | 1363     | 1361          | 1359            | tr                    | 0.01  | <b>0.01</b>  |
| $\alpha$ -Copaene                        | 96%                        | 1378     | 1374          | 1374            | -                     | 0.01  | <b>tr</b>    |
| Geranyl acetate                          | 96%                        | 1381     | 1380          | 1379            | -                     | 0.04  | <b>0.01</b>  |
| Methyl- <i>N</i> -methylantranilate      | 97%                        | 1408     | 1410          | 1405            | 0.22                  | 0.50  | <b>0.40</b>  |
| Dodecanal                                | 98%                        | 1410     | 1410          | 1408            | 0.01                  | 0.02  | <b>0.02</b>  |
| ( <i>E</i> )-Caryophyllene               | 97%                        | 1423     | 1424          | 1417            | 0.01                  | 0.08  | <b>0.06</b>  |
| ( <i>E,Z</i> )-2,6-Dodecadienal          | 97%                        | 1449     | 1448          | 1445            | -                     | 0.01  | <b>tr</b>    |
| $\alpha$ -Humulene                       | 96%                        | 1459     | 1454          | 1452            | tr                    | 0.01  | <b>0.01</b>  |
| 2-( <i>E</i> )-Dodecenal                 | 98%                        | 1471     | 1469          | 1464            | -                     | 0.02  | <b>0.01</b>  |
| Germacrene D                             | 94%                        | 1485     | 1480          | 1484            | -                     | tr    | <b>tr</b>    |
| $\alpha$ -Selinene                       | 98%                        | 1500     | 1501          | 1498            | 0.01                  | 0.03  | <b>0.02</b>  |
| ( <i>E,E</i> )- $\alpha$ -Farnesene      | 97%                        | 1505     | 1504          | 1505            | 0.02                  | 0.1   | <b>0.09</b>  |
| $\delta$ -Cadinene                       | 97%                        | 1522     | 1518          | 1522            | -                     | 0.01  | <b>tr</b>    |
| 2-( <i>E</i> )-Tridecenal                | 93%                        | 1571     | 1573          | 1569            | -                     | tr    | <b>tr</b>    |
| Tetradecanal                             | 98%                        | 1614     | 1614          | 1611            | -                     | 0.01  | <b>0.01</b>  |
| ( <i>Z,E</i> )-Farnesol                  | 95%                        | 1726     | 1726          | 1722            | -                     | tr    | <b>tr</b>    |
| $\alpha$ -Sinensal                       | 96%                        | 1757     | 1749          | 1755            | 0.12                  | 0.44  | <b>0.35</b>  |

Some important differences were noticed with the oils analyzed in a previous study by Lota *et al.* of 41 samples of *C. reticulata* Blanco [6l]. These authors clustered the oils obtained from different cultivars in France into two groups based on the composition of the volatile fraction. A great variability of the composition of these oils was evident, particularly with regards to the content of limonene (ranging between 52.2 and 96.2%),  $\gamma$ -terpinene (ranging between tr and 36.7%) methyl *N*-methylantranilate (detected only in two samples at 0.4 and 1.1%), and

$\alpha$ -sinensal (ranging between 0 and 0.7%). Pino *et al.*, in a study of Cuban mandarin (*C. reticulata* Blanco) [6g], reported high amounts of myrcene (5.3%) and limonene (78.3%) and low amounts of  $\gamma$ -terpinene (7.2%). Not determined in this study are the components identified by Feger *et al.* [6c] focused on germacrene content in some citrus essential oils. They reported for Italian, Greek, Cypriot and Argentinean mandarin oils bicyclogermacrene (tr-0.01%), germacrene A (tr-0.02), germacrene B (0-tr), and germacrene C (0-0.1%). Veriotti and Sacks [6m]

**Table 3:** Ranges of variation and average of the enantiomeric ratios determined in 27 samples of mandarin oils by Es-GC/FID compared with literature data.

| Compds                            | Range     | Average     | Liter.[7b,13a] |
|-----------------------------------|-----------|-------------|----------------|
| <i>1R,5R</i> (+)- $\beta$ -Pinene | 98.4-98.8 | <b>98.7</b> | 97.0-98.8      |
| <i>1S,5S</i> (-)- $\beta$ -Pinene | 1.6-1.2   | <b>1.3</b>  | 3.0-1.2        |
| <i>1R,5R</i> (+)-Sabinene         | 79.8-80.6 | <b>80.0</b> | 76.2-80.5      |
| <i>1S,5S</i> (-)-Sabinene         | 20.2-19.4 | <b>20.0</b> | 23.8-19.5      |
| <i>4S</i> (-)-Limonene            | 2.1-2.9   | <b>2.6</b>  | 2.0-2.3        |
| <i>4R</i> (+)-Limonene            | 97.9-97.1 | <b>97.4</b> | 98.0-97.7      |
| <i>3R</i> (-)-Linalool            | 17.7-21.0 | <b>18.5</b> | 13.1-19.8      |
| <i>3S</i> (+)-Linalool            | 82.3-79.0 | <b>81.5</b> | 86.9-80.2      |

reported *p*-cymene (16.1%), and  $\gamma$ -terpinene (5.8%) in commercial mandarin oil, but this is not in agreement with our results, nor with the data on the composition of industrially processed mandarin essential oils available in the literature.

Enantiomeric ratios (ER) of monoterpene hydrocarbons and linalool can be useful to reveal adulteration of mandarin oil [12]. The best approach for the determination of ER in complex matrices is multidimensional gas chromatography, with a chiral stationary phase in the second dimension of the system. Direct analysis is, however, simpler, although not always possible, due to interferences with the numerous components present in this complex matrix. It is possible to operate by direct Es-chiral GC applying the most appropriate chiral stationary phase selecting optimal experimental conditions, if limited to major components and to a limited number of enantiomeric pairs. For the determination of the ER of key components in mandarin oil, a direct enantioselective analysis was possible. Table 3 summarizes the ranges of variation and the averages determined for the single enantiomeric ratios in 27 samples of mandarin oils.

The results are in agreement with previous studies on mandarin essential oils [6e,7b,7c,13a]. Small differences are noticed for limonene, presenting a maximum value of the (-) isomer of 2.9%, slightly higher than the correspondent maximum (2.3%) previously reported [7b,13a]; the values of (-)/(+)-linalool (21.0/79.0) are determined in one sample with all the other enantiomers within the extremes of the ranges. For this sample, however, the composition of the volatile fraction, the non-volatiles, as well as the physico-chemical indices are compatible with a genuine mandarin oil. Excluding this sample from the set analyzed, the range of variation would be *3R*(-)-linalool (17.7 - 19.7) and *3S*(+)-linalool (82.3 - 80.3).

This investigation also included the determination by RP-HPLC of the polymethoxylated flavones. The

**Table 4:** Polymethoxylated flavones (g/L) in 27 samples of mandarin oils.

| Compds                              | Range       | Average | Liter. [13b] |
|-------------------------------------|-------------|---------|--------------|
| Sinensetin                          | n.q.d.      | n.q.d.  | 0.01-0.04    |
| Tetra- <i>O</i> -methylscutellarein | n.q.d.      | n.q.d.  | 0.03-0.11    |
| Nobiletin (cf. 0.675)               | 0.66 - 1.40 | 1.07    | 0.36-1.51    |
| Heptamethoxyflavone* (cf. 1.325)    | 0.29 - 0.92 | 0.62    | 0.16-0.69    |
| Tangeretin (cf. 0.475)              | 1.45 - 3.16 | 2.07    | 1.48-3.39    |

\* 3,3',4',5,6,7,8-heptamethoxyflavone; n.q.d.: not quantitatively determined; cf.: correction factor

results are summarized in Table 4. The analytical RP-HPLC method used is the result of a compromise in order to achieve good resolution with a single method for the highest number of the oxygen heterocyclic components present in the most common cold pressed citrus essential oil [9]. Thus, instead of the typical five components usually determined in this fraction of mandarin oil, only three (nobiletin, heptamethoxyflavone and tangeretin) were quantitatively determined, while sinensetin and tetra-*O*-methylscutellarein, which were present in very low amounts, were detected, but not quantitatively analyzed, due to the method limits. However, this procedure can provide sufficient information on the quality of mandarin essential oil.

In conclusion, the results of our study give a complete view of the composition of cold pressed mandarin essential oil industrially produced in Sicily from fruits of *C. deliciosa* Ten. cv. Tardivo di Ciaculli. We would like to stress the great importance of the exact indication of the botanical origin of the fruits used for the extraction, as well as the geographic area where these are cultivated. In fact, great confusion can be generated if this information is not clearly specified. The data available in the literature can also be affected by studies carried out on adulterated samples [6m]. The Citrus essential oil industry is still a significant source for the Sicilian economy, thus the high quality of mandarin oil produced in this area should be highlighted, particularly with regard to the higher content of methyl *N*-methylantranilate and  $\alpha$ -sinensal relative to mandarin oils of different origin. It is also worth mentioning that our results are consistent with previous studies from our laboratory and that only small differences have been detected in the composition of the volatile fraction. With regards to the ERs and the non-volatile PMFs the present study confirms what has been previously determined, although poor reference is available for deeper discussion of this matter. Further studies on the characterization of Sicilian mandarin essential oil are needed for a clear and unequivocal definition of this product.

## Experimental

**Oil samples:** A set of 27 samples of mandarin essential oil (*C. deliciosa* Tenore, cv. Tardivo di Ciaculli, produced in Sicily), kindly provided by a local producer (Simone Gatto, San Pier Niceto, ME, Italy), were collected during the productive season from October 2007 to January 2008. The essential oils were industrially extracted by the common techniques for cold pressed oils (Torchi and FMC) and kept refrigerated until analysis.

For Retention Indices determination, a hydrocarbon mixture (Supelco, PA, USA) ranging from *n*-heptane to triacontane was used. For quantitative determination of the oxygen heterocyclic compounds, coumarin (Fluka, Milan, Italy) was used as internal standard. All solvents (HPLC-grade) were purchased from Sigma-Aldrich (Milan, Italy). The physico-chemical indices were determined following the ISO 3528-1997(E) regulation [10a]. CD values were determined according to the ISO 4735-2002(E) regulation [10b].

**Oil analysis:** The volatile fraction of the samples was analyzed by HRGC/FID and HRGC/MS-LRI. The content of oxygen heterocyclic components (polymethoxyflavones) was analyzed by RP-HPLC/PDA. The enantiomeric distribution of some monoterpene hydrocarbons and alcohols was determined by Es-GC analyses.

**GC/FID** analyses were carried out using a GC-2010 system (Shimadzu, Japan), equipped with a SLB-5MS column (Supelco, Bellefonte, PA, USA), 30 m x 0.25 mm I.D. x 0.25  $\mu$ m film thickness. Oven temperature program: 50°C at 3°C/min to 250°C, held 10 min, at 10°C/min to 270°C, held 3 min. Samples: 1:10 in *n*-hexane; 1 $\mu$ L; split ratio: 1:50. Injector and FID temperatures: 280°C. Carrier gas: He, at 30.1 cm/s and pressure of 99.8 kPa. Data were processed through *GCsolution* software (Shimadzu, Japan).

**GC-MS analysis:** GC-MS analysis was carried out on a GCMS-QP2010 system (Shimadzu) equipped with an autosampler AOC-20is (Shimadzu) and a Equity-5MS column (Supelco, Bellefonte, PA, USA), 30 m x 0.25 mm I.D. x 0.25  $\mu$ m film thickness. Oven temperature program: 50°C at 3°C/min to 300°C, held 5 min. Samples: 1:10 in *n*-hexane, 1 $\mu$ L; split ratio of 1:50. Carrier gas: He at 32.4 cm/s. Mass spectrometer parameters were as follows: interface and source temperatures, 250°C and 200°C,

respectively; ionization mode, EI with a voltage of 0.94 kV applied; acquisition mass range, 40–400 *m/z* with a scan speed of 1666 amu/s and a scan interval of 0.25 s. Data handling was supported by the software *GCMSsolution*, ver. 2.51 (Shimadzu). For the identification of compounds, a GC-MS database dedicated to flavor and fragrance material (FFNSC ver.1.2; Shimadzu) was used, along with comparison of the linear retention indices (LRIs) with those reported by Adams' library [10c]. The FFNSC 1.2 library was developed in the same laboratory where this investigation has been carried out [11].

**Es-GC/FID** analyses were carried out using a GC-2010 system (Shimadzu, Japan), equipped with a DETTBSBETA-086 (diethyl-tert-butyl-silyl- $\beta$ -cyclodextrin) column (MEGA, Legnano, Italy), 30 m x 0.25 mm I.D. x 0.25  $\mu$ m film thickness. Oven temperature program: 45°C at 2°C/min to 200°C, held 10 min. Samples, 1  $\mu$ L of a 1:10 in *n*-hexane dilution, injected in split mode; split ratio: 1:100. Injector temperature: 250°C FID temperature: 220°C; make up gas (N<sub>2</sub>/air); Carrier gas: He, at 35.0 cm/s and a pressure of 96.6 kPa. Data were processed through *GCsolution* 2.4 software (Shimadzu, Japan).

**RP-HPLC** analyses were carried out using a Shimadzu HPLC system, equipped with two SCL-10-AVP pumps, a SCL-10-AVP controller, and a SPD-M10 AVP DAD detector; the column used was a discovery HS C18 (Supelco) (250 mm, 4.6 mm I.D., 5  $\mu$ m particle size). Prior to the analyses, each sample was diluted with acetonitrile (ACN) (1:20 w/v) and 50  $\mu$ L of a standard solution of coumarin in ACN (0.1% v/v) was added. Mobile phase: water (A) and ACN (B), gradient program: 0–1 min, 30% B; 1–5 min, 60% B; 5–8 min, 60% B; 8–30 min, 100% B held for 5 min. All solvents were of HPLC grade from Carlo Erba Reagenti. Injected volume: 10  $\mu$ L at a flow rate of 1 mL/min. Operative pressure: 170 bar; detector settings: 190 nm (initial wavelength) and 370 nm (final wavelength); sampling frequency: 1.5625 Hz. Quantitative determination was carried out by means of calibration curves with coumarin as internal standard, using the response factors obtained by injecting single standard solutions at different concentrations of nobiletin, heptamethoxyflavone and tangeretin isolated and purified in our laboratory, as previously reported [13b]. Correction factors can be found in Table 4.

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