Composition of Egyptian Nerolì Oil

**Ivana Bonaccorsi[a]*, Danilo Sciarrone[a], Luisa Schipilliti[a], Alessandra Trozzi[b],
Hussein A. Fakhry[c] and Giovanni Dugo[a]**

[a]*Dipartimento Farmaco-chimico, Università di Messina, V.le Annunziata, 98168 Messina, Italy*
[b]*Dipartimento Farmaco-biologico, Università di Messina, V.le Annunziata, 98168 Messina, Italy*
[c]*A. Fakhry & Co. 1081 Cornich El-Nil Cairo 11451, Egypt*

bonaccor@pharma.unime.it

The bitter orange flower oil (or nerolì) is an essential product, largely used in perfumery. Nerolì is obtained by hydrodistillation or steam distillation, from the flowers of bitter orange (*Citrus aurantium* L.). Since a long time nerolì production is limited and its cost on the market is considerably high. The annual production in Tunisia and Morocco is ca. 1500 Kg, representing more than 90% of the worldwide production. A small amount of nerolì is also produced in Egypt, Spain and Comorros (not exceeding 150 kg totally). Due to the high cost, the producers and the users have tried to obtain less expensive products, with odor characters close to that of nerolì oil to be used as substitute and sometimes as adulterants of the genuine oil. In this study are investigated five samples of Egyptian nerolì oils produced in 2008 and 2009, in the same industrial plant, declared genuine by the producer. For all the samples the composition was determined by GC/FID and by GC/MS-LRI; the samples were also analyzed by esGC to determine the enantiomeric distribution of twelve volatiles and by GC-C-IRMS for the determination of the $\delta^{13}C_{VPDB}$ values of some mono and sesquiterpene hydrocarbons, alcohols and esters. The analytical procedures allowed to quantitatively determining 86 components. In particular the variation of the composition seems to be dependent on the period of production. In fact, the amount of linalool decreases from March to April while linalyl acetate presents an opposite trend, increasing in the same period. The RSD determined for the $\delta^{13}C_{VPDB}$ are very small (max. 3.89%), ensuring the authenticity of all samples. The results are also discussed in function of the limits provided by the European Pharmacopoeia (EP) (2004), AFNOR (1995) and ISO (2002) regulations for genuine nerolì oils.

Keywords: Nerolì oil, *Citrus aurantium* L., GC, GC/MS-LRI, GC-C-IRMS, es-GC.

The oil of nerolì is obtained by hydrodistillation or by steam distillation of the flowers of bitter orange (*C. aurantium* L.).

Nerolì, rose and jasmine are often cited as "the three pearls of perfumery". Nerolì is the classic ingredient of the most famous and prestigious perfumes and eau de cologne. It is also used as flavor ingredient in food and beverages. In traditional Chinese medicine the extracts from bitter orange flowers are used to treat digestive problems and insomnia.

Nerolì is the product of a laborious work: the flowers, which bloom between the end of April and the beginning of June are collected manually during the first hours of the day; one worker can collect about 20 Kg per day for a period of 20 days; the flowers are hydro- or steam-distilled with a yield ranging from 0.08% at the beginning of the season to a maximum of 0.13% under the most favorable conditions. The production in the European Mediterranean Countries (mainly France) is subject to a strong decrease, mainly for the specialized working cost necessary to collect the flowers, and for the contraction of the cultivated fields of bitter orange.

The annual world production of nerolì is today less than 2000 Kg; most of it is concentrated in Morocco and in Tunisia. Small amounts of nerolì, about 150 Kg/year, are produced in Egypt, Spain, and Comorros. The market price of nerolì is considerably high and the organic product can be sold at more than 4,500 USD/Kg. It is therefore predictable that this oil can be subject to adulteration by the addition of less valuable natural products, such as the oils obtained from flowers of citrus different from *C. aurantium*, or by addition of leaf oils or of synthetic compounds. The adulteration of nerolì oil is not easily identifiable, mainly because the reference data available in literature, relative to oils produced industrially and extracted in laboratory [1-3], ranges widely and is also probably affected by the geographic origin of the trees. Based on the rules AFNOR (Association Francaise de Normalization) [4a], ISO (International Organization for Standardization) [4b] and EP (European Pharmacopeia) [5], some of the results available in literature should not indicate genuine samples. Very few results are available in literature on the enantiomeric distribution of volatiles determined in nerolì oils [6-10]. In the authors knowledge the IRMS analysis was never performed before on nerolì.

Usually essential oils quality assessment is obtained by traditional chromatographic techniques (GC-FID, GC-MS, Es-GC, HPLC) as recently reported by our research group [11], recognized for their validity in the quality control field. Advanced chromatographic techniques have been also exploited for different essential oils as fast GC/MS [12] and multidimensional GC-GC for quantitative [13] and enantiomeric ratio assessment [14,15]. Recently has gained importance the Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) that, determining small differences in the isotopic carbon composition of the matrices, can be exploited to discriminate between products of different origin [10,16-18a]. In this regards GC-C-IRMS can be an useful tool in the flavour and fragrance authenticity control, unveiling illicit essential oils production methods, such as the oils adulteration by the addition of synthetic or natural compounds, different from the genuine ones [18b,19,20].

To our knowledge nerolì oil was never investigated by IRMS. It is therefore particularly interesting to provide information useful for the genuineness assessment of nerolì, also in function of the geographic origin.

The present article reports the results relative to the composition of five samples of Egyptian nerolì produced in 2008 and 2009, to the enantiomeric distribution and the isotopic ratio of selected components.

The samples analyzed are described below:

Sample	Description
1	hydrodistilled from Egypt (2008)
2	steam-distilled March 23th 2009
3	steam-distilled March 28th 2009
4	steam-distilled April 7th 2009
5	steam-distilled April 9-11th 2009

Table 1 reports the composition determined by GC-FID, of the samples analyzed. To facilitate comparison with information already available in literature the results are here reported as raw peak area %. The correction factors (C.F.) for each class of substances determined by GC-FID are however reported in Table 1 to provide complete information to the reader. In the case of distilled oils, as nerolì, the volatile fraction should represent the whole oil. The quantitative results obtained from triplicates show CV% values always below 5%. The 86 components identified by GC/MS with the use of LRI as filters interactively applied during the mass spectral identification process [21] represent about 99% of the whole oils. In comparison with literature information this study led to the identification of numerous components (indicated by * in Table 1), while the presence of numerous minor components previously reported were not here confirmed.

Hydrocarbons range between 20-25%, oxygenated compounds vary between 73-78%; among these alcohols range from 58 to 70% and esters from 7 to 19%, while aldehydes are present at small amounts (0.16-0.26%). The main alcohol is linalool (44-53%), followed by α-terpineol

(5-6%) and by geraniol (3-4%). The sesquiterpene alcohols (*E*)-nerolidol and (*E,E*)-2,6-farnesol are also well represented ranging together between 2-5%. The main ester is linalyl acetate (2-15%) followed by geranyl acetate (about 3%) and neryl acetate (about 1.5%). The most abundant monoterpene hydrocarbon is limonene (8-12%) followed by (*E*)-β-ocimene (3-5%), and by β-pinene (2-4%); the main sesquiterpene hydrocarbon is β-caryophyllene (0.6-0.9%).

The sample produced in 2008 by hydrodistillation, compared to all the other oils obtained by steam distillation, has the highest amount of linalool and of total alcohols, and the lowest amount of linalyl acetate and of total esters.

In the samples produced in 2009 the composition varies gradually but significantly during the productive season. The total monoterpene hydrocarbons and the single components of this class of compounds, the total monoterpene alcohols and the single components of this class of compounds as well as the ratio linalool/linalyl acetate decrease during the season. Total esters and linalyl acetate present an opposite behavior, as well as the sesquiterpene hydrocarbons and aldehydes. Neryl and geranyl acetate remain constant during the whole season.

In Figure 1 are graphically described the seasonal variation of class of substances and some single components. The results confirm, as reported in literature, that the main components of nerolì oil are linalool, linalyl acetate and limonene. The amount of these components determined in this study fall in the ranges hitherto determined for nerolì oil. It should be however mentioned that in one Egyptian oil [1a] it was determined the 30% of linalool content and 1% of linalyl acetate; the highest value (74%) of linalool was reported for a Chinese oil [1a] which presented a very unusual low amount of limonene (1%); in some Spanish oils [3] were reported very low values of linalyl acetate (0.6%).

The results determined in the present study also confirm the presence of some *Key* compounds such as methyl anthranilate, methyl N-methyl anthranilate, phenyl ethyl alcohol, (*E*)-nerolidol, and some newly identified components such as the (*E,Z*)- and (*E,E*)-2,6-farnesals, useful for the characterization of this product.

Table 2 reports the enantiomeric distribution of some components determined by es-GC. The values are determined from triplicates with CV% never exceeding 5.5% with the exception of that relative to the (-)-α-thujene isomer which is 8.9% due to the chromatographic behavior of this component. Figure 2 shows the chiral chromatogram of one of the samples analyzed.

The enantiomeric ratios of camphene, sabinene, α- and β-phellandrene and citronellal were determined in nerolì oils for the first time. The values of the enantiomeric

Table 1: Composition of the five samples analyzed (% of peak areas) determined by GC-MS-LRI and GC-FID.

	LRI[a]	LRI[b]	1	2	3	4	5
α-Thujene	925	927	0.01	0.02	0.02	0.02	0.02
α-Pinene	933	933	0.15	0.26	0.22	0.22	0.23
Camphene	950	953	0.01	0.01	0.01	0.01	0.01
Sabinene	973	972	0.85	1.56	1.15	1.44	1.33
β-Pinene	979	978	1.89	3.70	3.44	3.29	3.04
6-Methyl-5-hepten-2-one	984	978	0.02	0.02	0.02	0.02	0.02
Myrcene	989	991	1.43	1.74	1.61	1.44	1.33
cis-Dehydrolinalol oxide*	1006	1006	0.02	0.02	0.02	0.02	tr
α-Phellandrene	1007	1002	0.01	0.02	0.01	0.01	0.02
δ-3-Carene	1010	1009	0.09	0.06	0.05	0.06	0.03
(E)-2-Hexenyl acetate	1014	1017	-	0.01	0.01	0.01	tr
α-Terpinene	1018	1018	0.04	0.13	0.09	0.07	0.04
o-Cymene*	1020	1022	tr	tr	tr	tr	tr
p-Cymene	1025	1025	0.13	0.09	0.06	0.13	0.13
Limonene	1031	1030	11.89	10.14	10.10	9.18	7.87
(Z)-β-Ocimene	1035	1026	0.48	0.61	0.58	0.50	0.47
(E)-β-Ocimene	1046	1046	3.31	5.11	4.81	4.09	3.40
γ-Terpinene	1058	1049	0.12	0.28	0.19	0.17	0.11
cis-Linalool oxide	1071	1069	0.13	0.17	0.17	0.18	0.25
Terpinolene	1087	1086	0.26	0.43	0.42	0.36	0.27
Linalool	1107	1101	53.33	45.58	45.31	43.80	43.69
4,8-Dimethyl-1,3(E),7-nonatriene* + Phenylethyl alcohol*	1114	1113	0.03	0.04	0.04	0.05	0.03
Fenchol°	1122	1123	tr	-	-	-	tr
trans-p-Menth-2,8-dienol	1124	1122	tr	-	-	-	tr
cis-p-Menth-2-en-1-ol	1127	1124	0.03	0.03	tr	0.02	0.02
trans-Limonene oxide*	1135	1142	0.01	0.09	0.09	0.10	0.02
trans-p-Menth-2-enol*	1144	1141	0.02	0.02	0.01	0.02	0.03
Terpinen-4-ol	1182	1177	0.44	0.79	0.52	0.57	0.59
p-Cymen-8-ol*	1189	1189	0.02	0.01	0.01	0.01	0.03
(Z)-3-Hexenyl butanoate*	1190	1184	0.01	tr	tr	0.01	0.02
α-Terpineol	1199	1195	6.22	6.17	5.90	5.50	4.89
trans-Piperitol	1211	1209	0.01	0.02	0.01	0.01	0.02
Nerol	1226	1229	1.28	1.12	1.06	1.01	0.95
cis-Carveol*	1234	1232	tr	-	-	-	-
Neral	1239	1238	0.03	tr	0.03	0.03	0.03
Carvone	1246	1246	0.01	0.02	0.02	0.03	0.01
Linalyl acetate	1250	1243	2.19	8.77	10.97	12.97	14.57
Geraniol	1253	1255	3.83	3.42	3.30	3.06	2.94
trans-Myrtanol*	1257	1261	tr	0.01	0.02	0.02	0.01
Geranial	1269	1264	0.07	0.05	0.05	0.07	0.07
Bornyl acetate*	1286	1287	tr	0.01	0.01	0.01	0.01
Geranyl formate*	1298	1298	0.03	0.01	0.02	tr	0.04
Methyl geranoate*	1321	1320	0.01	tr	tr	tr	0.02
Linalyl propanoate*	1331	1333	tr	-	-	-	0.02
Bicycloelemene*	1334	1338	tr	0.01	0.01	0.01	tr
δ-Elemene	1337	1335	0.03	0.05	0.06	0.06	0.06
Methyl anthranilate	1342	1337	0.04	0.10	0.12	0.09	0.06
α-Terpinyl acetate	1348	1349	0.05	0.06	0.07	0.06	0.07
Citronellyl acetate	1349	1353	0.02	-	-	-	0.02
Neryl acetate	1359	1361	1.45	1.45	1.43	1.44	1.45
Geranyl acetate	1379	1380	3.08	3.06	3.02	3.01	3.08
β-Elemene	1392	1391	0.09	0.11	0.09	0.12	0.18
(E)-Jasmone*	1395	1390	0.02	0.01	0.01	0.02	0.02
n-Tetradecane*	1400	1400	tr	0.01	tr	0.01	0.01
cis-α-Bergamotene*	1404	1411	0.01	tr	tr	0.01	
Methyl N-methyl anthranilate	1409	1405	0.04	0.02	0.02	0.02	0.01
β-Caryophyllene	1424	1424	0.56	0.60	0.68	0.82	0.94
Perillyl acetate*	1435	1435	-	tr	tr	tr	0.01
Aromadendrene	1443	1439	0.01	tr	tr	tr	0.01
Geranyl acetone*	1448	1453	0.04	0.03	0.03	0.04	0.04
(E)-β-Farnesene	1453	1452	0.09	0.13	0.13	0.18	0.22
α-Humulene	1460	1452	0.06	0.06	0.06	0.08	0.11
9-epi-β-Caryophyllene*	1464	1464	0.01	tr	tr	0.01	0.01
Geranyl propanoate*	1469	1471	tr	tr	tr	0.01	0.01
Germacrene D	1485	1479	0.03	0.07	0.07	0.08	0.08
Bicyclogermacrene*	1500	1497	0.10	0.14	0.16	0.14	0.05
α-Muurolene*	1502	1497	0.01	0.02	0.01	0.02	0.01
(E,E)-α-Farnesene	1505	1504	0.05	0.02	0.01	0.02	0.03
γ-Cadinene	1517	1513	tr	tr	tr	tr	0.01
δ-Cadinene	1522	1518	0.03	0.02	0.03	0.03	0.04
β-Sesquiphellandrene*	1526	1523	0.01	0.01	0.01	0.01	0.01
(E)-Nerolidol	1563	1561	2.35	1.15	1.16	2.04	3.21
Spathulenol	1582	1576	0.05	0.03	0.03	0.05	0.05
Caryophyllene oxide°	1587	1587	0.04	0.02	0.02	0.04	0.04
Globulol	1591	1592	0.02	0.01	0.01	0.01	0.02
Cadin-4-en-10-ol*	1660	1659	0.02	0.02	0.02	0.01	0.01
n-Tetradecanol*	1677	1680	0.02	0.02	0.02	0.03	0.03
2,3-Dihydrofarnesol*	1688	1688	0.02	0.01	0.01	0.01	0.04
β-Sinensal*	1695	1699	0.09	0.09	0.09	0.08	0.08
(E,Z)-2,6-Farnesal*	1711	1714	0.02	0.01	0.01	0.02	0.03
(E,E)-2,6-Farnesol	1718	1716	2.03	1.17	1.29	1.59	1.66
(E,E)-2,6-Farnesal*	1739	1737	0.03	0.02	0.02	0.03	0.04
α-Sinensal*	1752	1749	0.02	0.02	0.02	0.02	0.01
Farnesyl acetate°	1833	1832	0.04	0.02	0.02	0.03	0.03
(E,E)-Geranyl linalol*	2024	2026	0.02	0.02	0.02	0.03	0.03
	C.F.		Sum of uncorrected % of peak areas				
HYDROCARBONS	**1.0**		**21.79**	**24.95**	**23.97**	**22.57**	**20.10**
Monoterpene			20.67	24.16	22.76	20.99	18.30
Sesquiterpene			1.09	0.74	1.17	1.52	1.77
Aliphatic			0.03	0.05	0.04	0.06	0.03
ALDEHYDES	**1.3**		**0.26**	**0.16**	**0.22**	**0.25**	**0.26**
Monoterpene			0.10	0.05	0.08	0.10	0.10
Sesquiterpene			0.16	0.14	0.14	0.15	0.16
KETONES	**1.3**		**0.09**	**0.08**	**0.08**	**0.11**	**0.09**
Monoterpene			0.05	0.05	0.05	0.07	0.05
Aliphatic			0.04	0.03	0.03	0.04	0.04
ALCOHOLS	**1.3**		**69.72**	**59.58**	**58.68**	**57.81**	**58.19**
Monoterpene			65.21	57.17	56.14	54.07	53.17
Sesquiterpene			4.49	2.39	2.52	3.71	4.99
Aliphatic			0.02	0.02	0.02	0.03	0.03
ESTERS	**1.6**		**6.88**	**13.39**	**15.55**	**17.55**	**19.35**
Monoterpene			6.83	13.36	15.52	17.50	19.30
Sesquiterpene			0.04	0.02	0.02	0.03	0.03
Aliphatic			0.01	0.01	0.01	0.02	0.02
OXIDES + ETHERS	**1.5**		**0.22**	**0.30**	**0.32**	**0.37**	**0.34**
OTHERS			0.10	0.14	0.16	0.14	0.10
ALL			**99.06**	**98.60**	**98.98**	**98.80**	**98.43**

Notes a: LRI measured on SLB-5MS column; b: Reference LRI reported in literature (*FFNSC 1.3GC-MS library*, Shimadzu, Japan; or Adams RP. *Identification of essential oil components by gas chromatography/mass spectrometry*, 4th Edn. Carol Stream, IL, USA: Allured Publishing Corp; 2007; or Hochmuth, D.H., Joulain, D., König, W.A., 2002. *MassFinder Software and Data Bank*, University of Hamburg); tr: ≤ 0.005; C.F. Correction Factor (FID response) for class of compounds; * identified, in the authors knowledge, for the first time in nerolì oils; ° correct isomer not identified.

Table 2: Enantiomeric distribution of some volatile components in the samples analyzed.

	1	2	3	4	5
S-(+)-α-Thujene	59.02	48.80	33.53	56.53	30.98
R-(-)-α-Thujene	40.98	51.20	66.47	43.47	69.02
R-(+)-α-Pinene	41.37	36.77	32.96	34.70	n.d.
S-(-)-α-Pinene	58.63	63.23	67.04	65.30	n.d.
1S,4R-(-)-Camphene	n.d.	n.d.	n.d.	n.d.	90.85
1R,4S-(+)-Camphene	n.d.	n.d.	n.d.	n.d.	9.15
R-(+)-β-Pinene	2.05	2.04	1.72	1.76	2.08
S-(-)-β-Pinene	97.95	97.96	98.28	98.24	97.92
R-(+)-Sabinene	80.89	81.40	76.72	81.93	81.14
S-(-)-Sabinene	19.11	18.60	23.28	18.07	18.86
R-(-)-α-Phellandrene	44.55	27.42	n.d.	14.37	32.01
S-(+)-α-Phellandrene	55.45	72.58	n.d.	85.63	67.99
R-(-)-β-Phellandrene	39.17	27.75	53.74	36.50	49.31
S-(+)-β-Phellandrene	60.83	72.25	46.26	63.50	50.69
S-(-)-Limonene	1.65	2.54	2.42	2.58	2.60
R-(+)-Limonene	98.35	97.46	97.58	97.42	97.40
S-(-)-Citronellal	n.d.	47.14	45.09	n.d.	n.d.
R-(+)-Citronellal	n.d.	52.86	54.91	n.d.	n.d.
R-(-)-Linalyl acetate	98.95	99.39	99.47	99.41	99.28
S-(+)-Linalyl acetate	1.05	0.61	0.53	0.59	0.72
R-(-)-Linalol	78.25	78.51	78.54	78.48	78.55
S-(+)-Linalol	21.75	21.49	21.46	21.52	21.45
S-(+)-Terpinen-4-ol	62.19	62.74	60.00	61.68	62.13
R-(-)-Terpinen-4-ol	37.81	37.26	40.00	38.32	37.87
S-(-)-α-Terpineol	28.77	28.98	28.88	29.21	28.99
R-(+)-α-Terpineol	71.23	71.02	71.12	70.84	70.79

n.d.: not determined.

distribution of limonene, linalol, terpinen-4-ol and α-terpineol here determined are in good agreement with literature results relative to genuine nerolì oils [6,7a]; however, if compared to literature, the enantiomeric excess of (-)-β-pinene is slightly lower, that of (-)-α-pinene
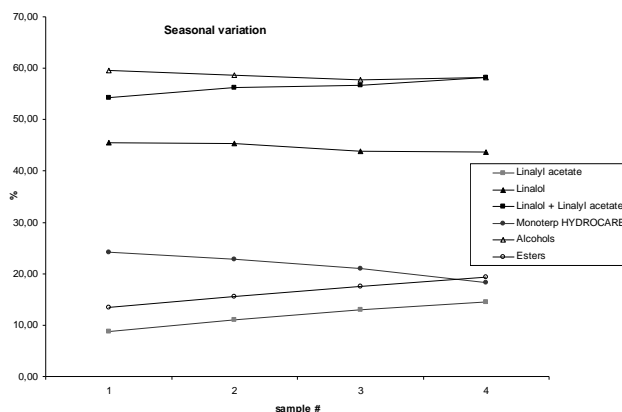
Table 3: (-)$\delta^{13}C_{VPDB}$ values calculated for the samples analyzed, average and relative standard deviation% (RSD).

	1	2	3	4	5	Ave	RSD
β-Pinene	25.24	25.75	25.16	25.24	25.48	**25.37**	**0.95**
Myrcene	24.77	26.26	25.05	24.25	24.30	**24.92**	**3.28**
Limonene	27.29	27.78	27.20	27.84	27.32	**27.48**	**1.09**
Linalol	25.64	25.85	25.80	26.08	26.06	**25.89**	**0.72**
Terpinen-4-ol	28.10	27.74	27.01	27.68	27.81	**27.67**	**1.46**
α-Terpineol	26.84	27.14	26.57	26.93	27.22	**26.94**	**0.96**
Nerol	27.05	27.39	26.27	26.89	28.19	**27.16**	**2.59**
Neryl acetate	28.14	27.86	27.48	28.42	28.52	**28.09**	**1.51**
Geranyl acetate	27.42	28.06	27.75	28.54	28.41	**28.03**	**1.65**
Caryophyllene*	25.74	25.15	23.18	25.04	24.61	**24.74**	**3.89**
(E)-Nerolidol	30.60	30.43	30.12	30.79	30.13	**30.41**	**0.96**
Farnesol**	30.18	29.3	29.64	29.58	29.79	**29.71**	**1.03**

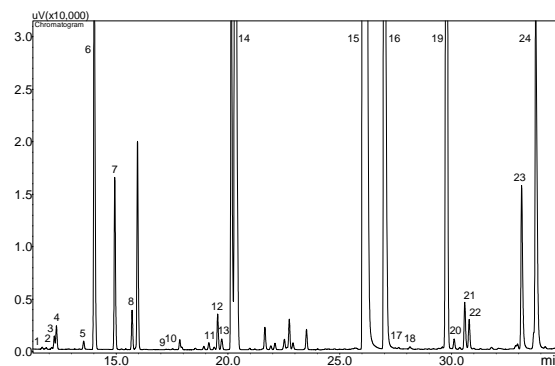Correct isomer identification: *β-; **(2E,6E)-

Table 4: Comparison between the limits reported in the regulations [4,5] and the results experimentally obtained for the five samples of Egyptian Nerolì (peak area %).

	AFNOR 1995	ISO 3517:2002	EP 2006	Range
Limonene	9-18	9-18	9.0-18.0%	7.87-11.89
Myrcene	1-4	1-4	-	1.33-1.74
(E)-β-Ocimene	3-8	3-8	-	3.31-5.11
α-Pinene	max. 2	tr-2	-	-
β-Pinene	7-17	7-17	7.0-17.0	1.89-3.70
Sabinene	-	tr-3%	-	0.85-1.44
Linalool	28-44	28-44	28.0-44.0	43.31-53.33
α-Terpineol	2-5.5	2-5.5	2.0-5.5	4.89-6.22
(E,E)-Farnesol	1-4	1-4	0.8-4.0	1.29-2.03
(E)-Nerolidol	1-5	1-5	1.0-5.0	1.15-3.21
Linalyl acetate	3-15	3-15	2.0-15.0	2.19-14.57
Geranyl acetate	1-5	1-5	1.0-5.0	3.0-3.08
Neryl acetate	max. 2.5	tr-2.5	max 2.5	1.43-1.45
Methyl anthranilate	-	-	0.1-1.0	0.04-0.12
Chiral purity				
(+)-Linalol	-	-	max 30	21.45-21.75
(+)-Linalyl acetate	-	-	max 5	0.53-1.05

Figure 1: Seasonal variation of class of components, of linalool and linalyl acetate in the four samples of nerolì produced during 2009.

Figure 2: Chiral chromatogram of one sample of neroli oil. Peak identification: 1. (+)-α-thujene; 2. (-)-α-thujene; 3. (+)-α-pinene; 4. (-)-α-pinene; 5. (+)-β-pinene; 6. (-)-β-pinene; 7. (+)-sabinene; 8. (-)-sabinene; 9. (-)-α-phellandrene; 10. (+)-α-phellandrene; 11. (-)-β-phellandrene; 12. (-)-limonene; 13. (+)-β-phellandrene; 14. (+)-limonene; 15. (-)-linalol; 16. (+)-linalol; 17. (-)-citronellal; 18. (+)-citronellal; 19. (-)-linalyl acetate; 20. (+)-linalyl acetate; 21. (+)-terpinen-4-ol; 22. (-)-terpinen-4-ol; 23. (-)-α-terpineol; 24. (+)-α-terpineol.
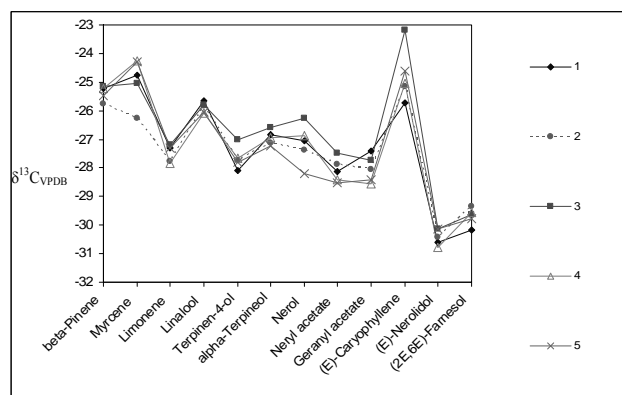
Figure 3: Diagram obtained for $\delta^{13}C_{VPDB}$ values for each components in function of the period of production. For sample description see table in text.

by plotting the $\delta^{13}C_{VPDB}$ values for each component analysed in function of the period of production. The relative standard deviation of the $\delta^{13}C_{VPDB}$ values range for the samples analyzed between 3.52 ((E)-β-caryophyllene) and 0.96 (α-terpineol). These low values indicate very narrow ranges of variation, therefore it is posible to assume that the $\delta^{13}C_{VPDB}$ can be considered characteristic of authenticity and of the geographic origin of the samples.

Table 4 provides a comparison of the ranges determined from the present results with the limits provided by the AFNOR, ISO and EP regulations [4,5]. Some of the results fall within these limits; others fall only slightly outside them; in three of the samples analyzed linalol is present at levels sensibly higher than the limits provided by the aformentioned regulations; β-pinene is always below the minima reported for nerolì oils. These behavior could be due to the geographic origin of the oils analyzed.

Considering the high commercial value of nerolì, its limited production in different geographic areas and the high possibility that this product can be subject to adulteration, it is necessary to fix quality parameters in

extremely lower and that of (-)-linalyl acetate is higher. With the exception of α-pinene, the results here obtained for the components analyzed are overall in good agreement with the enantiomeric purity previously determined by Mosandl [6] considered characteristic of genuine nerolì oils.

Table 3 reports the (-)$\delta^{13}C_{VPDB}$ values calculated for the nerolì oils samples. This study reports for the first time the GC-C-IRMS analysis of selected volatile components in nerolì oil. It is impossible to compare these values with literature information. Figure 3 shows the graph obtained

consideration of its variability among different geographic areas. To accomplish this, for a correct evaluation, not only the indices given by these regulations should be taken into account, but also the $\delta^{13}C_{VPDB}$ of selected compounds and the chiral purity of more compounds than the two already indicated, thus providing adequate tools for quality and genuineness assessment.

Experimental

Analysis of the essential oils: On the five samples described in text the following analytical investigations have been carried out: GC/FID, GC/MS of the volatile fraction; direct enantio-GC for the determination of the enantiomeric distribution of some volatiles. Each analysis was performed in triplicates. Results are expressed as average peak area %.

GC/FID: The volatile fraction was analyzed by HRGC/FID as described. Gas chromatograph: Shimadzu GC2010 equipped with a Flame Ionization Detector, a split/splitless injector and an AOC-20i series auto-injector. Capillary column: 30 m x 0.25 mm I.D. 0.25 μm d_f coated with SLB-5MS [silphenylene polymer, virtually equivalent in polarity to poly (5% diphenyl/95% methyl)siloxane)] (Supelco, Milan, Italy); column temperature, 50-250°C (10 min) at 3°C/min; injector temperature: 250°C; detector temperature: 280°C; carrier gas, He at 99.5 kPa (30.0 cm/s); injection mode: split; split ratio, 1:100; injected volume, 1.0 μL of diluted oil. Data handling was made by means of *GCsolution* software.

GC/MS Analysis: Samples were analyzed by GC/MS (EI) on a GCMS-QP2010 system equipped with commercially available libraries (see notes to Table 1) including the commercial version of the FFNSC ver. 1.3 (Shimadzu, Japan) database (created in the authors' laboratory) consisting of about 2000 reference standards and their relative linear retention indices determined on apolar column, interactively used as filters for the spectral interpretation. GC conditions: capillary column and temperature program as in GC/FID; carrier gas, He delivered at a constant pressure of 30.6 kPa (30.1 cm/s); 1.0 μL of solution (1/10, v/v, essential oil/hexane) injected on a split/splitless injector; injector temperature, 250°C; injection mode, split; split ratio, 1:50. MS scan conditions: source temperature, 200°C; interface temperature, 250°C; *E* energy 70eV; mass scan range, 40-400 amu. Data was handled through the use of *GCMSsolution* software.

Enantio-GC: Shimadzu GC2010 gas chromatograph equipped with a Flame Ionization Detector, a split/splitless injector and an AOC-20i series autoinjector. Capillary chiral column was a Megadex DETTBS-β (diethyl-*tert*-butil-silyl β-cyclodextrin) 25 m x 0.25 mm I.D. x 0.25μm d_f (Mega, Legnano, Italy). Temperature program: 50°-200°C at 2°C/min. Inlet pressure 96.6 kPa (220°C), split mode 1:20 (gas carrier He); injected volume, 1.0 μl; linear velocity, 30 cm/sec (constant). Data handling was made by means of *GCsolution* software

GC-C-IRMS device and analyses: Trace GC Ultra equipped with a TriPlus autosampler, retrofitted to the combustion interface GC/CIII and hyphenated to the isotope ratio mass spectrometer Delta V Advantage (all purchased from Thermo Fisher Scientific, Milan, Italy).
GC: column: SLB-5ms (silphenylene polymer) 30 m x 0.25 mm i.d., 0.25 μm d_f (Supelco, Milan, Italy.); temperature program: 50°C to 230°C at 3°C/min; split/splitless injector (250°C). Inlet pressure: 167 kPa; column flow: 2.0 ml/min (constant flow mode); carrier gas: He.
GC/C III: ox. reactor (Cu/Ni/Pt): 980°C; red. reactor: 640°C; He: 1 bar; O_2: 0.8 bar; CO_2: 0.5 bar.
IRMS: EI; electron voltage: 123.99 eV; electron current: 1.5 mA; 3 Faraday cup collectors at *m/z* 44, 45, and 46; peak center pre-delay and post-delay: 15 s, cup 3; reference: 60-80 s, 100-120 s, 140-160 s, 180-200 s; split: open; evaluation type: CO_2_SSH, ref. time: 155.90 s, $\delta^{13}C/^{12}C$ -60.300‰; integration time 0.2 s.

GC-C-IRMS instrument achieves highly precise measurement of carbon isotopic abundance, converting the eluted volatile components, in CO_2 and water into an oxidation chamber. After removing water, just behind the furnace, by a capillary-shaped phase separator, CO_2 reaches an ionization chamber where it will be transformed into three ion traces for the different isotopomers: $^{12}C^{16}O_2$, $^{13}C^{16}O_2$ and $^{12}C^{18}O^{16}O$, with their corresponding masses at *(m/z)* 44, 45, 46. The three ion beams are registered simultaneously by an Universal Faraday collector that detects the different contributions of ionic fragments obtained. Isotopic ratios, 45/44 and 46/44, are expressed in ‰ and are related to a certified standard (VPDB-standard) of known value [20]. Exploiting the GC-Combustion backflush, the most concentrated components were not introduced into the combustion chamber.

The samples dilutions and the GC-Combustion conditions were as follows: concentration 1:10 (*v/v*), 1 μL split injection, 1:100 split ratio, backflush: off, for the determination of $\delta^{13}C_{VPDB}$ of limonene and linalool.

Concentration 1:10 (*v/v*), 1 μL split injection, 1:50 split ratio, backflush open: 780-830 s and 970-1060 s, for the determination of $\delta^{13}C_{VPDB}$ of *β*-pinene, myrcene, terpinen-4-ol, *α*-terpineol, nerol, neryl acetate, geranyl acetate, (*E*)-caryophyllene, nerolidol, (2*E*,6*E*)-farnesol. Data are collected in triplicate, by using the Isodat 2.5 software (Thermo Fisher Scientific).

CO_2 reference gas cylinder calibration: The attained carbon isotope ratio of the unknown sample is compared to that of a calibrated CO_2 reference. The CO_2 reference gas was calibrated by injecting 1 μL of a carbon stable isotope ratio reference alkanes mixture comprising C_{16} to C_{30} (Indiana University, Bloomington, U.S.A.), calibrated against VPDB standard with a defined ^{13}C content. Isotope ratios were expressed as δ values (‰), versus a standard.

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

Tricosane (C$_{23}$) was arbitrarily chosen as reference alkane.

References

[1]   Peyron, L. (**2002**) Production of bitter orange nerolì and petitgrain oils. In *Citrus,* G. Dugo, and A. Di Giacomo (Eds.). Taylor & Francis, London and New York, 148-152; (b) Zhu L-F, Li Y-H, Li B-L, Lu B-Y, and Xia N-H. (**1993**) Aromatic plants and essential constituents. South China Institute of Botany, Chinese Academy of Sciences, Hai Feng Publish. Co. distributed by Peace Book Co. Ltd. Hong Kong. In Lawrence, B.M. (**2003**) Progress in Essential Oils. *Perfumer and Flavorist*, **28**, 56-58.

[2]   Boussaada O. (**1995**) Variation of essential oil yield and composition of *Citrus aurantium* var. amara. M. Sc. thesis Mediterranean Agronomic Institute, Chania, Greece. In Lawrence, B.M. (**2003**) Progress in essential oils. *Perfumer and Flav*orist, **28**, 56-58.

[3]   Boelens MH. (**1997**) Differences in chemical and sensory properties of orange flower and rose oils obtained from hydrodistillation and from supercritical CO$_2$ extraction. *Perfumer and Flavorist*, **22**, 31-35.

[4]   (a) Association Francaise de Normalisation (AFNOR) (**1995**) Huile essentielle de nerolì bigaradier (Huile essentielle de fleurs d'orange bigaradier) (*Citrus aurantium* L. subsp. *aurantium* syn. *Citrus aurantium* L. subsp. Amara var. *pumilia*), NF T 75-202, 473-481; (b) International Organization for Standardization (ISO) (**2002**) Oil of nerolì (*Citrus aurantium* L. ssp. *aurantium* syn. *Citrus aurantium* L. ssp. Amara var. *pumilia*) ISO 3517, 2002(E).

[5]   European Pharamacopoeia. (**2004**) 5th Edition. Suppl. 5. 3, January (**2006**) 3562-3563

[6]   Mosandl A, Hener U, Kreis P, Schmarr H-G. (**1990**) Enantiomeric distribution of *α*-pinene, *β*-pinene and limonene in essential oils and extracts. Part 1. Rutaceae and Gramineae. *Flavour and Fragrances Journal*, **5**, 193-199.

[7]   (a) Juchelka DA. Steil D, Witt K, Mosandl A. (**1996**) Chiral compounds of essential oils. XX. Chirality evaluation and authenticity profiles of nerolì and petitgrain oils. *Journal of Essential Oil Res*earch, **8**, 487-497; (b) Ravid U, Putievsky E, Katzir I. (**1995**) Determination of the enantiomeric composition of α-terpineol in essential oils. *Flavour and Fragrance Journal*, **10**, 281-284.

[8]   Hener U, Hollnagel A, Kreis P, Maas B, Schmarr HG, Schubert V, Rettinger K, Weber B, Mosandl A. (**1990**) Direct enantiomer separation of chiral volatiles from complex matrices by multidimensional gas chromatography. In *Flavour Science and Technology*, Y. Bessiere, and A.F. Thomas (Eds.) Wiley and Sons, Chichester, West Sussex, England, 25-28.

[9]   Hener U, Kreis P, Mosandl A. (**1990**) Enantiome ric distribution of *α*-pinene, *β*-pinene, and limonene in essential oils and extracts. Part 2. Oils, perfumes and cosmetics. *Flavour and Fragrance Journal*, **5**, 201-204.

[10]   Mosandl A. (**1995**) Enantioselective capillary gas chromatography and stable isotope ratio mass spectrometry in the authenticity control of flavours and essential oils. *Food Reviews International*, **11**, 597-664.

[11]   Dugo G, Sciarrone D, Costa R, Bonaccorsi I, Dugo P, Mondello L, Santi L, Fakhry H A. (**2011**) Characterization of oils from the fruits, leaves and flowers of the bitter orange tree. *Journal of Essential Oil Research*, **23**, 45-59.

[12]   Mondello L, Sciarrone D, Casilli A, Tranchida PQ, Dugo P, Dugo G. (**2007**) Fast Gas Chromatography-full Scan Quadruple Mass Spectrometry for the Determination of Allergens in Fragrances. *Journal of Separation Science*, **30**, 1905-1911.

[13]   (a) Sciarrone D, Ragonese C, Carnovale C, Piperno A, Dugo P, Dugo G, Mondello L. (**2010**) Evaluation of tea tree oil quality and ascaridole: A deep study by means of chiral and multi heart-cuts multidimensional gas chromatography system coupled to MS detection. *Journal of Chromatography A*, **1217**, 6422–6427; (b) Sciarrone D, Costa R, Ragonese C, Tranchida PQ, Tedone L, Santi L, Dugo P, Dugo G, Joulain D, Mondello L. (**2011**) Application of a multidimensional GC system with simultaneous mass spectrometric and FID detection to the analysis of sandalwood oil. *Journal of Chromatography A*, *1218*, 137-142

[14]   Sciarrone D, Schipilliti L, Ragonese C, Tranchida PQ, Dugo P, Dugo G, Mondello L. (**2010**) Thorough evaluation of the validity of conventional enantio-gas chromatography in the analysis of volatile chiral compounds in mandarin essential oil: a comparative investigation with multidimensional gas chromatography. *Journal of Chromatography A*, *1217*, 1101-1105.

[15]   Lo Presti M, Sciarrone D, Crupi ML, Costa R, Ragusa S, Dugo G, Mondello L. (**2008**) Evaluation of the volatile and chiral composition in Pistacia lentiscus L. essential oil. *Flavour Fragrance Journal*, **23**, 249-257.

[16]   Ruff C, Hor K, Weckerle B, Konig T, Schereier P. (**2002**) Authenticity Assessment of Estragole and Methyl Eugenol by On-Line Gas Ghromatografy-Isotope Ratio Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, **50**, 1028-1031.

[17]   (a) Kelly S, Parker I, Sharman M, Dennis J, Goodall I. (**1997**) Assessing the authenticity of single seed vegetable oils using fatty acid stable carbon isotope ratios.(^{13}C/^{12}C). *Food Chemistry*, **59**, 181-186; (b) Pupin AM, Parker MJ, Kelly S, Bigwood T, Toledo MCF. (**1998**) Use of isotopic analysis to determine the authenticity of Brazilian orange juice.(*Citrus sinensis*). *Journal of Agricultural and Food Chemistry*, **46**, 1369-1373; (c) Juchelka D, Mosandl A. (**1996**) Authenticity profiles of bergamot oil. *Pharmazie*, **51**, 417-422; (d) Faulhaber S, Hener U, Mosandl A. (**1997**) GC-IRMS analysis of mandarin essential oils. 2. δ^{13}C$_{PBD}$ values of characteristic flavour components. *Journal of Agricultural and Food Chemistry*, **45**, 4719-4725.

[18]   (a) Schipilliti L, Tranchida PQ, Sciarrone D, Russo M, Dugo P, Dugo G, Mondello L. (**2010**) Genuineness assessment of mandarin essential oils employing Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). *Journal of Separation Science*, *33*, 1–6; (b) Schipilliti L, Dugo P, Dugo G, Santi L, Mondello, L. (**2010**)Authentication of bergamot essential oil by Gas-Chromatography-Combustion-Isotope Ratio Mass Spectrometer (GC-C-IRMS). *Journal of Essential Oil Research, 23*, 60-71.

[19]   Schipilliti L, Dugo P, Bonaccorsi I, Mondello L. (**2011**) Authenticity control on lemon essential oils employing Gas-Chromatography-Combustion-Isotope Mass Spectrometry (GC-C-IRMS). Submitted to *Food Chemistry.*

[20]   Russe K, Valkiers S, Taylor PDP. (**2004**) Synthetic isotope mixtures for the calibration of isotope amount ratio measurements of carbon. *International Journal of Mass Spectrometry*, **235**, 255-262.

[21]   Mondello L, Dugo P, Basile A, Dugo G. (**1995**) Interactive use of linear retention indices, on polar and apolar columns, with a MS library for reliable identification of complex mixtures. *Journal of Microcolumn Separations*, **7**, 581-591.