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Evaluation of Gas Chromatography−Combustion−Isotope Ratio Mass Spectrometry (GC-C-IRMS) for the Quality Assessment of Citrus Liqueurs

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ABSTRACT: Citrus liqueurs are alcoholic beverages obtained by maceration. The European Parliament protects these alcoholic beverages, forbidding the addition of nature-identical flavoring substances. However, for economical and technological reasons, producers often add natural and/or synthetic flavors to the alcoholic syrup, obtaining artificial spirit drinks. The aim of this study is to investigate the authenticity of Italian liqueurs, of lemon, bergamot, and mandarin (locally known as "limoncello", "bargamino", and "mandarinetto"), comparing the carbon isotope ratios with values determined in genuine cold-pressed peel oils. Authenticity assessment was performed using headspace−solid phase microextraction coupled to gas chromatography−combustion−isotope ratio mass spectrometry. Additional analyses were performed by direct enantioselective gas chromatography to determine the enantiomeric distribution of selected chiral volatiles and by gas chromatography−mass spectrometry for the qualitative analyses of the samples. The method allowed confirmation of genuineness. Enantioselective gas chromatography analyses confirmed the results, demonstrating the reliability of the method.

KEYWORDS: *carbon isotope ratio mass spectrometry, enantiomeric distribution, citrus liqueurs, authenticity control*

INTRODUCTION

Citrus essential oils are appreciated natural flavoring materials and are applied in an enormous number of products of the food, beverage, cosmetics, and perfumery industries. They are obtained from the citrus fruit peel by cold extraction. These essential oils are rich in volatile compounds and plant secondary metabolites, mainly mono- and sesquiterpene hydrocarbons and their oxygenated derivatives.

Among the numerous food products obtained from citrus fruits are the traditional Italian citrus liqueurs, prepared by alcoholic maceration of the fruit peel of lemon, mandarin, and bergamot in ethanol, locally known as "limoncello", "mandarinetto", and "bergamino", respectively. These are appreciated for their fresh taste and digestive properties and, particularly the Italian ones, are registered with recognized Protected Designation of Origin (PDO).

Regulation (EC) No. 110/2008 of the European Parliament and of the Council standardizes the production and labeling of these products, forbidding the addition of nature-identical flavorings or synthetic substances. However, for economical reasons, producers often illegally add flavoring substances to confer or fortify the desired citrus flavor. In some circumstances commercial products are obtained by the addition of reconstituted oils free of the monoterpene hydrocarbons. This procedure helps to prevent the undesired formation of a "collar" at the neck of the bottle ("collarino" effect), due to the separation of monoterpene hydrocarbons during storage at low temperatures, and prevents the formation of off-flavors due to oxidation of monoterpene hydrocarbons induced by photochemical phenomena.[1,2]

Isotope ratio mass spectrometry (IRMS) coupled to gas chromatography (GC) has been widely applied to determine the origin and to evaluate the authenticity of numerous natural products, citrus essential oils,[3−5] and different food flavors.[5,6] This technique was optimized to develop analytical methods for food quality control, detecting the addition of synthetic or natural compounds[6−8] and/or differentiating the botanical and geographical origin of the raw material from which the components were isolated.[9,10]

Carbon isotope ratio mass spectrometry (GC-C-IRMS) enables the measurement of the natural abundance of carbon isotope ratios from an internationally established standard (primary standard), Vienna Pee Dee Belemnite (VPDB), selected for its constant and well-assessed $^{13}C/^{12}C$ ratio. For practical reasons in the laboratory it is, however, more convenient to adopt a "working" standard, calibrated against a secondary standard with a defined $^{13}C/^{12}C$ value related to the VPDB isotope ratio. For this purpose a cylinder of calibrated CO_2 is employed to send gas pulses directly to the isotope ratio mass spectrometer. The main steps in a GC-C-IRMS analysis consist of (i) achieving the best chromatographic separation through the GC capillary column, (ii) converting each separated component in CO_2, through the combustion chamber, and (iii) collecting the CO_2 ionized contributes into the mass spectrometer and comparing the $^{13}C/^{12}C$ values with those relative to working standard. The values are expressed as nondimensional quantities (δ) indicating the isotope ratio of a specific analyte and are expressed as parts per thousands (‰).[11] When GC-C-IRMS is performed, it

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is fundamental to quantitatively transfer highly pure compounds to the mass spectrometer, thus avoiding isotopic discrimination. Proper performance of the analyses requires optimal chromatographic peak resolution ($R > 1.5$); the entire peak area must be carefully integrated (v/v), and peak intensity must be comparable to the operating standard pulses.[12−14]

Citrus fruits are classified as C3 plants, and it has been reported that the carbon isotope ratios of their secondary metabolites may overlap with those of synthetic compounds derived from fossil sources or with those obtained from the metabolites of CAM plants. To avoid this inconvenience, the use of an internal standard (i-std) has been proposed.[5] Using the approach suggested by Mosandl's research group,[5] it is possible to neglect the contribution of the carbon isotopic fractionation, which occurs during the primary metabolism of the plant, influenced by the geographic origin, the soil characteristics, and climate conditions. Thus, it is possible to evaluate purely the contribution of the carbon isotope ratio linked to the enzymatic reactions of the secondary biogenetic pathway, which is characteristic of the plant.

Recently a large number of genuine citrus peel oils, industrially cold-extracted in Italy, have been the subject of a systematic study of this research group carried out by GC-C-IRMS.[8,9,15−18] Ranges of authenticity of selected volatiles were determined relative to $\delta^{13}C$ values by GC-C-IRMS and to the enantiomeric excesses (EE) by direct enantioselective GC (Es-GC) and by multidimensional GC (MDGC). These values were used to assess the genuineness of different citrus essential oils.[8,9,15,16] The results obtained for lemon, bergamot, and mandarin cold-pressed peel oils are used in the present study to define useful parameters of genuineness to evaluate homemade and commercial samples of lemon, bergamot, and mandarin liqueurs.

Eighteen volatiles (monoterpene and sesquiterpene hydrocarbons, monoterpene alcohols, aldehydes and esters, and one aliphatic aldehyde) extracted from the liqueurs by headspace−solid phase microextraction (HS-SPME) were analyzed by GC-C-IRMS, and six enantiomeric pairs were separated by Es-GC.

MATERIALS AND METHODS

Sample and Sample Preparation. Lemon liqueurs comprised three homemade samples (L1−L3) and five commercial samples (LC1−LC5). Bergamot liqueurs included four commercial samples (BC1−BC4). Mandarin liqueurs consisted of one homemade sample (M1) and three commercial samples (MC1−MC3).

Homemade samples were prepared by infusion of organic citrus peels in ethanol, for 10 days, and then diluted with water and sugar to the final alcoholic grade of 40°, without the addition of any kind of aromas, according to the Regulation (CE) No. 110/2008 of the European Parliament and of the Council on the definition, description, presentation, labeling, and protection of citrus spirit drinks. Commercial samples were purchased in a local supermarket.

Measurements of pH were performed using a Mettler Toledo pH-meter LE 409 (Novate Milanese, Italy) in all samples. Values ranged between 4.0 and 5.5.

Three and a half milliliters of citrus liqueurs were diluted with 1.5 mL of NaCl-saturated water, and the volatile components were extracted from a 10 mL half-filled SPME vial (with silicone/PTFE septa).

All of the analyses were carried out in triplicate with the exception of those performed by GC-C-IRMS, which were reproduced five times.

(HS-SPME) Extraction Parameters. The fiber was polydimethylsiloxane (PDMS) (Supelco) of 100 μm thickness. It was conditioned prior to use at 250 °C for 30 min. The manual extraction and the injector exposure times were optimized as described in detail below for each analytical setup. All of the samples were stirred at 2000 rpm.

GC-C-IRMS Device and Analyses. The GC-C-IRMS was a Trace GC Ultra equipped with a TriPlus autosampler, hyphenated to a combustion interface GC/CIII and to an isotope ratio mass spectrometer Delta V Advantage (all purchased from Thermo Fisher Scientific, Milan, Italy).

The column was an SLB-5 ms (silphenylene polymer), 30 m × 0.25 mm i.d., 0.25 μm film thickness (Supelco, Milan, Italy). The temperature program was as follows: from 40 to 125 °C at 3 °C/min and at 5 °C/min until 250 °C. The split/splitless injector was in splitless mode at 250 °C (splitless time = 1 min) and the inlet pressure at 101 kPa. The carrier gas was He, with a column flow of 2.0 mL/min (constant flow mode).

The oxidation reactor (GC/CIII) consisted of a ceramic tube filled with three wires of CuO/NiO/Pt, maintained at 980 °C. The CO_2 produced by combustion of each component is transferred to the mass spectrometer, and the resulting water is eliminated through a Nafion tube.

Gas pressures were as follows: He, 1 bar; O_2, 0.8 bar; CO_2, 0.5 bar.

The isotope ratio mass spectrometer (IRMS) electron voltage was 123.99 eV and the electron current, 1.5 mA. The IRMS was equipped with three Faraday cup collectors set at m/z 44, 45, and 46. The peak center predelay and postdelay were set at 15 s on cup 3.

The four CO_2 reference pulses were programmed at 60−80, 100−120, 140−160, and 180−200 s, in the mode split open. The evaluation type was CO_2_SSH, and the reference time was set at 155.91 s relative to the calibrated CO_2 isotope ratio ($\delta^{13}C/^{12}C = -60.300‰$). The integration time was 0.2 s.

Data were collected in triplicate by Isodat 2.5 software (Thermo Fisher Scientific).

The experimental conditions applied to extract and inject the samples varied as a function of the characteristic composition of the samples. To analyze β-pinene and limonene in lemon liqueurs, β-pinene, limonene, γ-terpinene, and linalool in bergamot liqueurs, and limonene and γ-terpinene in mandarin liqueurs, a 10 mL vial was half filled with 3.5 mL of liqueur plus 1.5 mL of water saturated with NaCl. The exposure time of the fiber was 10 s at 30 °C, and the GC injector was set at 250 °C with a desorption time of 1 min in splitless mode. The backflush option was off. To analyze neral, geranial, neryl acetate, geranyl acetate, β-caryophyllene, *trans*-α-bergamotene, and β-bisabolene in lemon liqueurs, linalyl acetate, neryl acetate, geranyl acetate, β-caryophyllene, α-beragmotene, and β-bisabolene in bergamot liqueurs, and α-pinene, β-pinene, myrcene, terpinen-4-ol, α-terpineol, decanal, and methyl *N*-methylanthranilate in mandarin liqueurs, the exposure time of the fiber was 20 min at 30 °C. The fiber desorption in the GC injector was for 2 min at 250 °C, in splitless mode.

Backflush was opened for 1200 s for lemon and bergamot liqueurs, whereas for mandarin liqueurs the backfush option was used between 250 and 950 s.

All samples were analyzed five consecutive times with a resulting relative standard deviation of the $\delta^{13}C$ values always <3%.

CO_2 Reference Gas Cylinder Calibration. The CO_2 reference gas was calibrated by injecting 1 μL (70 ppm) of an alkane mixture (C_{16}−C_{30}) with a well-assessed carbon stable isotope ratio (Indiana University, Bloomington, IN, USA); tricosane (C_{23}) was arbitrarily chosen as reference.

Isotope ratios were expressed as δ values (‰), versus a standard.

$$\delta^{13}C_{VPDB} = \frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{standard}}{(^{13}C/^{12}C)_{sample}} \times 1000$$

GC-MS Analyses. A Shimadzu QP2010 GC-MS was used with an SLB-5 ms fused silica capillary column, 30 m × 0.25 mm, 0.25 μm film thickness (Supelco, Milan, Italy). The temperature program was as follows: 40−125 °C at 3 °C/min, then to 250 °C at 5 °C/min, and to 330 °C at 10 °C/min. The carrier gas was He at a constant linear velocity (30.0 cm/s), and the injector was set at 250 °C in splitless mode (2 min). Mass spectrometry conditions were as follows: ion source temperature, 200 °C; interface temperature, 250 °C; scan

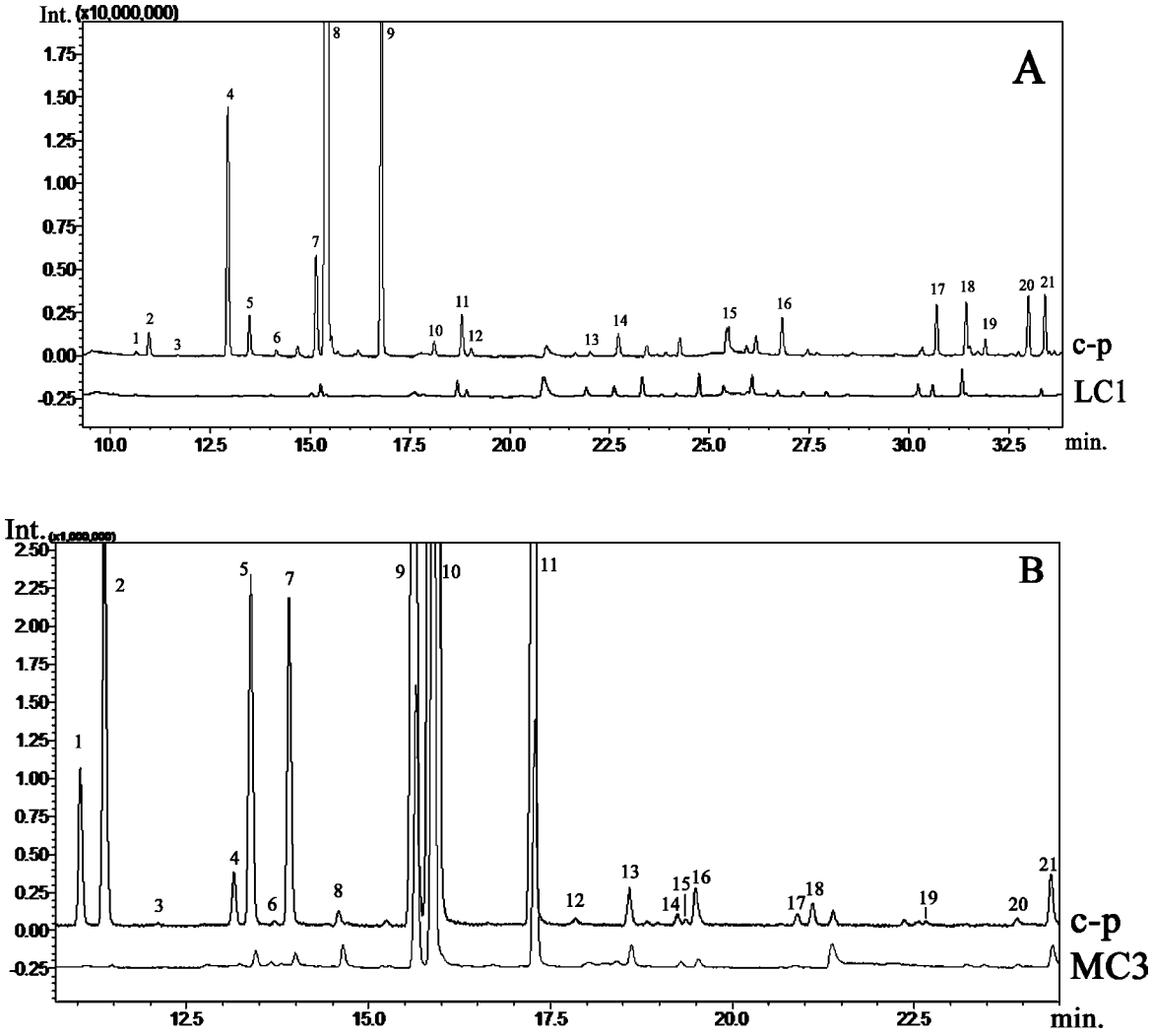

Figure 1. Comparison of the TIC profiles of (A) cold-pressed (c-p) lemon oil with sample LC1 and (B) c-p mandarin oil with sample MC3. (A) Peaks: 1, *α*-thujene; 2, *α*-pinene; 3, camphene; 4, *β*-pinene; 5, myrcene; 6, octanal; 7, *p*-cymene; 8, limonene; 9, *γ*-terpinene; 10, terpinolene; 11, linalool; 12, nonanal; 13, linalool ethyl ester; 14, terpinen-4-ol; 15, neral; 16, geranial; 17, neryl acetate; 18, geranyl acetate; 19, *β*-elemene; 20, *β*-caryophyllene; 21, *trans*-*α*-bergamotene. (B) Peaks: 1, *α*-thujene; 2, *α*-pinene; 3, camphene; 4, sabinene; 5, *β*-pinene; 6, 6-methyl-5-hepten-2-one; 7, myrcene; 8, octanal; 9, *p*-cymene; 10, limonene; 11, *γ*-terpinene; 12, *cis*-sabinene hydrate; 13, terpinolene; 14, linalool; 15, *trans*-sabinene hydrate; 16, nonanal; 17, *cis*-limonene oxide; 18, *trans*-limonene oxide; 19, terpinen-4-ol; 20, *α*-terpineol; 21, decanal.

range, m/z 40−400, with a scan interval of 0.25 s. The detector voltage was set at 0.94 kV. Data were collected by the GCMSsolution software (Shimadzu). Identification was performed by means of spectral similarity with the MS library FFNSC 2 − Flavor and Fragrance Natural and Synthetic Compounds (Shimadzu Corp., Japan) with the use of linear retention indices (LRI) interactively. The LRI were calculated by injecting a C_7−C_{30} homologous series of alkanes under identical chromatographic conditions.

Es-GC Analyses. The system consisted of a Shimadzu GC2010 gas chromatograph. Data were collected by GCsolution software (Shimadzu).

The column was a Megadex DETTBS-*β* (diethyl-*tert*-butyl-silyl-*β*-cyclodextrin), 25 m × 0.25 mm i.d., 0.25 *μ*m d_f (Mega, Legnano, Italy). The temperature was programmed from 50 to 200 °C at 2 °C/min and held for 5 min. The injector temperature was 220 °C, in splitless mode (2 min). The carrier gas was He at constant linear velocity (35 cm/s); the FID detector temperature was 220 °C, and the acquisition sampling rate was 80 ms.

Analyses were carried out in triplicate; the relative standard deviation determined for retention times and peak areas was always <5%. The enantiomer elution order was based on literature data.[8,9,15,16]

RESULTS AND DISCUSSION

HS-SPME hyphenated to gas chromatography mass spectrometry (GC-MS) was first employed to elucidate the qualitative composition of the volatile fraction in all of the samples investigated.

For the qualitative evaluation of the samples we assumed as reference the chromatographic profile obtained by analyzing the corresponding citrus genuine peel oil, under the same GC-MS operating conditions and SPME extraction procedure with the PDMS fiber.

The chromatographic profiles obtained for the samples of homemade lemon and mandarin liqueurs (L1−L3 and M1) were almost identical to those relative to the genuine cold-pressed lemon and mandarin peel oils. Figure 1 shows the GC-MS profiles of a commercial lemon liqueur compared to the HS-SPME-GC-MS profile of a genuine cold-pressed lemon oil (A) and the comparison of a commercial mandarin liqueur compared to the HS-SPME-GC-MS profile of a genuine cold-pressed mandarin oil (B).

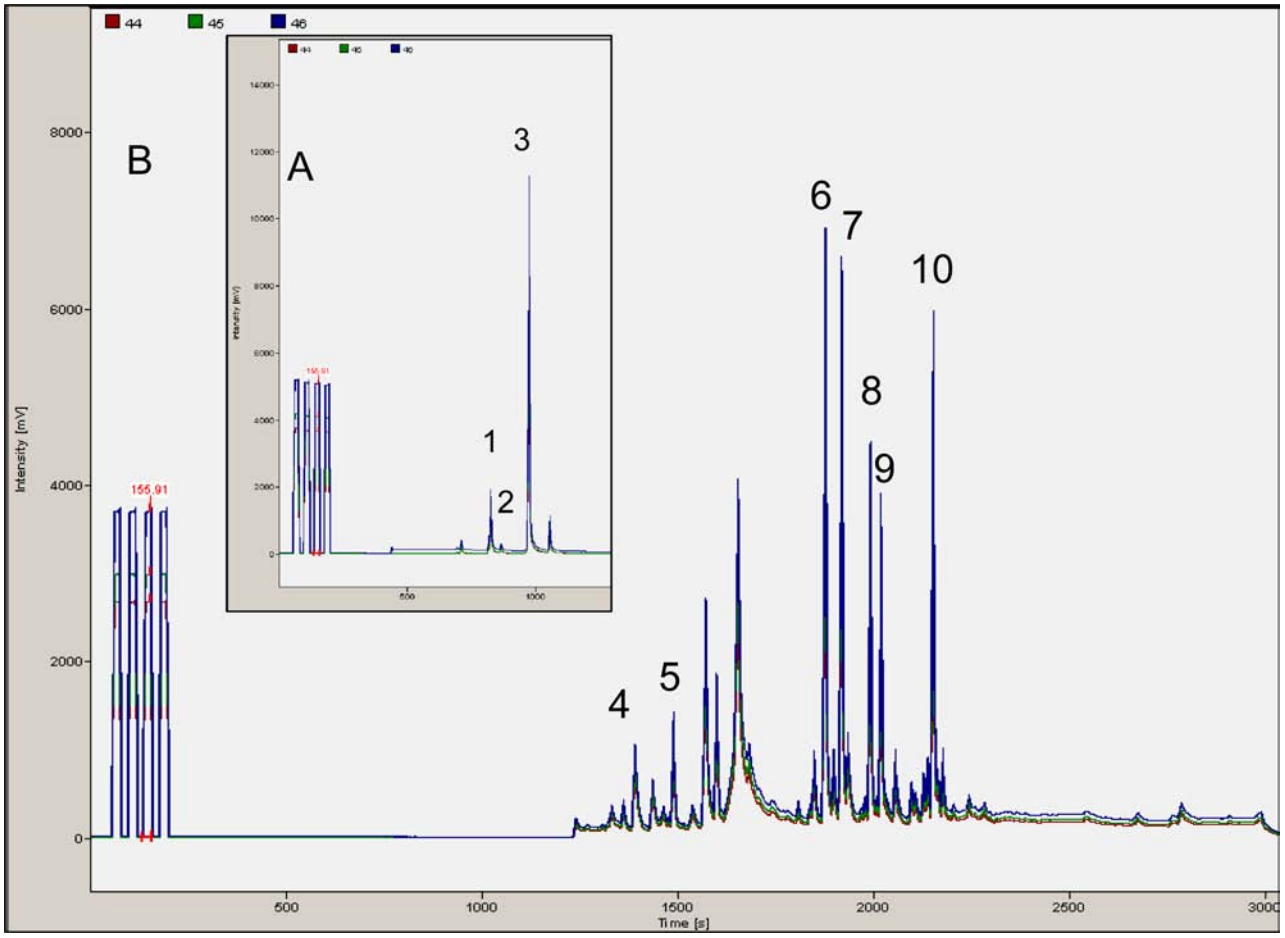

Figure 2. HS-SPME GC-C-IRMS profiles of a homemade limoncello. (A) Peaks: 1, β-pinene; 2, myrcene; 3, limonene. (B) Peaks: 4, neral; 5, geranial; 6, neryl acetate; 7, geranyl acetate; 8, β-caryophyllene; 9, *trans*-α-bergamotene; 10, β-bisabolene.

Under the conditions here applied quantitative analyses were not performed. However, some differences emerged, in terms of peak intensities between the commercial samples and the cold-pressed genuine oils analyzed under identical conditions. Sample LC1 was characterized by an extremely low content of the monoterpene hydrocarbons, although the sesquiterpene fraction was similar to the cold-pressed oil. The major volatiles were linalool, nonanal, linalool ethyl ester, terpinen-4-ol, citronellyl acetate, neryl acetate, and geranyl acetate.

The GC-MS profiles of samples LC2, LC3, and LC4 were similar to those of the genuine cold-pressed lemon peel oil, with limonene, γ-terpinene, and β-pinene as major compounds. Sample LC5 was characterized by low intensities of the sesquiterpene hydrocarbons.

The GC-MS analysis of the commercial bergamot liqueurs revealed for sample BC1 low intensities of α-pinene, β-pinene, myrcene, γ-terpinene, and the sesquiterpene compounds. In this sample linalyl acetate was not detected.

Sample BC2 showed a chromatographic profile similar to that of sample BC1, but in this sample linalyl acetate and the sesquiterpene hydrocarbons β-caryophyllene and β-bisabolene were detected. Sample BC3 was characterized by very low monoterpene hydrocarbons and by the total absence of linalyl acetate and sesquiterpene hydrocarbons. The main compounds detected were linalool, linalool ethyl ether, neryl acetate, and geranyl acetate. Sample BC4 showed a chromatographic profile similar to that of sample BC3, but in this case linalyl acetate, *trans*-α-bergamotene, and β-bisabolene were detected.

Sample MC1 showed a GC-MS profile similar to that of the genuine cold-pressed mandarin peel oil, although methyl *N*-methylanthranilate was quite low. In sample MC2 was detected the presence of δ-3-carene. This compound is normally absent or present at only trace levels in genuine mandarin oiln and it is a typical marker used to reveal adulteration by the addition of sweet orange oil.[19,20] Moreover, terpinen-4-ol and α-terpineol were quite low. The sesquiterpene hydrocarbons α-cubebene, α-copaene, β-caryophyllene, α-humulene, β-elemene, germacrene D, and δ-cadinene were detected. In sample MC3 were observed the unusual absence of α-pinene and very low intensities of β-pinene and myrcene.

Selected volatiles were then analyzed by means of gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS). The chromatogram of a lemon liqueur obtained by HS-SPME GC-C-IRMS is reported in Figure 2. The carbon isotope ratios of the selected markers were compared with the authenticity ranges previously determined in the corresponding Italian citrus peel oils[8,9,15,16,21] on large sets of genuine cold-pressed lemon, mandarin, and bergamot essential oils. For each citrus species, the markers were chosen on the basis of their abundance and their characteristic aroma, as well as the appropriate chromatographic resolution. The analyses were carried out five times for each sample, and the results are expressed as mean values. The relative standard deviation determined for the $\delta^{13}C_{VPDB}$ values was <3% for all of the components analyzed. The average values corresponding to each sample analyzed are reported in Table 1.

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Table 1. Carbon Isotope Ratio Average Values Obtained for Volatile Components in Liqueur Samples: Reference $\delta^{13}C_{VPDB}$ Authenticity Range Relative to the Corresponding Genuine Cold-Pressed Citrus Oils

	$\delta^{13}C_{VPDB}$ mean values																		
	lemon liqueurs									bergamot liqueurs					mandarin liqueurs				
volatile component	LC1	LC2	LC3	LC4	LC5	L1 home-made	L2 home-made	L3 home-made	ref 9	BC1	BC2	BC3	BC4	ref 16	MC1	MC2	MC3	M1 home-made	ref 15
α-pinene															−28.85	−26.93		−30.21	−32.37 to −30.28
β-pinene		−25.71	−27.50	−27.90	−25.47	−27.50		−24.80	−27.95 to −25.22	−25.94	−28.19		−26.41	−28.34 to −24.28	−28.59	−27.48		−29.12	−31.70 to −29.46
myrcene		−26.21	−25.91	−26.60	−25.44	−25.88	−27.48	−25.25	−27.35 to −24.72	−25.32	−24.20	−26.55	−26.89	−26.74 to −24.18	−27.40	−26.61	−30.45	−27.68	−29.51 to −27.04
limonene	−27.29	−25.22	−26.15	−27.47	−25.64	−26.48	−27.63	−25.51	−28.53 to −25.29	−26.09	−26.23	−25.61	−26.26	−28.58 to −25.18	−29.01	−28.05	−29.71	−28.63	−30.94 to −28.65
γ-terpinene										−26.34	−27.42	−29.72	−27.80	−30.71 to −27.09					
linalool										−23.91	−24.04	−25.58	−25.60	−28.93 to −25.92					
terpinen-4-ol															−30.31		−31.71	−30.54	−33.17 to −28.26
α-terpineol															−30.04		−29.81	−30.27	−31.57 to −28.15
decanal															−27.91	−28.81	−29.67	−29.44	−31.99 to −27.52
neral	−27.33	−26.20	−26.84	−27.43	−27.20	−25.67	−25.83		−27.36 to −24.76										
geranial	−26.67	−24.45	−25.95	−26.00	−25.33	−26.29	−26.60		−26.85 to −24.52										
linalyl acetate											−22.83		−24.03	−29.50 to −26.39					
neryl acetate	−28.08	−28.66		−27.51	−27.46	−28.84	−30.57	−27.27	−31.87 to −28.76	−25.71	−22.85	−27.32	−27.51	−28.50 to −26.32					
geranyl acetate	−29.96	−27.49	−28.51	−31.25	−28.06	−29.00	−30.24	−28.86	−31.22 to −28.09	−25.04	−21.79	−26.71	−27.21	−28.31 to −26.23					
MNM[a]																−31.52		−31.49	−34.46 to −31.71
β-caryophyllene	−29.43	−28.57		−29.03	−27.84	−28.33	−28.53	−27.27	−30.89 to −28.02		−24.58		−24.99	−27.66 to −24.84					
trans-α-bergamotene	−28.56	−29.50		−30.95	−29.28	−29.50	−31.32	−27.97	−32.09 to −28.58		−25.08		−27.17	−27.61 to −25.13					
β-bisabolene	−27.03	−27.71		−27.23	−25.91	−27.48	−29.30	−26.33	−30.26 to −26.44		−23.75		−25.43	−26.67 to −24.49					

[a]Methyl *N*-methylanthranilate.

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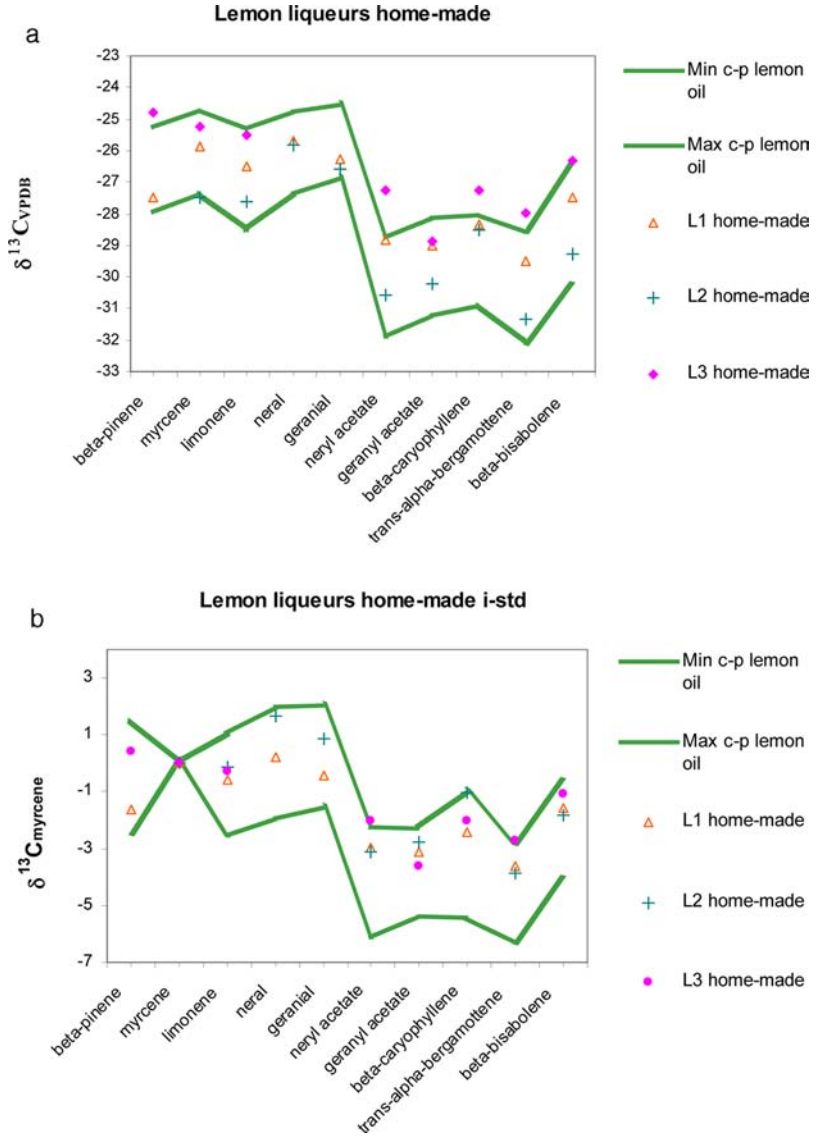

Figure 3. Isotope ratios determined in homemade lemon liqueurs by GC-C-IRMS: (A) direct comparison with the range of authenticity determined in lemon peel oils; (B) comparison with the range of authenticity determined in lemon peel oils using myrcene as internal standard.

The $\delta^{13}C_{VPDB}$ values are the consequence of the carbon fractionation occurring during the primary and secondary biogenetic pathways of the plant. The introduction of an internal standard (i-std) is mandatory to directly compare the isotope ratios determined in the samples with those used to build the range of authenticity, thus eliminating the environmental or climatic factors linked to the primary biogenetic pathways, such as CO_2 fixation, plant geographic origin, and eventually the differentiation of Calvin, Hatch Slack, or CAM photosynthetic cycles (C3, C4, CAM). The values obtained with the use of an internal standard will thus refer only to the carbon fractionation occurring during the secondary metabolite biosynthesis in the plant cells within the methylerythritol phosphate (MEP) and mevalonate (MVA) pathways.[22]

Among the volatiles analyzed, myrcene was chosen as internal standard for all of the citrus liqueurs on the basis of the following requisites: it is a genuine compound of less sensorial relevance; it is available in sufficient amount and is free of isotopic fractionation during sampling and analysis; and it is biogenetically related to the other volatiles and is not a legally allowed additive.[5]

In Figure 3 are plotted the carbon isotope ratio values for homemade lemon liqueurs, without (A) and with (B) the internal standard myrcene (i-std), respectively, versus the range of authenticity determined in genuine Italian cold-pressed lemon peel oils.[9] Samples L1 and L2 are in agreement with the range, whereas the slight deviations relative to sample L3 were resolved in graph B by introducing the i-std as expected.

The values relative to commercial lemon liqueurs are plotted in Figure 4. The values obtained without the i-std reported in Table 1 slightly deviate from the authenticity ranges observed for the $\delta^{13}C_{VPDB}$ values of neryl acetate in samples LC1, LC4, and LC5; of geranyl acetate in sample LC2; and of β-caryophyllene and β-bisabolene in sample LC5. The use of the internal standard minimizes the differences of these samples in comparison with the range obtained for authentic lemon oils. Sample LC5 plotted in Figure 4A is now inside the range, although samples LC2 and LC4 still present values outside the range for geranyl and neryl acetates, respectively. This could be explained by the addition of these compounds to fortify the lemon flavor. Among the commercial samples only LC3 presented all of the $\delta^{13}C$ values of the analyzed components in excellent agreement with the range of

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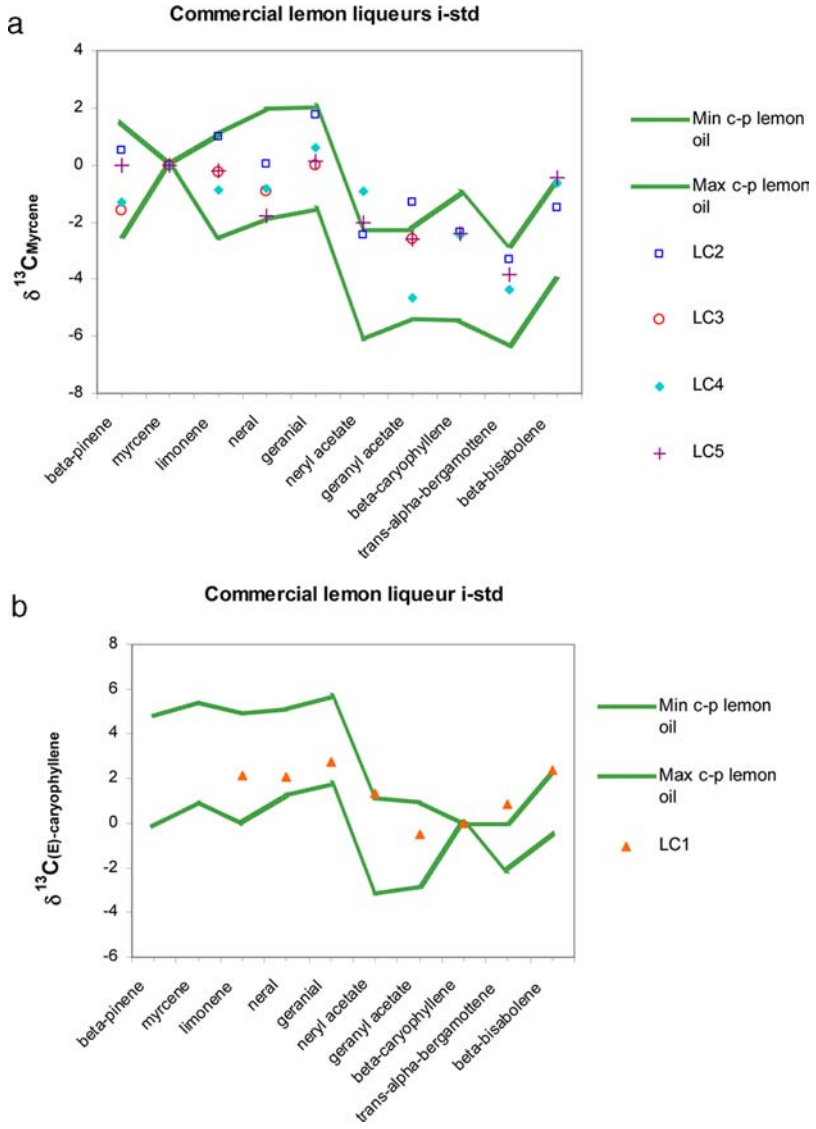

Figure 4. Isotope ratios determined in commercial lemon liqueurs by GC-C-IRMS: (A) comparison with the range of authenticity determined in lemon peel oils with myrcene as internal standard; (B) direct comparison with the range of authenticity determined in lemon peel oils with (*E*)-caryophyllene as internal standard.

authenticity. Figure 4B shows the $\delta^{13}C_{\beta\text{-caryophyllene}}$ values relative to sample LC1, because myrcene was not detected in this limoncello. In fact, from the volatile profile this sample was probably obtained with monoterpene-free lemon oil. This hypothesis is proved by the fact that isotope ratios are in good agreement with the authenticity range of lemon cold-pressed oil.

Figure 5 shows the bergamot liqueur $\delta^{13}C$ values. In these samples several deviations are observed from the authentic cold-pressed bergamot peel oil.[8,16] Sample BC1 presents anomalous values of γ-terpinene, neryl acetate, and geranyl acetate; in sample BC2 linalool, linalyl acetate, and neryl and geranyl acetate are evidently outside the values of the range of authenticity and β-caryophyllene and β-bisabolene, to a lesser extent, are outside their ranges. Sample BC3 showed deviation for linalool, and sample BC4 deviates for the range of authenticity for the values of linalyl acetate. The differences between these samples and the range of authenticity confirm the addition of nature-identical or artificial flavorings.

The $\delta^{13}C_{myrcene}$ values determined in madarinetto are shown in Figure 6. The commercial mandarinetto liqueurs (samples

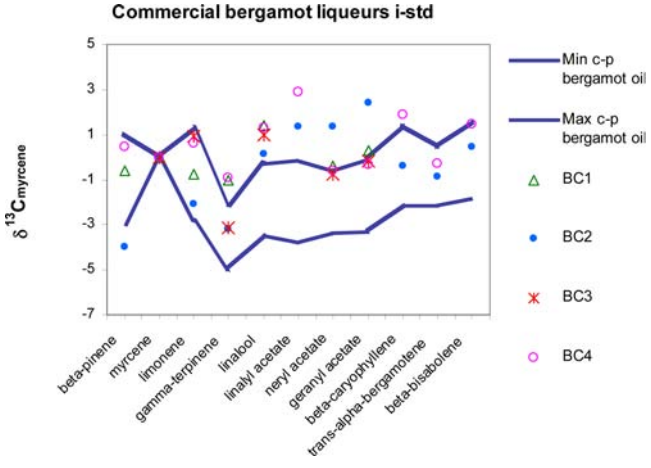

Figure 5. Isotope ratios determined in commercial bergamot liqueurs by GC-C-IRMS compared with the range of authenticity determined in bergamot peel oils using myrcene as internal standard.

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Table 2. Enantiomeric Distribution of Chiral Volatile Components in Citrus Liqueurs: Reference Literature Data Relative to the Corresponding Genuine Citrus Cold-Pressed Peel Oils

	lemon liqueurs									bergamot liqueurs					mandarin liqueurs				
chiral volatile component	LC1	LC2	LC3	LC4	LC5	L1 home-made	L2 home-made	L3 home-made	ref 9	BC1	BC2	BC3	BC4	ref 8	MC1	MC2	MC3	M1 home-made	ref 15
R-(+)-α-pinene	24.04	20.97	26.59	34.09	26.89	26.01	35.71	25.07	25.5−38.0		26.00		27.84	26.0−38.4	33.23	29.60	27.85	43.15	41.7−54.5
S-(−)-α-pinene	75.96	79.03	73.41	65.91	73.11	73.99	64.29	74.93	74.5−62.0		74.00		72.16	74.0−61.6	66.77	70.40	72.15	56.85	58.3−45.5
R-(+)-β-pinene	5.85	5.74	5.87	8.76	6.82	5.44	5.59	6.17	4.2−7.0	9.74	8.79	14.73	9.29	6.8−10.3	98.41	77.89	36.33	98.77	86.0−98.8
S-(−)-β-pinene	94.15	94.26	94.13	91.24	93.18	94.56	94.41	93.83	95.8−93.0	90.26	91.21	85.27	90.71	93.2−89.7	1.59	22.11	63.67	1.23	14.0−1.2
R-(+)-sabinene	14.68	16.90	17.01	15.09	14.61	14.70	15.11	16.27	12.4−15.5	17.20	16.11			13.7−19.8		75.90	47.91	80.39	70.3−81.7
S-(−)-sabinene	85.32	83.10	82.99	84.91	85.39	85.30	84.89	83.73	87.6−84.5	82.80	83.89			86.3−80.2		24.10	52.09	19.61	29.7−18.3
S-(−)-limonene	1.84	1.72	1.43	1.25	1.84	1.46	1.24	1.60	1.0−2.6	1.86	2.14	5.45	1.69	1.2 to <3.0	1.49	1.89	1.77	1.65	tr−2.6
R-(+)-limonene	98.16	98.28	98.57	98.75	98.16	98.54	98.76	98.40	99.0−97.4	98.14	97.86	94.55	98.31	98.8 to >97.0	98.51	98.11	98.23	98.35	100−97.4
R-(−)-linalool		60.90			61.13	74.73	53.42	71.66	49.5−74.5	89.32	71.17	86.19	68.91	99.0−100	28.54	28.64			13.0−21.0
S-(+)-linalool		39.10			38.87	25.27	46.58	28.34	50.5−25.5	10.68	28.83	13.81	31.09	1.0−0	71.46	71.36			87.0−79.0
(−)-linalyl acetate											78.18		72.55	99.0−100					
(+)-linalyl acetate											21.82		27.45	1.0−0					

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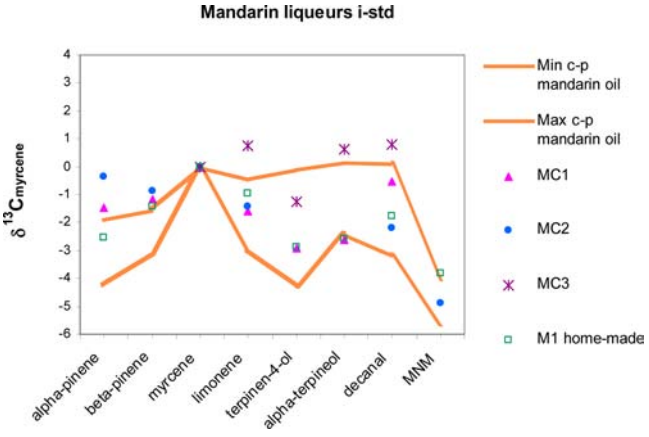

Figure 6. Isotope ratios determined in homemade (A) and commercial (B) mandarin liqueurs by GC-C-IRMS compared with the range of authenticity determined in mandarin peel oils using myrcene as internal standard.

MC1−MC3) show numerous values of their $\delta^{13}C$ outside the range of authenticity. This confirms the suspected addition/contamination with sweet orange or lemon oils already arisen from the GC profile of the whole volatile fraction. In fact, the $\delta^{13}C_{myrcene}$ values of α- and β-pinene in samples MC1 and MC2 as well as the values of limonene, α-terpineol, and decanal in sample MC3 fall outside the range, implying the addition of nature-identical, artificial flavoring or contamination/addition with other citrus oils, such as sweet orange or lemon.

To better elucidate the nature of flavorings in our samples and to prove the results obtained from GC-MS and GC-C-IRMS analyses, the samples underwent enantioselective gas chromatography (Es-GC) to determine the enantiomeric distribution of selected volatiles as reported in Table 2.

Most of the homemade liqueurs were in good accordance with the enantiomeric distributions reported in the literature for genuine cold-pressed lemon, bergamot, and mandarin oils of secure origin and surely genuine. In particular, the chiral distribution in lemon liqueurs was in excellent agreement with the range determined for genuine lemon peel oils. In the case of the homemade lemon liqueurs (samples L1−L3), the enantiomeric distributions determined in these samples confirm the IRMS results, proving that these liqueurs were prepared according to the preparation procedure dictated by Regulation (CE) No. 110/2008 of the European Parliament and of the Council. The results obtained on the chiral distribution of samples LC1, LC3, and LC5 confirm the IRMS results, although some concerns arise in view of the GC-MS profile of samples LC1 and LC5. These two samples could be considered not fully in agreement with the CE regulation, as they appear to be obtained by the addition of lemon essential oil fractions. This procedure is often preferred for the industrial production to avoid the previously mentioned "collarino" effect, produced from the terpenes under refrigeration, and the unpleasant taste, due to the terpenic oxidation.

The enantiomeric ratios determined in bergamot liqueurs (BC1−BC4) are in accordance with the isotopic ones. More in detail, in sample BC1 the enantiomeric distribution and the $\delta^{13}C$ value of linalool fall outside the ranges relative to genuine bergamot peel oils determined by both techniques. Similar cases are samples BC2 and BC4 with deviations from the carbon isotope ratios and the enatiomeric distributions of several compounds. In sample BC3 the enantiomeric distribution as well as the isotope ratio of linalool is not in agreement with that of genuine bergamot oils, whereas linalyl acetate is absent. This indicates the probable blending of these liqueurs with artificial flavorings to enhance or reproduce the characteristic bergamot aroma. This is confirmed by the lack or low level of the mono- and sesquiterpene hydrocarbons detected in these samples by GC-MS.

All of the commercial mandarin liqueurs analyzed presented values of the chiral distribution not compatible with genuine cold-pressed mandarin oil, confirming the results obtained by the evaluation of the carbon isotope ratios previously discussed.

In conclusion, we can assert that the determination of the $\delta^{13}C$ values of selected volatiles can be useful to evaluate the authenticity of citrus liqueurs or to define the type of adulteration. However, it must be emphasized that more conventional analytical approaches (GC-MS or Es-GC) can lead to the same results. The importance of the isotopic ratio evaluation cannot be, however, disregarded when the other two techniques produce uncertain results (very subtle adulterations). However, this simple analytical approach, based on the evaluation of the carbon isotope ratios of a few selected compounds, can unveil adulteration of commercial citrus liqueurs and confirm the genuineness of homemade ones by itself as confirmed by direct Es-GC analyses and by GC-C-IRMS, demonstrating the reliability of the method.

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Notes

The authors declare no competing financial interest.

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