Isolation of Seseli *Bocconi* Guss. Volatile Oil By Supercritical Carbon Dioxide Extraction

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Isolation of the volatile concentrate from dried leaves of Seseli *bocconi* Guss. subsp. *praecox* Gamisans were obtained by supercritical extraction with carbon dioxide. Leaves from different zones of Sardinia (Italy) were treated. Compositions of samples were analyzed by GC/MS. The volatile concentrate of *S. bocconi* from Buggerru was found to contain: himachalol (16.4%) sabinene (14.8%), β-phellandrene (8.1%), cis-sabinene hydrate (4.5%). β-Phellandrene (29.2%), undecane (9.6%) α-pinene (6.1%) and β-guaiene (5.7%) were the main constituents of the volatile extract of *S. bocconi* from Carloforte. The volatile concentrate of *S. bocconi* of Ogliastra inland, was composed chiefly by α-humulene (17.7%), γ-himachalene (9.3%), β-phellandrene (8.0) and bicyclogermacrene (7.7%). The yields of extraction were in the range (0.13-0.60%). A comparison with the hydrodistilled oil revealed in each case a remarkable difference in composition .

INTRODUCTION

Supercritical extraction (SFE) is a promising technique for the production of flavours and fragrances from vegetable matter. New and improved fragrance formulation can be obtained compared to traditional techniques, like steam distillation (SD), hydrodistillation (HD) and solvent extraction (SE). Indeed, using SFE, it is possible to avoid thermal degradation and solvent pollution of the extracts.

Several studies have been devoted to the extraction of essential oils and of related products by supercritical CO_2, as reviewed by Stahl et al.[1] and by Reverchon [2].

At low extraction pressure it is possible to obtain an extract consisting mainly of essential oil which is comparable to the extracts from the hydrodistillation.

An alternative procedure might to use a higher extraction pressure allowing a faster solubilization of the cuticular waxes following by a two-stage decompression to separate the waxes from volatile oil [3] and to obtain an extract devoid these unwanted compounds [4]. The value of these waxes and costs of compression and comminution may dictate the choice of procedures. Losses due to the comminution process should also be minimised as they may be significant.

The aim of this study was to apply supercritical CO_2 extraction plus multistage separation to a *Seseli bocconi* Guss. subsp. *praecox* Gamisans leaves from different zones of Sardinia and to compare the SFE products to those obtained by hydrodistillation.

This species belonging to the Apiaceae family is an endemism of central eastern and south-western Sardinia (Ogliastra and Sulcis-Iglesiente). It is a rupicolous and evergreen

chamaephyte; it blooms from August to October. Analyzed specimens were gathered from large populations from Ogliastra and Iglesiente. In coastal stations S. *bocconi* lives indifferently either in sea cliffs or in the rear plateaus intensely beaten from winds, it is indifferent to the substrate. In the Sardinian folk medicine this specie is cited rarely. Few reports have showed its hypothetical use like carminative, while in the familiar use is frequently used as food in the preparation of salads. Surveys on *S. bocconi* are included in a wider program of phytochemical analysis on Apiaceae in Sardinia [5-7].
No studies have been found in literature concerning the composition of the oil obtained by supercritical CO_2 extraction and by hydrodistillation on leaves of *S. bocconi.*

I-MATERIALS AND METHODS

Supercritical CO_2 extraction: SFE were performed in a laboratory apparatus [8], equipped with a 320 mL extraction vessel and two separators operating in the single-pass mode of CO_2 through the fixed bed of ground material. The extraction was carried out in a semi-batch mode; batch charging of vegetable matter and continuous flow solvent. The extraction conditions were as follows: 90 bar and 50°C in the extraction vessel, 90 bar and –10°C in the first separator and 15-20 bar and 10-20°C in the second one. Traces of waxes were found in the first separator. A pale yellow volatile oil was recovered in the second separator. For each test, the extractor was charged with about 100 g of vegetable matter. Carbon dioxide flow rate, ϕ_{CO2}, was 1.0 kg h^{-1} in all experiments.

Materials. Leaves of *S. bocconi* were collected during full blossom from three different sites: Buggerru (Sulcis-Iglesiente SW-Sardinia) sea-area; Carloforte, S. Pietro Island (Sulcis-Iglesiente, SW-Sardinia) sea-area; Ogliastra, (Central-eastern Sardinia) mountain-area. After harvesting leaves were air-dried in the shade. Their final moisture content was 10% by weight on dry basis. Before utilization the matrices were ground to a mean particle size of about 500 μm. The mean diameter of the vegetable particles was determined by mechanical sieving. CO_2 (purity 99%) was supplied by SIO (Società Italiana Ossigeno, Cagliari, Italy).

Hydrodistillation. Hydrodistillations were performed in a circulatory Clevenger-type apparatus, up to the point where the oil contained in the matrix were exhausted. About 100 g of material belonging to the same batch employed in SFE were charged.
GC-MS Analysis. A Hewlett-Packard (Palo Alto, USA) 5890 series II gas chromatograph, GC, was employed. It was equipped with a split-splitless injector and a DB5-MS fused silica column; 5% phenyl-methyl-poly-siloxane, 30 m × 0.25 mm i.d., film thickness 0.25 μm. The used GC conditions were: programmed heating from 60 to 280 °C at 3 °C/min followed by 30 min under isothermal conditions. The GC was fitted with a quadrupole mass spectrometer, MS, model HP 5989 A. The injector was maintained at 250 °C. Helium was the carrier gas at 1.0 mL/min; the sample (1 μL) was injected in the split mode (1:20). The GC conditions were as follows: ionization energy 70 eV, electronic impact ion source temperature 200 °C, quadrupole temperature 100 °C, scan rate 1.6 scan/s, mass range (40-500) u. The software adopted to handle mass spectra and chromatograms was ChemStation. NIST98, FLAVOUR, and LIBR(TP) mass spectra libraries were used as references. Samples were run diluted in chloroform with a dilution ratio of 1: 100. The chromatographic results (Tables 1-2) were expressed as area percentages calculated without any response factor as a function of Kováts'

Indices, I_K. Identifications were made by matching their mass spectra and I_K with those reported in the literature.

CONCLUSION

Leaves employed came from three different Sardinia's sites: one was located in a mountain-area and the others were in a sea-area. Leaves were subjected to identical drying process and grinding treatment.

The leaf matrix, on account nature herself, always contains cuticular waxes that do not give any contribution to odour but strongly contribute to the viscosity of extract. So, to isolate the volatile extract we adopted a stepwise separation technique employing two separators in series. Fractionation conditions were determined on the basis of CO_2 solubility of terpenic and paraffinic compounds [1, 7]. On the basis of previous results on extraction of similar matrixes [7-10] 90 bar and 50°C in the extraction section were selected. The co-extracted paraffins were removed from the oil inducing supersaturation and following precipitation by a decrease in temperature inside the first separator (- 10°C). At these conditions essential oil compounds show very high CO_2 solubilities, and they are carried to the second separator working at 15 bar and 15°C where take place the separation of essential oil. Operating conditions in the second separator were chose to minimize the loss of essential oil in the gaseous CO_2 stream at the exit of the apparatus.

The *S. bocconi* volatile oil of leaves of Buggerru, Carloforte and Ogliastra total yield was respectively 0.13, 0.30 and 0.60% by weight of the charged material, according with the result of hydrodistillation (HD). All compounds identified in all samples and their percentages were reported in Table 1. These results confirm that the obtained volatile concentrates were wax-free and contained only traces of high molecular weight compounds.

Among the constituents of the oils we identified four classes. Hydrocarbon monoterpenes (HM), oxygenated monoterpenes (OM), hydrocarbon sesquiterpenes (HS) and oxygenated sesquiterpenes (OS) on the basis of their chemical structure or retention time for non terpenoids or non identified compounds. We observe the most important differences to compare SFE extracts (richer in oxygenated terpenes, mono- and sesqui-) with the HD one (richer in hydrocarbon terpenes, mono- and sesqui-).

The GC results (Table 1) indicate that large differences in oil composition take place with respect to geographical area. It is possible note that in the volatile oil of leaves from Buggerru the main constituents were sabinene (14.8% versus 20.1% in the SFE and HD oil, respectively), β-phellandrene (8.1% versus 12.5%), himachalol (16.4% versus 4.4%) and cis-sabinene hydrate (4.5% versus 0.5%). In the volatile oil of leaves from Carloforte the compounds present in the biggest quantity were α-pinene (6.1% versus 16.7%), myrcene (4.1% versus 5.6), β-phellandrene (29.2% versus 37.9%) and undecane (9.6% versus 8.7%).

Whereas the volatile concentrate from Ogliastra was composed chiefly by α-humulene (17.7% versus 20.1%), γ-himachalene (9.3% versus 0%), β-phellandrene (8.0% versus 10.4% and biclyclogermacrene (7.7% versus 8.3%).

A search in literature reveals that some papers are available for the chemical composition of essential oils from different *Seseli* species growing in Iran and Tukey. Habibi et al. [11] reported the chemical composition of hydrodistilled oil of *S. tortuosum* from Iran: α-pinene, β-phellandrene and sabinene were the main constituents. Tosun et al. [12] reported the composition of the essential oils from *Seseli* species (*gummiferum* subsp. *gummiferum* and

subsp. *corymbosum*). Bicyclogermacrene, germacrene B and spathulenol were the main constituents.

As reported by Tosun et al.[13], *Seseli gummiferum* also other species of the genus *Seseli* could contain pyranocoumarins. The mild extraction conditions (P=90 bar, T=50°C; ρ=0.287 g. cm^{-3}) and a two-stages separation, adopted in our study, chosen to obtain an high recovery of the volatile oil, allowed us to obtain an extract essentially devoid of undesired compounds of higher molecular weight as coumarins, psoralens, dyes, long chain hydrocarbons and FAME's. Only a small amount of iso-bergaptene (0.6%) was present in the volatile concentrate from Ogliastra. Extraction at higher pressures (> 200 bar) i.e. higher solvent density would permit to extract from the plant material such classes of compounds.

Biological activity The cytotoxic activity was evaluated on VERO cells (a line of green monkey kidney cells from ICN-Flow), grown on Dulbecco's modified MEM with 2 % foetal calf serum (both from Gibco). The assays were performed in duplicate in 24-well plates with about 5×10^4 cells per well. Cytotoxicity was read after 48 h of incubation in an atmosphere of 5% CO_2 at 37 °C. In a similar manner was scored the inhibition of cell multiplication in the presence of decreasing amounts of the compounds under study, with the use of a light microscope. The Maximal Non Toxic Dose ($MNTD_{50}$) was considered to be the dose of the sample, as mg/ml, which reduced cell multiplication no more than 50%, as compared to controls. Antimicrobial activity was tested on: *Staphylococcus aureus ATCC25923, E. coli ATCC25922, Candida albicans 5M, Candida tropicalis CA44, Cryptococcus neoformans CA1*. Antimicrobial activity was detected with an agar dilution method, using Mueller-Hinton agar for bacteria and Casitone agar for the yeasts. The inoculum was standardized at 10^5 CFU/ml for all microorganisms. The inhibition of microorganism growth was evalued after 48 h of incubation at 37 °C and expressed as the Minimal lnhibitory Concentration (MIC) in mg cm^{-3}. All the extracts obtained were assayed for biological activity but none of the samples resulted active.

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Table 1. Kovats' indices and area percentages of compounds identified in the extracts of leaves of *S. bocconi*.

I_K	Buggerru SFE Area %	Buggerru HD Area %	Carloforte SFE Area %	Carloforte HD Area %	Ogliastra SFE Area%	Ogliastra HD Area %	COMPOUND
855	-	-	0.6	-	-	-	N-HEXANOL
874	-	-	-	-	-	0.4	N-NONANE
905	2.1	2.3	-	-	-	0.4	TRYCICLENE
913	1.4	2.3	6.2	16.7	2.1	4.9	α-PINENE
928	-	-	0.7	2.4	-	-	CAMPHENE
953	14.8	20.1	0.5	0.5	3.2	4.8	SABINENE
956	1.9	2.1	-	1.2	0.6	1.0	β-PINENE
965	1.1	1.8	4.1	5.5	0.9	1.7	MYRCENE
977	-	-	1.3	2.3	-	-	N-OCTANAL
981	-	1.6	1.5	3.8	2.4	2.8	α-PHELLANDRENE
985	-	-	-	-	-	1.6	Δ-3-CARENE
991	0.6	2.2	1.5	1.6	-	0.4	α-TERPINENE
999	5.1	3.0	2.3	2.9	0.6	0.9	PARA-CYMENE
1006	8.1	12.5	29.2	37.9	8.0	10.4	β-PHELLANDRENE
1010	-	-	-	-	-	1.1	(Z) -β-OCIMENE
1013	-	-	0.4	0.7	-	-	DIHYDRO TAGETONE
1030	1.1	4.4	-	-	1.4	1.9	γ-TERPINENE
1041	4.5	0.5	-	-	-	-	CIS-SABINENE-HYDRATE
1054	-	1.1	-	-	-	0.4	TERPINOLENE
1068	1.8	-	9.6	8.7	-	0.4	N-UNDECANE
1070	-	0.3	0.8	1.5	-	-	ISOPENTYL-ISOVALERATE
1121	-	-	0.2	-	-	-	(Z)-3-HEXENYL ISOBUTYRATE
1148	3.5	6.3	-	-	-	0.4	TRANS-β-TERPINEOL
1153	1.5	-	3.5	2.1	-	-	META-CYMEN-8-OL
1163	0.9	0.5	1.2	-	-	-	α-TERPINEOL
1169	0.8	1.1	1.0	0.6	-	-	DIHYDRO CARVEOL
1175	-	-	1.8	-	-	-	N-DECANAL

1206	-	-	0.5	0.4	-	-	HEXYL 2-METHYL BUTYRATE
1212	-	-	0.7	-	-	-	CUMIN ALDEHYDE
1218	-	-	0.7	0.6	-	-	CARVOTANACETONE
1243	0.5	-	-	-	-	-	DEC-9-EN-1-OL
1306	-	0.4	-	-	1.0	0.9	δ-ELEMENE
1310	1.5	4.1	0.6	-	3.1	3.1	TERPIN-4-OL ACETATE
1349	0.8	0.9	0.4	-	0.7	0.6	α-COAPENE
1357	-	-	-	-	1.1	1.0	β-BOURBONENE
1363	0.6	0.9	-	-	0.9	1.2	β-ELEMENE
1386	-	0.5	2.7	1.0	1.4	1.9	β-LONGIPINENE
1388	-	0.6	-	-	1.6	1.7	1,7-DI-EPI-β-CEDRENE
1399	1.6	2.2	-	-	1.9	1.9	(E)-CARYOPHILLENE
1404	-	-	0.8	-	-	-	LAVANDULYL ISOBUTYRATE
1411	1.9	2.8	-	-	1.0	-	CIS-THUJOPSENE
1415	0.9	0.9	-	-	-	1.4	AROMADENDRENE
1418	2.2	1.7	1.4	-	-	-	CIS-MUUROLA-3,5-DIENE
1424	-	1.0	-	-	17.7	20.1	α-HUMULENE
1446	2.1	2.1	-	-	1.0	-	α-ACORADIENE
1449	1.0	1.3	-	-	9.3	-	γ-HIMACHALENE
1457	0.8	1.2	-	-	-	7.9	GERMACRENE D
1462	-	-	2.0	0.9	-	-	CITRONELLYL ISOBUTYRATE
1470	-	-	-	-	7.7	8.3	BICYCLOGERMACRENE
1473	0.5	1.6	5.7	2.0	-	0.5	TRANS-β-GUAIENE
1479	0.9	1.6	3.5	1.6	-	1.0	(Z)-α-BISABOLENE
1482	-	-	-	-	-	0.7	γ-CADINENE
1488	-	-	-	-	0.7	0.8	(Z)-γ- BISABOLENE
1493	1.4	1.7	-	-	0.8	0.9	β-SESQUIPHELLANDRENE
1523	1.1	3.7	0.4	-	2.0	2.4	GERMACRENE B
1543	-	-	1.6	0.7	1.9	2.9	SPATHULENOL
1552	1.6	1.0	-	-	-	0.8	GLOBULOL
1567	0.8	-	-	-	0.7	1.5	(Z)-SESQUILAVANDULOL
1584	2.4	1.1	0.7	-	0.5	-	γ-EUDESMOL
1588	-	-	-	-	-	1.0	β-ACORENOL
1590	16.4	4.4	0.8	-	-	0.7	HIMACHALOL
1682	-	-	-	-	-	1.1	(Z,Z)-FARNESOL
1908	-	-	-	-	0.6	-	PHYTOL
2016	-	-	-	-	0.6	-	ISO-BERGAPTENE
2063	-	-	-	-	1.8	-	OCTADECANOL

Table 2. Overall chromatographic area percentages of hydrocarbon monoterpenes (HM), oxygenated nonoterpenes (OM), hydrocarbon sesquiterpenes (HS) and oxygenated sesquiterpenes (OS) in *S. bocconi* extracts.

SESELI	**HM**		**OM**		**HS**		**OS**	
	HD	**SFE**	**HD**	**SFE**	**HD**	**SFE**	**HD**	**SFE**
BUGGERRU	53	38	13	13	26	16	8	33
CARLOFORTE	82	56	10	15	7	16	1	13
OGLIASTRA	33	19	4	3	53	49	10	29