

Essential Oil Composition of *Citrus meyerii* Y. Tan. and *Citrus medica* L. cv. Diamante and Their Lemon Hybrids

ANTONELLA VERZERA,^{*,†} ALESSANDRA TROZZI,[‡] MARIO ZAPPALÁ,[§]
 CETTINA CONDURSO,[†] AND ANTONELLA COTRONEO^{||}

Dipartimento di Chimica Organica e Biologica, Università di Messina,
 Salita Sperone 31, 98168 Messina, Dipartimento di Scienze Farmaco-biologiche, Università di
 Catanzaro, Roccelletta di Borgia, 88021 Catanzaro, Dipartimento di OrtoFloroArboricoltura e
 Tecnologie Agroalimentari (DOFATA), Università di Catania, Via S. Sofia 98, 95124 Catania, and
 Dipartimento Farmaco-chimico, Facoltà di Farmacia, Università di Messina,
 SS. Annunziata, 98168 Messina, Italy

In this paper we report the volatile fraction composition of *Citrus meyerii* Y. Tan. and *Citrus medica* L. cv. Diamante and two new lemon hybrids obtained by cross-breeding them with the tetraploid *Citrus limon* Burm. cv. Cavone. Both parent and hybrid oils were laboratory-extracted from the peel fruits and analyzed by HRGC–MS and HRGC–FID. Sixty-three components were fully characterized by mass spectra, linear retention indices, and injection of standards. The average composition as single components for all the oils analyzed is reported. Moreover, the data obtained were statistically analyzed. Since limonene is by far the main component of all the essential oils examined, analysis of variance and multivariate analysis gave interesting information on the similarities and differences between the oils analyzed. The new hybrid oils analyzed have potential commercial value because they could be an acceptable alternative to the valuable lemon oil.

KEYWORDS: *Citrus meyerii* Y. Tan. (Meyer lemon); *Citrus medica* L. cv. Diamante (Diamante citron); *Citrus limon* Burm.; hybrids; volatile fraction composition; HRGC–MS; HRGC-FID; ANOVA; multivariate analysis

INTRODUCTION

The hybridization project represents one of the most important research lines in citrus genetic improvement. The aim is to create or select the finest cultivars that can satisfy both consumers and industries; the selected cultivars should have the following qualities: rapid growth but small in size; better resistance to diseases, drought, and frost; abundant, early, and steady fructification (in the second to third year since they were planted); uniform and homogeneous size of fruits; resistance to transportation; fine colored fruits; mostly seedless; easily removable peel; good quality and amount of juice and/or essential oil.

As regards the lemon, the early crosses, which eventually led to the genesis of new species, were performed to obtain cultivars resistant to the disease mal secco, which was for the first time reported in 1918 in the Province of Messina, Sicily, after unchecked infected goods were imported from Greece; afterward the disease widely spread. The mal secco agent in citrus is the *Phoma tracheiphila* fungus that lives in the ligneous

vessels of the host plant, thus causing the typical tracheomycosis syndrome. In the Mediterranean region, *P. tracheiphila* is the most destructive fungal disease of lemons; up to 100% of trees in a lemon orchard of a susceptible cultivar can be affected. Because of the numerous losses in lemon groves of diseased plants or because of a drastic pruning of their diseased parts, from an economic point of view, this disease caused a major decline in the growing of lemons, particularly in the Mediterranean area of Italy. The disease reduces the quantity and quality of lemon production in the areas where the pathogen is present, and limits the use of susceptible species and cultivars (1–3).

Since 1978, the Istituto Sperimentale per l'Agrumicoltura (Acireale, Sicily, Italy) has conducted an extensive program for the selection of seedless cultivars via the production and the selection of triploid plants which yield seedless fruits and, in the case of lemon, have a good tolerance to mal secco (4). Recently, new hybrids, by cross-breeding the diploids *Citrus meyerii* Y. Tan. (Meyer lemon) and *Citrus medica* L. cv. Diamante (Diamante citron) with the tetraploid *Citrus limon* Burm. cv. Cavone (Cavone lemon), have been obtained.

Meyer lemon is a natural hybrid between lemon and orange, originally found near Peking, China. Fruits are similar in shape and size to those of lemon but differ in lower citric acid content, in the flavor of the essential oil, and in the orange-yellow peel.

* To whom correspondence should be addressed. Phone: 39-90-6765240. Fax: 39-90 393895. E-mail averzera@pharma.unime.it.

[†] Dipartimento di Chimica Organica e Biologica, Università di Messina.

[‡] Università di Catanzaro.

[§] Università di Catania.

^{||} Dipartimento Farmaco-chimico, Università di Messina.

Because of its resistance to mal secco and its high productivity, it is also used as an ornamental plant and can be considered as a parent in crosses to obtain hybrids resistant to the disease (5).

Diamante citron was the first citrus to become known to the European civilization; nowadays its cultivation is limited to a few areas, in Calabria, Crete, Corsica, and Israel. Ripe fruits are big, with a thin, smooth, and lemon-yellow peel. The main products obtained from citron extracts are candy and liqueurs, while the oil is a minor product, used as a flavoring in sweets and beverages (1).

Cavone lemon was discovered in 1940 in a group of tangerine plants grafted with lemons in the Cavone farmland (Syracuse, Sicily, Italy). It is a plant called autotetraploid and, as others of the same class, is important for genetic improvement as it produces hybrids with a triploid chromosome complement which gives seedless fruits. It is not used for marketing purposes as its fruits are unrefined and characterized by unusual dimensions, irregular form and thickness of the peel. Moreover, the flavedo oleiferous glands that contain the essential oil are prominent and large, although not numerous (6).

The lemon hybrids recently obtained have potential commercial value because they have shown interesting agrobiological characteristics, particularly a good resistance to the disease mal secco. Although the programs of genetic improvement of lemon in Sicily are mainly aimed at the fresh fruit market, the economic relevance of the essential oil should not be disregarded; cold-pressed Sicilian lemon oil is highly valued and is able to find an outlet on the international market owing to the superior quality of its odor. Lemon oil is used in a wide variety of applications, mainly in the food and confectionery industries as a flavoring for liqueurs, teas, toffees, candies, ice creams, and soft drinks. The composition of lemon oil has been widely studied (7–10); moreover, some papers on lemon hybrid oils have been published (11–17).

Since the quality of products, such as juice and essential oil, is fundamental to promote the citrus industrial processing, the aim of this study is the characterization of the volatile fraction of these new hybrid oils; the oil composition of the parents, for which the information reported in the literature is very scant (18–21), has also been studied. The data obtained were statistically analyzed by analysis of variance (ANOVA) and multivariate analysis to obtain information on the similarities and differences between the oils examined.

MATERIALS AND METHODS

Plant Material and Hybridization. The hybrids have been obtained by manual pollination between *C. meyerii* Y. Tan. (Meyer lemon) (hybrid 1) and *C. medica* L. cv. Diamante (Diamante citron) (hybrid 2) as female parents and the tetraploid *C. limon* Burm cv. Cavone as the male parent.

Immature embryos, which were incompletely developed and therefore unsuitable for natural germination, were taken out of the seeds of four month old fruits (22). These embryos were grown on standard MS substrate, agar-solidified medium (23) and the seedlings planted in peat mould and then kept at 27 °C in an environment at saturated humidity. After one month the young seedlings were transferred into a greenhouse, and their growth was observed for two years. When the young plants were well established, some scions were cut from the apical part of the plants and grafted upon mature bitter orange in the experimental field of the Istituto Sperimentale per l'Agrumicoltura (Reggio Calabria, Calabria, Italy). After three years, the fruits obtained were found to resemble ripe lemons with an oval shape and a smooth peel.

For each hybrid and parent three different plants were selected; for each plant, 12 fruits were collected following the cardinal points and 4 ripe, regular, sound fruits were selected for each essential oil.

Extraction of the essential oil was carried out in the laboratory by applying manual pressure on the rind to cause the breaking of the utricles and the release of the oil itself, which was collected on a watch glass, transferred to a test tube, centrifuged, and analyzed. The volatile fraction of the oils was studied by HRGC–FID and HRGC–MS; the data obtained were statistically analyzed.

HRGC–MS Analysis. For the identification of volatile components, each sample was analyzed with a Shimadzu QP 5000 equipped with Adams' library (24) on two different columns: (1) fused silica capillary column, 30 m × 0.25 mm i.d., coated with Mega 5 MS, 0.25 μm film thickness (Mega, Legnano (MI), Italy); column temperature, 40 °C (2 min) to 240 °C at 3.0 °C/min; carrier gas, He, 90 kPa; linear gas velocity, 42.7 cm/s at 40 °C; (2) fused silica capillary column, 30 m × 0.25 mm, coated with Megawax, 0.25 μm film thickness (Mega, Legnano (MI) Italy); column temperature, 40 °C (6 min) to 220 °C (10 min) at 2.0 °C/min; carrier gas, He, 90 kPa; linear gas velocity, 42.8 cm/s at 40 °C. For following conditions were used for both columns: injector temperature, 250 °C; injection mode, split; split ratio, 1:100; volume injected, 1 μL of a solution 1/20 in pentane of the oil. The following MS scan conditions were used: transfer line temperature, 250 °C; interface temperature, 250 °C; source temperature, 200 °C; ionization technique, electronic impact (EI) at 70 eV; acquisition range, 30–300 m/z; scan rate, 1 amu/s. The compound identification was based on comparison of their linear retention indices (LRIs), on apolar and polar columns, with those of authentic compounds, computer matching with mass spectral libraries, and comparison with spectra of authentic samples or literature data. Linear retention indices of the sample components were determined on the basis of homologue *n*-alkane hydrocarbons analyzed under the same GC conditions.

HRGC–FID Analysis. For quantitative results of the volatile fraction, each sample was analyzed by HRGC on a Fisons Mega Series 5160 gas chromatograph equipped with a Shimadzu data processor C-R3A [fused silica capillary column, 30 m × 0.32 mm i.d. coated with SE-52, 0.40–0.45 μm film thickness (Mega, Legnano (MI), Italy); column temperature, 45 °C (6 min) to 200 °C at 3 °C/min; injection mode, split; split ratio, 1:100; detector, FID; injector and detector temperature, 250 °C; carrier gas, He, 95 kPa; injected volume, 1 μL of neat oil. The quantitative composition was obtained by peak area normalization, the response factor for each component was considered equal to 1, and three replicates of each sample were made.

Statistical Analysis. Statgraphic plus software, 5.1 version, was used to perform statistical analysis of the data. One-way ANOVA and Duncan's multiple range test were applied to the data to determine significant differences between the analyzed oils; the model was statistically significant with a *P* value of less than 0.05. Multivariate analysis using principal components and discriminant analysis were performed to verify the contribution of the parent to the hybrid oils.

RESULTS AND DISCUSSION

Sixty-three volatile components have been identified and quantified in the two new hybrids and their parent essential oils, as listed in **Table 1**; the components identified, which represented about 99.9% of the whole volatile fraction of each oil analyzed, are reported in the table together with their LRIs calculated on Mega 5 MS and Megawax columns.

Characteristics of the Female Parent Oils. *C. meyerii* Y. Tan. Fifty-nine components were identified, and the following for the first time: octanal, δ-3-carene, *cis*-sabinene hydrate, camphor, dihydrocarveol, carvone, methyl geranate, valencene, bicyclogermacrene. Most of the components identified are typical of the lemon oils, but otherwise the Meyer volatile composition showed peculiar characteristics which determine the unusual odor for cold-pressed lemon oil. In fact, comparing the composition of Meyer with that of genuine cold-pressed Italian lemon oil (7), it had a higher content of alcohols, especially due to the presence of dihydrocarveol and thymol, and a lower content of esters and carbonyl compounds. The high amount of thymol ($\bar{X} = 1.77\%$), a typical compound of

Table 1. Volatile Compounds^a in Essential Oils from Lemon Hybrids and Their Parents^b

compound ^c	IRL		hybrid 1	hybrid 2	Meyer lemon	Cavone lemon	Diamante citron
	Mega 5 MS	Megawax					
tricyclene	913	998	– ^d	<0.01 a	–	<0.01 a	–
α-thujene	920	1012	0.47 a	0.61 ab	0.42 a	0.43 a	0.97 b
α-pinene	925	1008	1.77 a	1.96 a	1.33 a	2.19 a	2.25 a
camphene	936	1043	0.03 ab	0.04 b	0.01 a	0.07 c	0.01 a
sabinene	964	1105	1.10 bc	1.21 c	0.18 a	2.59 d	0.35 ab
β-pinene	964	1085	6.25 c	16.90 d	1.04 a	14.68 d	2.01 b
myrcene	987	1152	1.88 a	1.74 a	2.09 a	1.62 a	1.45 a
octanal	997	1270	0.05 a	0.05 a	0.05 a	0.04 a	0.06 a
α-phellandrene	997	1146	0.05 b	0.09 c	–	0.06 bc	0.01 a
δ-3-carene	1002	1128	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
α-terpinene	1009	1160	0.26 a	0.35 ab	0.19 a	0.2 a	0.53 b
p-cymene	1016	–	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
limonene	1021	1181	71.12 bc	62.84 ab	79.7 c	62.8 ab	51.95 a
(Z)-β-ocimene	1036	1224	0.03 a	0.28 a	0.04 a	0.03 a	1.60 b
(E)-β-ocimene	1045	1239	0.08 a	0.42 a	0.14 a	0.07 a	2.21 b
γ-terpinene	1051	1225	11.85 ab	16.00 b	8.56 a	8.93 a	27.71 c
cis-sabinene hydrate	1058	1447	0.06 ab	0.04 a	0.03 a	0.08 b	0.05 ab
terpinolene	1079	1260	0.57 ab	0.69 ab	0.84 bc	0.38 a	1.07 c
trans-sabinene hydrate	1088	1526	0.07 a	0.07 a	0.05 a	0.06 a	0.07 a
linalool	1094	1537	0.16 a	0.11 a	0.36 b	0.14 a	0.09 a
nonanal	1098	1372	0.08 ab	0.13 b	0.01 a	0.12 b	0.08 ab
cis-limonene oxide	1122	1403	–	–	<0.01 a	<0.01 a	–
trans-limonene oxide	1127	1423	–	–	–	<0.01 a	0.01 a
camphor	1127	1467	0.02 a	0.01 a	0.11 b	0.01 a	–
citronellal	1146	1457	0.56 b	0.18 a	0.36 ab	0.14 a	0.24 a
borneol	1152	1674	0.01 a	–	<0.01 a	0.01 a	0.01 a
terpinen-4-ol	1165	1575	0.03 ab	0.04 ab	0.03 ab	0.02 a	0.05 b
α-terpineol	1179	1670	0.23 a	0.24 a	0.20 a	0.23 a	0.21 a
dihydrocarveol	1182	1702	0.06 a	–	0.48 b	–	–
decanal	1199	1476	0.03 ab	0.05 b	<0.01 a	0.04 ab	0.04 ab
octyl acetate	1210	1461	0.01 a	–	–	–	–
nerol	1222	1830	0.07 ab	0.1 b	0.03 a	0.09 b	0.07 ab
carvone	1229	1684	0.03 a	–	0.08 b	–	–
neral	1231	1645	0.30 a	1.3 b c	0.05 a	1.03 b	1.7 c
piperitone	1239	1678	–	–	<0.01 a	<0.01 a	–
geraniol	1251	1783	0.01 a	0.03 a	<0.01 a	0.04 a	0.02 a
perillaldehyde	1257	1729	–	–	–	–	–
geranial	1262	1697	0.49 a	2.14 b c	0.10 a	1.69 b	2.79 c
thymol	1289	2164	0.49 a	–	1.77 b	–	–
undecanal	1300	1580	0.02 a	0.02 a	0.01 a	0.02 a	0.07 b
nonyl acetate	1308	1361	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
methyl geranate	1316	–	–	<0.01 a	0.03 b	–	<0.01 a
citronellyl acetate	1349	1349	0.04 b	0.04 b	0.01 a	0.03 ab	0.04 b
neryl acetate	1360	1705	0.11 a	0.57 b c	0.03 a	0.64 c	0.39 b
geranyl acetate	1379	1738	0.05 a	0.23 b c	0.22 b c	0.2 b	0.36 c
dodecanal	1402	–	0.01 a	<0.01 a	<0.01 a	0.01 a	<0.01 a
decyl acetate	1404	1662	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
β-caryophyllene	1404	1555	0.16 bc	0.23 c	0.08 a	0.15bc	0.11 b
trans-α-bergamotene	1427	1560	0.25 ab	0.42 b	0.13 a	0.27 ab	0.34 b
α-humulene	1437	1624	0.01 a	0.01 a	0.01 a	<0.01 a	0.01 a
cis-β-santalene ^e	1449	1617	–	–	–	–	–
(E)-β-farnesene	1452	1649	0.2 b	0.06 a	0.23 b	0.04 a	0.02 a
germacrene D	1466	1664	0.22 b	0.02 a	0.24 b	0.01 a	0.02 a
valencene	1479	1676	0.01 a	<0.01 a	0.02 ab	0.04 b	<0.01 a
bicyclogermacrene	1482	1688	0.04 a	<0.01 a	0.18 b	<0.01 a	<0.01 a
(Z)-α-bisabolene	1495	1702	0.06 b	<0.01 a	<0.01 a	0.03 ab	<0.01 a
β-bisabolene	1500	1702	0.35 ab	0.6 b	0.17 a	0.4 ab	0.48 ab
γ-elemene	1532	1607	0.01 a	<0.01 a	0.01 a	0.01 a	0.06 a
tetradecanal	1605	2110	<0.01 a	0.01 a	<0.01 a	0.01 a	<0.01 a
2,3-dimethyl-3-(4-methyl-3-pentenyl)-2-norbomanol ^e	1638	–	0.01 ab	0.02 b	<0.01 a	0.01 ab	0.01 ab
campherenol	1654	2245	0.01 ab	0.03 c	<0.01 a	0.01 ab	0.02 bc
α-bisabolol	1657	2186	0.01 a	0.03 b	0.01 a	0.02 ab	0.02 ab
nootkatone	1780	2432	0.01 a	<0.01 a	<0.01 a	<0.01 a	0.01 a

^a Average quantitative composition by peak area normalization; the response factor for each component was considered equal to 1. For each citrus oil, five samples and three replicates for each sample were used. ^b Different letters in the same row represent significant differences at $P = 0.05$ by Duncan's multiple range test. ^c Listed in increasing retention index on an apolar capillary GC column. Component identification by mass spectra, linear retention indices (LRIs), and standard injection. The LRIs were calculated according to the Van der Dool and Kratz equation (29). ^d Dash = not detected. ^e Tentative identification.

mandarin oils (7) but never reported in lemon oils, and the low content of neral and geranial agree with what was observed by Moshonas et al. and Lota et al. and give the oil a strong distinctive thymol-like aroma (18, 19).

C. medica L. cv. Diamante. Fifty-five components were identified in the oils analyzed. The oils were found to be rich in monoterpene hydrocarbons. The main component was limonene ($\bar{X} = 51.95\%$), followed by γ -terpinene ($\bar{X} = 27.71\%$). According to Cotroneo et al. (21) and Poiana et al. (20), the high content of γ -terpinene and α -terpinene characterizes this citron cultivar. The sesquiterpene fraction was less represented; the main components were β -bisabolene ($\bar{X} = 0.48\%$) and *trans*- α -bergamotene ($\bar{X} = 0.34\%$). Among oxygenated compounds, carbonyl compounds showed the highest amount ($\bar{X} = 4.99\%$), with neral and geranial as the main components; their average content is similar to that of genuine cold-pressed lemon oils. Alcohols and esters are less represented; their amount was less than 1%.

Characteristics of the Male Parent Oil. *Citrus limon* Burm cv. Cavone. Its qualitative and quantitative composition agreed with that of industrial genuine oils previously analyzed (7–10). On the whole, this oil could be considered a valuable product due to the content of citral; with the term "citral" we mainly mean neral and geranial, substances that determine the lemon oil flavor peculiarity to the extent that the market value of the oil is defined according to its citral content.

Hybrid Oil Composition. Fifty-eight and fifty-five components were identified in hybrid oils 1 and 2, respectively. Comparison of the average values in **Table 1** for volatile compounds found in hybrids 1 and 2 to those found in the parent fruit showed most of the components quantified in these hybrids to be present at levels intermediate between those of the two parents. The examination of the quantitative information on individual constituents such as summarized in the table was not enough to evaluate the similarities and differences between the oils considered. The statistical elaboration of the data obtained was thus performed using all the samples analyzed. Statistically significant differences were found between the average content of some components in hybrid and parent oils by ANOVA and Duncan's multiple range test (**Table 1**). Examples are α -thujene, α -pinene, sabinene, β -pinene, α -terpinene, limonene, γ -terpinene, terpinolene, linalool, citronellal, nerol, neral, geranial, neryl acetate, geranyl acetate, β -caryophyllene, *trans*- α -bergamotene, and β -bisabolene. These components constituted more than 92% of the volatile fraction of all samples analyzed; among these, the main components of the lemon oils (limonene, β -pinene, and γ -terpinene), together with the substances which characterize its flavor, neral and geranial, are present.

A multivariate pattern recognition approach should be more effective in recognizing differences among the samples analyzed (25). Multivariate analysis techniques have been recently used to detect patterns of differences among a group of citrus juices and peel and leaf oils (19, 26, 27). Discriminant analysis (28) was performed using the volatile compounds which showed significant statistical differences by ANOVA and Duncan's multiple range test. The results are illustrated in **Figure 1**; the figure shows the two hybrid oil samples differ from each other and from their parents. Functions 1 (68.17%) and 2 (21.78%) represented 90% of the total variance.

The regression analysis was performed for selecting those components most important in differentiating samples; significant compounds resulted: neryl acetate, β -caryophyllene, geranyl acetate, limonene, α -terpinene, β -pinene, and nerol. Forward stepwise discriminant analysis (seven steps) afforded the separa-

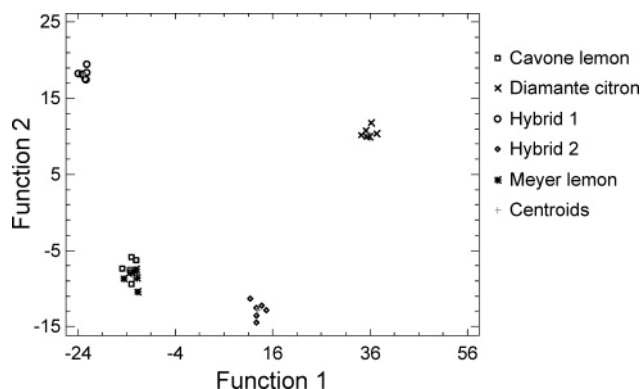


Figure 1. Discriminant analysis involving the significant constituents of the oils analyzed by the ANOVA test.

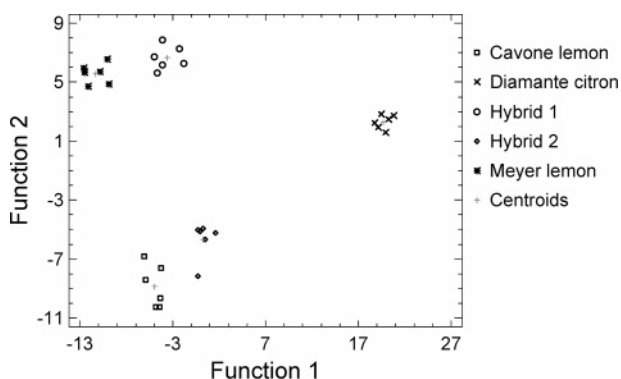


Figure 2. Forward stepwise discriminant analysis (seven steps) of the oils analyzed.

Table 2. Principal Components Loading Autoscaled Data

compound	loading 1	compound	loading 2	compound	loading 3
neryl acetate	0.462506	β -pinene	-0.546024	β -caryophyllene	0.673526
limonene	-0.450731	α -terpinene	0.438376	geranyl acetate	-0.526396
nerol	0.441667	β -caryophyllene	0.428537	β -pinene	0.431343
geranyl acetate	0.422207	neryl acetate	-0.370629	limonene	-0.247483
α -terpinene	0.387757	nerol	-0.330903	nerol	-0.138556
β -pinene	0.176103	geranyl acetate	0.221519	α -terpinene	-0.0521989
β -caryophyllene	0.168026	limonene	-0.173513	neryl acetate	0.00649852

tion shown in **Figure 2**. This analysis confirmed that the two hybrid oils were different from each other and from Diamante citron oils; moreover, hybrid 1 samples were clustered in a small area with Meyer lemon oils, whereas hybrid 2 samples were clustered with Cavone lemon oils.

Principal component analysis (PCA) was thus performed to calculate the total variance contained in the seven constituents considered for all the samples analyzed. Principal components 1 (PC1), 2 (PC2), and 3 (PC3) represented 90% of the total variance, 44.0% of the total variance for PC1, 34.0% for PC2, and 12.4% for PC3. **Table 2** reports the significant component loadings in decreasing order of importance for PC1–3. As listed in **Table 2** the constituents that contribute most to PC1 were limonene, neryl acetate, and nerol, to PC2, sabinene, β -pinene, α -terpinene, and β -caryophyllene, and to PC3, β -caryophyllene, geranyl acetate, sabinene, and β -pinene. A graphic display of loadings for PC1 and PC2 is shown in **Figure 3**. The figure shows the hybrid 1 oil grouped with Meyer oils because of limonene and hybrid 2 oils with Cavone lemon oils because of nerol, neryl acetate, and β -pinene; moreover, α -terpinene, geranyl acetate, and β -caryophyllene distinguish Diamante citron

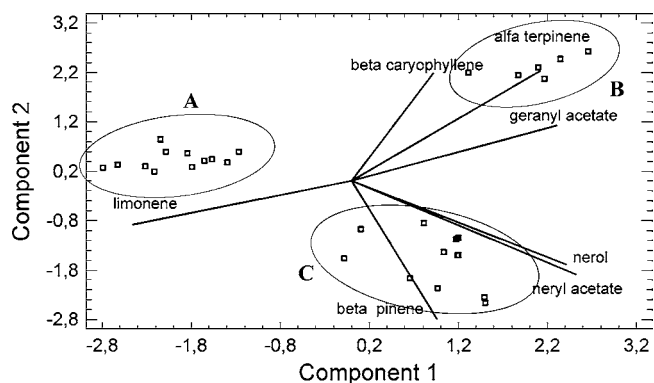


Figure 3. Principal component (PC) analysis (PC1 and PC2) involving quantities of seven constituents in the samples analyzed: A = Meyer lemon and hybrid 1; B = Diamante citron; C = Cavone lemon and hybrid 2.

oils from the other analyzed oils. On the whole, multivariate analysis techniques aided in the interpretation of chemical data obtained for volatile components of citrus hybrids and their parents. Differences among individual components provided less useful information, mainly because there is often a wide variation in the volatile fraction composition from different samples of the same oils (7); since multivariate analysis involves variability of several or all of the components, it seems less affected by such variation.

In this research the two new hybrid oils and their parents have been characterized; the results statistically evaluated have demonstrated that the two hybrid oil compositions were different from those of the parents even though some components allowed us to correlate the hybrids with at least one of the parents. These hybrids have potential commercial value because both could be an acceptable alternative to the valuable lemon oil. This was expected, since the flavor of hybrid 2 oil was comparable to that of lemon, and that of hybrid 1 oil reminiscent of lemon but altogether lighter and it could be used when mild flavoring properties are desired.

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