SUPPLEMENTARY MATERIAL

Essential oil components of orange peels and antimicrobial activity

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In this study the orange peel of twelve cultivars of *Citrus sinensis* from central-eastern Sicily was employed to obtain essential oils and extracts. The ones were extracted through steam distillation, the others through extraction in hexane. Chemical constituents were evaluated in terms of qualitative and quantitative analysis by gas chromatography/mass spectrometry (GC/MS). 54 components were identified in the steam essential oils, 44 in the extracts. In all the cultivars the main component is d-limonene (73.9-97%); discrete percentages of linalool, geraniol and nerol were also found. Cluster analysis based on essential oils composition showed a certain degree of affinity between cultivars of the same type. The antimicrobial activity was investigated against three microorganisms (*Staphylococcus aureus*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*). ‘Sanguinello’ and ‘Solarino Moro’ essential oils are significantly active against *L. monocytogenes* while ‘Valencia’ hexanic extract against all the tested microorganisms.

Keywords: *Citrus sinensis* cultivars, Sicily, Volatile compounds, Gas chromatography-Mass spectrometry, Essential oils.

3. Experimental

3.1. Fruit material

The fruit material of twelve cultivars of sweet orange (*C. sinensis*) was harvested between November 2013 and March 2014. They were originally from Central-Eastern Sicily and some of them were cultivated in Experimental fields of the University of Palermo (SAF). In particular, the examined cultivars of sweet orange were classified into three groups according to the criteria reported in the literature (Calabrese 1973; Vacante & Calabrese 2009). See Table S1. After being washed, the oranges underwent a flavedo peeling process through a manual orange peeler, when they were still fresh.

3.2. Chemicals

Standards of *n*-alkanes mixture (C6–C20) were purchased from Sigma Chemical Company. The reference aroma compounds were obtained from the following sources: α -pinene, β -pinene D-limonene, sabinene, β -myrcene, γ -terpinene, α -terpinolene, geranyl acetate, linalool, nerol, geraniol, dihydrocarvone, terpinen-4-ol, valencene, ethyl linolenate, δ -cadinene, carvone, hexanoic acid, nerol, geraniol, (Z)-carveol, β -sinensal, (Sigma-Aldrich, Milan, Italy). *n*-Hexane and anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany).

3.3. Hydrodistillation of essential oil

100 g of fresh orange peel (only flavedo portion) were submitted to hydrodistillation by a Clevenger-type apparatus according to the *European Pharmacopoeia*. The essential oil was collected, dried over anhydrous sodium sulphate and stored at -4°C in darkness until analyses. Hydrodistillation of each cultivar was performed at least three times and the mean values were reported. The yield of essential oil obtained by the steam distillation was 1.2%.

3.4. Hexanic extract preparation

A weighed amount (50 g) of flavedo was cut in small pieces and then introduced into a flask in which 100 mL of *n*-hexane were added and left for 24 hours. After the extraction was completed, *n*-hexane was removed at 50°C under reduced pressure. The obtained oil was stored at 4°C until further analysis.

3.5. GC/MS analysis and identification of the components of the essential oils and hexanic extracts

Identification of the components of the essential oil and hexanic extracts was carried out using a GC/MS Thermo Focus GC-DSQ II equipped with a ZB-5 fused silica capillary tubes column (30 m × 0.25 mm × 0.25 µm). The volume of the injection was 1 µL, using helium as carrier gas at the flow of 1 mL/min with a split ratio of 1:50. The initial oven temperature of 60 °C was maintained for 5 min; it was raised to 310 °C at the rate of 10 °C/min and finally kept for 1 min. Other settings were: interface temperature 245 °C, ion source temperature 280 °C, electron impact ionization (EI) 70 eV. Mass spectra were analysed in the Full Scan mode over the range of 35 to 550 amu. Retention indices (KI) of all the constituents were determined by the Kovats method by co-injection of the samples with a solution containing the homologous series of n-alkanes (C6-C20). The identification of the components was performed by visual interpretation, comparing their retention indices and mass spectra with data published in the literature (Adams 2007). and matching their recorded mass spectra with reference spectra in the computer library (NIST® Database). Some structures were further confirmed by authentic standards, analyzed under the conditions described above. Quantification was computed as the percentage contribution of each compound to the total amount.

3.6. Statistical analysis

In order to estimate the degree of affinity among the cultivars, on the basis of the content of essential oils extracted by steam distillation, we used Jaccard's similarity index and a dendrogram was constructed by means of the Unweighted Pair Group Method with Arithmetic Averaging Algorithm (UPGMA).

3.7. Antimicrobial assay

3.7.1. Determination of MICs

Minimum inhibitory concentrations (MICs) against planktonic strains were determined by an agar dilution method using Tryptic Soy Agar (TSA, Sigma). A suitable volume of essential oils solution in DMSO was added to 20 ml of molten medium at 50 °C to obtain the required concentrations from 100 to 2.5 mg/ml and the resulting mixture was poured onto plates and allowed to solidify. The plates were inoculated with bacterial suspensions from a 24 h culture containing $\sim 10^6$ cfu/ml of each tested strain. The plates were incubated at 37 °C and the MIC was considered the lowest concentration of the sample, which yields no visible growth.

3.7.2. Microbial strains

The microbial reference strains were: *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442 - the reference strain in official tests for antibacterial evaluation *in vitro* (UNI EN European Standard) - and *Listeria monocytogenes* ATCC 7644. An isolate of *L. monocytogenes* from the Istituto Zooprofilattico Sperimentale, Sicily (IZS), obtained from a food sample, was also used in this study.

References

- Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectroscopy. 4th edition. Allured Publishing Corporation, Carol Stream.
- Calabrese F. 1973. Considerazioni sulla classificazione botanica delle Aurantioideae (Rutaceae). *Webbia*, 28(1):161-187.
- Vacante V, Calabrese F. 2009. Trattato di agrumicoltura. Edagricole, Milano.

Figure S1. Dendrogram obtained from Jaccard's similarity coefficient on the basis of essential oil composition (Table S2).

Table S1 *Citrus sinensis* cultivars included in this study.

ID *	CULTIVARS	PLACE OF HARVEST	COLLECTION PERIOD	CLASSIFICATION GROUP
1	‘Navelina’	Scillato (PA)	February 2014	Navel or umbilicate oranges
2	‘Thomson Navel’	Experimental Field of SAF (PA)	November 2013	
3	‘Valencia’	Experimental Field of SAF (PA)	April 2014	Blonde pulp oranges
4	‘Salustiana’	Experimental Field of SAF (PA)	February 2014	
5	‘Tarocco tradizionale’	C.da Margi Francofonte (SR)	December 2013	Pigmented pulp oranges
6	‘Tarocco dal muso’	C.da Cuticchia Francofonte (SR)	December 2013	
7	‘Tarocco giallo’	C.da Nipotelle Paternò (CT)	January 2014	
8	‘Sanguinello’	C.da Nipotelle Paternò (CT)	January 2014	
9	‘Sanguinello moscato’ 1 st sample	C.da Nipotelle Paternò (CT)	February 2014	
10	‘Sanguinello moscato’ 2 nd sample	Experimental Field of SAF (PA)	January 2014	
11	‘Moro’	Experimental Field of SAF (PA)	February 2014	
12	‘Moro’ Solarino	Solarino (SR)	January 2014	

*The *exsiccata* are deposited in the herbarium of the “Museo naturalistico Francesco Minà Palumbo” in Castelbuono (PA), numbered from 7231 to 7242.

Table S2 Percentage composition of chemical constituents of essential oils from the 12 varieties of orange grown in Sicily.

		NAVEL		BLOND PULPE		NAVEL PIGMENTED PULP								
Compounds	KI	1	2	3	4	5	6	7	8	9	10	11	12	*
Hydrocarbons														
<i>Monoterpenes</i>														
α -Pinene	939	005	-	015	017	008	026	006	012	021	015	012	012	0.28-0.55
Sabinene	976	06	011	027	025	009	018	01	008	011	076	099	009	0.13-0.93
β -Pinene	982	-	-	-	-	-	-	-	-	001	004	004	-	0.06
β -Myrcene	992	064	032	065	093	065	1.15	07	083	1.11	093	078	07	0.93-2.05
α -Phellandrene	1004	-	-	009	002	-	002	002	-	004	002	001	006	0.02-0.09
δ -3-Carene	1007	002	-	-	001	-	007	014	-	026	-	001	005	0-0.22
<i>cis-p</i> -Mentha-2.8-dienol	1133	-	-	-	-	-	-	-	-	-	-	017	-	-
D-Limonene	1036	739	927	905	967	967	976	967	97.1	957	955	901	9643	8618-968
<i>cis</i> Ocimene	1054	-	004	001	002	-	-	002	-	-	004	005	-	0.01
γ -Terpinene	1068	-	003	-	001	-	-	001	-	-	-	006	002	0.18
α -Terpinolene	1107	-	-	-	-	003	002	004	003	006	-	-	-	0.08
<i>Sesquiterpenes</i>														
α -Copaene	1384	-	023	-	002	003	002	001	002	-	002	005	002	tr
Calarene	1398	-	025	-	003	-	-	-	002	003	003	007	002	0.01
<i>trans</i> -Caryophyllene	1434	102	043	-	002	-	-	-	001	-	003	004	001	0.04
epi- <i>bicyclo</i> Sesquiphellandrene	1444	-	013	-	002	-	-	-	001	001	-	004	001	-
α -Humulene	1472	-	004	-	-	-	-	-	-	-	-	-	-	tr
(E, E)- α -Farnesene	1506	-	009	-	-	-	-	-	-	-	-	006	-	0.01-0.03
Valencene	1508	158	185	184	028	039	005	007	007	009	016	057	01	0.31
δ -Cadinene	1525	058	032	001	003	003	002	002	002	003	-	004	002	0.02-0.03
α -Panasinene	1536	-	008	-	001	-	-	-	-	001	-	002	-	-
Aldehydes														
<i>Monoterpenes</i>														
Citronellal	1159	-	008	001	002	-	001	002	001	001	002	003	001	-
Z-Citral	1240	044	-	-	003	002	002	005	003	002	004	012	004	-
E-Citral	1275	056	-	001	005	004	003	007	004	003	006	017	005	-
Perilla aldehyde	1355	-	-	-	-	-	-	001	-	-	-	-	-	0-0.01
<i>Sesquiterpenes</i>														
β -Sinensal	1818	-	009	-	-	003	-	-	-	-	-	-	001	0.01-0.05
α -Sinensal	1853	-	01	-	-	003	-	-	-	-	-	003	001	0-0.06
<i>Aliphatics</i>														
Octanal	1006	002	002	-	007	004	005	004	005	002	009	011	006	0.09-0.68
Nonanal	1115	-	004	-	002	002	001	001	002	001	004	004	001	tr-0.10
Decanal	1210	217	055	022	019	009	005	-	008	004	024	029	011	0.14-0.43
Dodecanal	1413	124	016	001	002	-	-	-	001	001	003	005	002	0.02-0.06
Ketons														
Carvone	1253	-	-	-	-	-	-	-	-	-	-	026	-	tr
Nootkatone	1815	-	006	012	002	-	-	-	-	-	-	-	-	0.01-0.04
Alcohols														
<i>Monoterpenes</i>														
Linalool	1109	419	071	411	082	103	027	137	105	19	119	304	16	0.31-2.56
<i>cis-p</i> -Menthen-1-ol	1152	-	-	-	-	-	-	-	-	-	-	002	-	-
Terpinen-4-ol	1187	177	014	042	004	004	002	005	002	002	007	075	003	tr-0.31
α -Terpineol	1224	242	012	072	012	034	01	023	015	015	028	095	016	tr-0.25
Carveol	1225	-	-	-	-	-	-	-	-	001	-	009	-	tr-0.01
Geraniol	1227	215	-	001	002	005	002	005	004	002	004	017	005	tr-0.09
Citronellol	1228	-	012	-	-	-	-	-	-	-	-	-	-	tr-0.02
Nerol	1255	155	-	-	002	003	002	004	003	001	-	009	004	0-0.02
<i>Sesquiterpenes</i>														
Nerolidol	1556	-	004	-	-	-	-	-	-	-	-	-	-	tr-0.01
Elemol	1559	-	004	001	001	-	-	-	-	-	-	-	-	tr
7(11)-Eudesmen-4-ol	1707	-	002	-	001	-	-	-	-	-	-	-	001	-
Farnesol	1830	-	001	-	-	003	-	-	-	-	-	-	001	-
<i>Aliphatics</i>														
1-Octanol	1080	131	006	067	008	009	005	005	01	-	014	031	012	tr-0.24
Nonanol	1175	-	-	-	-	-	001	-	-	-	-	-	-	tr
1-Decanol	1277	084	014	-	-	-	-	-	-	-	-	-	001	tr
Esters														
Limonen-10-ylacetate	1300	112	-	012	011	007	-	003	003	-	003	-	006	-
α -Terpinil acetate	1355	-	007	-	-	-	-	-	-	-	-	-	-	tr-0.01
Neryl acetate	1362	-	005	-	-	-	-	-	-	-	-	-	-	tr-0.01
Geranyl acetate	1380	-	006	-	-	-	-	-	-	-	-	-	-	tr-0.02
Oxides														
<i>trans</i> Limonene oxide	1146	001	006	002	001	-	001	002	-	001	002	018	001	-
Caryophyllene oxide	1598	-	009	-	-	-	-	-	-	-	-	-	-	tr
Others														
Oxacycloheptadec-8-en-2-one	1551	004	035	-	-	-	-	-	001	-	-	003	002	-

1: 'Navelina'; 2: Thomson Navel'; 3: 'Valencia'; 4: 'Salustiana'; 5: 'Tarocco tradizionale'; 6: 'Tarocco dal muso'; 7: 'Tarocco giallo'; 8: 'Sanguinello'; 9: 'Sanguinello moscato' 1st sample; 10: 'Sanguinello moscato' 2nd sample; 11: 'Moro'; 12: 'Moro' Solarino. KI: Retention indice; ^[1]comparison with Dugo & Mondello, 2011

Table S3 Percentage composition of chemical constituents identified in the hexanic extracts of 5 orange varieties

COMPOUND	KI	NAVELINA SCILLATO	VALENTIA	SALUSTIANA	SANGUINELLO MOSCATO1	MORO SOLARINO
Hydrocarbons						
<i>Monoterpenes</i>						
α -Pinene	939	0.19	0.24	0.13	0.14	0.16
Sabinene	976	0.19	0.12	0.14	0.1	0.16
β -Pinene	977	0.01	-	-	-	0.01
β -Myrcene	992	0.59	0.96	0.88	0.99	0.93
α -Phellandrene	1010	0.03	0.01	0.01	0.02	0.02
δ -3-Carene	1013	0.18	0.01	0.07	0.07	0.08
D-Limonene	1036	89.5	96.47	97.1	97.04	96.52
<i>cis</i> -Ocimene	1054	0.02	0.07	0.01	-	-
α -Terpinolene	1107	-	-	0.02	-	0.02
<i>Sesquiterpenes</i>						
α -Copaene	1384	0.01	0.01	0.03	0.03	0.02
Calarene	1398	0.02	0.02	0.04	0.03	0.03
<i>trans</i> -Caryophyllene	1434	0.02	0.01	-	-	0.01
<i>epi-bicyclo</i> Sesquiphellandrene	1444	0.01	0.02	0.01	-	-
Germacrene D	1497	-	-	0.01	0.01	0.01
Valencene	1508	0.94	1.04	0.31	0.06	0.13
δ -Cadinene	1525	0.11	0.02	0.04	0.04	0.02
α -Panasinsene	1536	-	-	0.02	-	-
<i>Aliphatics</i>						
Decane	1002	0.17	-	-	-	-
Aldehydes						
<i>Monoterpenes</i>						
Citronellal	1159	-	0.02	0.01	0.01	0.02
Z-Citral	1240	0.02	0.02	0.02	0.01	0.04
E-Citral	1275	0.03	0.03	0.03	0.02	0.06
Perilla aldehyde	1355	-	-	-	0.01	-
<i>Sesquiterpenes</i>						
β -Sinensal	1818	-	-	-	-	0.01
α -Sinensal	1853	-	-	-	-	0.01
<i>Aliphatics</i>						
Octanal	1006	0.09	0.1	0.05	0.03	0.06
Nonanal	1115	-	-	0.01	0.01	0.01
Decanal	1210	0.14	0.12	0.12	0.05	0.07
Dodecanal	1413	0.02	0.02	-	-	0.01
9,17-Octadecadienal	1552	-	-	-	0.04	-
Ketons						
Nootkatone	1815	-	0.47	0.02	-	-
Alcohols						
<i>Monoterpenes</i>						
Linalool	1109	3.18	0.48	0.47	1.03	1.2
α -Terpineol	1225	2.2	0.06	0.09	0.11	0.12
Geraniol	1227	0.33	0.02	0.03	0.02	0.04
Nerol	1255	0.02	-	0.02	0.02	-
<i>Sesquiterpenes</i>						
Elemol	1559	-	0.01	0.02	-	-
7(11)-Eudesmen-4-ol	1707	-	-	0.02	-	0.01
Farnesol	1830	-	-	-	-	0.01
<i>Aliphatics</i>						
1-Octanol	1080	0.93	0.07	0.08	0.06	0.05
1-Decanol	1277	0.33	-	0.02	-	-
Esters						
<i>Monoterpenes</i>						
Linalyl acetate	1253	-	0.01	-	-	0.03
Limonen-10-ylacetate	1300	0.62	0.01	0.01	0.02	0.07
Oxides						
<i>Monoterpenes</i>						
Trans limonene oxide	1146	0.01	0.02	0.01	0.01	0.01
Others						
Azulene	1295	-	-	0.18	-	-
Oxacycloheptadec-8-en-2-one	1551	0.03	0.02	-	-	0.04