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Brassica fruticulosa Cyr. and *Brassica incana* Ten. (Brassicaceae) as Mediterranean traditional wild vegetables: a valuable source of bioactive compounds

G. Tripodi[a], A. Verzera[a]*, G. Dima[a], C. Condurso[a] and S. Ragusa[b]

[a]*Department of Organic and Biological Chemistry, University of Messina, Messina, Italy;* [b]*Department of Health Sciences, University 'Magna Graecia' of Catanzaro, Catanzaro, Italy*

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Two species of Brassicaceae (Crucifer) family, used and appreciated as traditional wild vegetables, including *Brassica fruticulosa* Cyr. and *Brassica incana* Ten., were examined as potential source of bioactive volatile compounds. The volatile constituents released by the chopped leaves and roots were extracted and analyzed by solid-phase microextraction–gas chromatography–mass spectrometry (SPME–GC–MS). A large number of volatile constituents were identified: alcohols, aldehydes, esters, acids, ketones, terpenes, C_{13}-norisoprenoides and sulfur compounds. Volatiles included isothiocyanates with a well known anticancer activity – the largest amount resulted in the roots, with 3-butenyl isothiocyanate the most represented in both species; of great interest also the good amount revealed in the leaves of *Brassica fruticulosa* Cyr. The revaluation of these plants, a vegetable source of high antioxidant power, will be interesting for consumer health by the production of new commercial herbal products and/or dietary supplements of high quality and low cost.

Keywords: *Brassica fruticulosa* Cyr.; *Brassica incana* Ten.; volatile constituents; isothiocyanates; SPME–GC–MS

Introduction

Brassicaceae are believed to originate in the Mediterranean–Middle Eastern area with a secondary center of origin and differentiation of *Brassica rapa* L. and *Brassica juncea* Coss. in China. The genus *Brassica* belongs to the Brassicaceae family or mustard family, which includes many economically important edible and industrial oilseed, vegetable, condiment and fodder crop species. Many *Brassica* species are also important vegetable crops; those cultivated are represented by six interrelated species, *Brassica nigra* (L.) Koch, *Brassica oleracea* L., *B. rapa* L., *Brassica carinata* Braun., *B. juncea* Coss. and *Brassica napus* L. The family is also known for its more than 120 weedy species, several of which are important cosmopolitan agricultural weeds (e.g. wild mustard, *Sinapis arvensis* L., and stinkweed, *Thlaspi arvense* L.) while others form crop–weed complexes (e.g. *Raphanus raphanistrum* Cav.). Many genera of the Brassicaceae have been studied for their chemical composition, especially for variation in oil content and for the fatty acid and glucosinolate (GLS) amount of the seeds; GLS and their hydrolysis products, mainly isothiocyanates, provide the characteristic odors and flavors of crucifers. Their pharmacological role in the prevention of disease and in chemical defense against pathogens, herbivores and weeds is attracting increasing attention (1, 2). Several GLSs have been reported in the Brassicaceae family, and many of these are unique to certain species and genera (3–11).

Many species have potential interest and could be grown for their value-added traits or production of pharmaceuticals as happens, for example, with *Lepidium draba* L. This species contains glucoraphanin, an alkenyl GLS, which hydrolyzes to form the enzyme inducer sulforaphane, used as a dietary additive for cancer and high blood pressure treatments; sulforaphane is also effective against pathogens such as bacteria, yeasts, fungi, mycoplasm, protozoans, nematodes and viruses (12).

The potential presence of volatile isothiocyanates, which are typical constituents of *Brassica* vegetables with well-known cancer chemo-protective attributes (13), aroused our particular interest in *Brassica fruticulosa* Cyr. and *B. incana* Ten., two wild species widely spread in the Mediterranean area and highly appreciated as vegetables.

Brassica fruticulosa Cyr. (Mediterranean cabbage) is an herbaceous species, seldom biennial. It is 20–60 cm high and it presents a suffruticose aspect with a woody stem at the basis. The basal leaves are long and petiolate, lobed or whole and arranged as a rosette; the cauline leaves are reduced or missing. It is widely spread in southern Italy, from 0 to 1200 m above the sea level, and we can find it in untilled lands, on walls

*Corresponding author. Email: averzera@unime.it

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and debris. It blooms from January to December and it has flowers with violet sepals of 3–5 mm and yellow petals of 6–7 mm. Its fruit is a peduncled siliqua with a 'beak' of 5 mm (14, 15).

Brassica incana Ten., 'whitish cabbage' or 'velvety cabbage', is a suffruticose plant grows on marine cliffs, among sandy and barren rocks and land, and also in unfavorable environmental conditions (16). Its stem is woody at the base and its branches are herbaceous in the florescence scape only. Its basal leaves have a 'winged' petiole; the leaf lamina has a lanceolate shape, pubescent especially on the lower surface and along the keels, and shows a complete margin, crenated or with asse or two lobes in the lower half; the cauline leaves have a dentate margin and basal, embracing auricles. The flowers, with yellow petals, are gathered in a rich racemose florescence, long and narrow. Its fruit is a bent siliqua 40–80 mm, terete, convoluted and cylindrical, gradually attenuate into beak (14, 15).

The interest of our research mainly regards the rediscovering of a vegetable source of high antioxidant power for human health, and the possibility of producing new commercial herbal products and/or dietary supplements of high quality and low cost. For the two *Brassica* species, *B. fruticulosa* and *B. incana*, a headspace solid-phase microextraction (HS-SPME) method in combination with gas chromatography–mass spectrometry (GC–MS) has been used for the extraction, identification and quantification of the leaf and root volatile constituents.

Experimental

Plant material

The plants of *B. fruticulosa* Cyr. (Brassicaceae) were collected in Spartà (Messina, Sicily, Italy), those of *B. incana* Ten. in Mongiove di Patti (Messina, Sicily, Italy) in October 2009 and 2010. The fresh leaves and roots were harvested manually from plants at beginning of flowering. Five samples for year were analyzed by HS-SPME/GC–MS as described in the following, each sample in triplicate; the samples consisted of a batch of grinded and homogenized fresh leaves and roots from at least ten different plants. The voucher specimens, numbered 35/09 (*B. fruticulosa*) and 22/10 (*B. incana*), have been deposited at the Herbarium of the Pharmacobiological Department of the University of Messina (Italy).

Volatile extractions: development of the SPME method

The volatile components were extracted by the HS-SPME method. SPME was performed with a commercially available fiber housed in its manual holder (Supelco, Bellefonte, PA, USA). Plant leaves being a complex matrix, rather than optimizing the analytical technique, different factors were considered, namely sample volume, sample headspace volume, sample heating temperature, extraction time, and so on, which affect the sensitivity of the technique. Each measurement was repeated three times. The criteria of the efficiency was the desorption peak area (total ion current chromatogram) and the coefficient of variation (CV%) of the measurements.

Several types of coating fibers are currently available; we used divinylbenzene/carboxen/polydimethy lsiloxane (DVB/CAR/PDMS), since mixed fibers contain DVB copolymers and CAR (a porous activated carbon support) increases the retention capacity due to the mutually effects of adsorption and distribution to the stationary phase (17). The use of a 'mininert' vial, which is septumless, avoided the formation of extraneous peaks due to possible septum bleeding. Each sample was suspended in water to favor the magnetic stirring, which plays an important role in increasing the reproducibility of SPME and shortening the equilibrium time (18).

Different amounts of leaves, roots and water were tested; 2 g of leaves or roots and 15 mL of water with a headspace/sample volume ratio of about 1:1, according to Pawliszyn (19), provided the highest peak areas for most of the extracted compounds. In the present study, different sample heating temperatures were tested (25°, 30° and 35°C) for the extraction of the cheese. An increase in the peak areas was observed from 25° to 30°C. Trials were not carried out at higher temperatures that can adversely affect the adsorption by the coating as a result of the decrease of the partition coefficient. A sampling time of 25 minutes and an equilibrium step of 30 minutes proved to be optimal for the maximum amount of the components of interest. The recommended injector temperature by the manufacturer for the DVB/CAR/PDMS fiber ranges between 230° and 270°C; under our conditions, we observed the highest peak areas at 260°C, which is approximately equal to the boiling point of the least volatile analyte – benzyl isothiocyanate.

Volatile extraction: SPME procedure

All extractions were carried out using a DVB/CAR/PDMS fiber, 50/30 μm film thickness (Supelco, Bellefonte, PA, USA). Using a 40-mL vial, about 2 g, exactly weighed, of each sample was dissolved in 15 mL of water. Extraction was performed in the headspace keeping the vial at 30°C. The sample was equilibrated for 30 minutes and the extraction time was 25 minutes; during the extraction, the sample was continuously stirred with a magnetic stir bar on a stir plate revolving at 750 rpm. The fiber was carefully placed in the same location for each exposure to the headspace in order to obtain maximum repeatability. After

sampling, the SPME fiber was introduced onto the GC/MS injector. The fiber was kept in the splitless injector, maintained at 260°C for 3 minutes for the thermal desorption of the analytes.

Volatile analysis: GC/MS

A Varian 3800 gas chromatograph, directly interfaced with a Varian 2000 ion trap mass spectrometer (Varian Spa, Milan, Italy), was used to analyze the headspace components. Two different fused silica capillary columns were used: (i) Mega 5 MS, 60 m × 0.25 mm, 0.25 μm film thickness (Mega, Legnano, Milan, Italy); GC oven temperature, 40°C, held for 2 minutes then increased to 240°C at a rate of 3.0°C/minute; carrier gas, He at a constant pressure of 90 kPa. (ii) CP-Wax 52 CB, 60 m, 0.25 mm, 0.25 μm film thickness (Chrompack, Milan, Italy); GC oven temperature, 45°C, held for 5 minutes, then increased to 150°C at a rate of 3°C/minute, and to 240°C at 10°C/minute; carrier gas, He at constant pressure of 90 kPa. For both columns: injector temperature, 260°C; injection mode, splitless. MS scan conditions: interface temperature, 250°C; ionization technique, electronic impact (EI) at 70 eV; acquisition range, 30–200 *m/z*; scan rate, 1 m/second. The compound identification was based on comparison of their linear retention indices (LRI) with those of authentic compounds, computer matching with mass spectral libraries (NIST'05, NIST/EPA/NIH, version 2.0 USA), comparison with spectra of authentic samples or literature data and injection of standards; LRI of the sample components were determined on the basis of homologue *n*-alkane hydrocarbons, analyzed under the same GC conditions according to the Van der Dool and Kratz (20) equation on both a polar and apolar column. The coefficient of variation (CV%) for the three replicates of the same sample was inferior to 12.0, for all the analyzed compounds.

Volatile analysis: GC–FID

For quantitative results, each sample was analyzed by GC–FID on a Dani 1000 gas chromatograph. Capillary column, Equity-5, 60 m, 0.25 mm, 0.25 μm film thickness (Supelco, Milan, Italy); GC oven temperature, 45°C held for 5 minutes then increased to 150°C at a rate of 3.0°/minute, and to 240°C at 10°/minute; carrier gas, He at a constant pressure of 90 kPa. Injection mode, split; split ratio, 1:100; injector and detector temperature, 250°C. Quantitative results were obtained using the method of internal standard: aliquots of an aqueous solution of ethyl isothiocyanate (1 mg/ml) were added in the slurry prior to extraction.

Results and discussion

SPME–GC–MS analyses were performed to identify and quantify the volatile compounds in the root and leaves of the two *Brassica* species. The applied technique allowed the identification of a large number of compounds belonged to the following classes of substances: alcohols, aldehydes, esters, acids, ketones, terpenes, C_{13}-norisoprenoides and sulfur compounds such as thiocyanates, isothiocyanates and nitriles. The identified compounds are listed in Table 1 with their amount and LRI calculated on polar and apolar column. In all the analyzed samples, the most abundant classes were aldehydes, alcohols and sulfur compounds even if different qualitative and quantitative composition between the two species and, within the same species, between leaf and root resulted. The major compounds found in the leaves of both *Brassica* species were 3-butenyl isothiocyanate, (*E*)-3-hexenol, (*E*)-2-hexenal and hexanal, whereas 3-butenyl isothiocyanate prevailed in the two root species. Carbonyl compounds, (*E*)-2-hexenal and hexanal constituted 89.4% of the total aldehydes in *B. fruticulosa* Cyr. leaves and 92.7% in *B. incana* Ten. ones even if numerous aldehydes, aromatic and aliphatic, saturated and unsaturated, were identified. (*E*)-3-hexenol constituted 19.7% in *B. fruticulosa* Cyr. and 32.1% in *B. incana* Ten. leaves of the total alcohol content. Alcohols and aldehydes are common volatiles in processed vegetables, and are formed by oxidative breakdown of free fatty acids by the action of lipoxygenase (21) The typical fresh and pleasant top notes of crushed fruits and many green parts of plants, often referred as 'green' note, arise from volatile C_6-compounds, such as (*E*)-2-hexanal (leaf aldehyde) and (*Z*)-3-hexenol (leaf alcohol). These C_6-volatiles compounds are widely used in food and beverage industry (22) also due to their anti-microbial activities (23). In *B. incana* Ten. leaves the amount of β-ionone, 5,6-epoxy-β-ionone and β-cyclocitral that can be produced by β-carotene degradation through an attack by enzyme-generated free radicals and a cleavage at the C_{9-10} bond was interesting; these compounds constitute the essential aroma notes and suggest the presence of a possible greater amount of carotenoids in *B. incana* than in *fruticulosa*; otherwise, the content in carotenoids such as β-carotene and lutein is different within the different *Brassica* species (24).

In the two root species alcohols, aldehydes but also esters, ketones and acids were less represented compared with their leaf amount. This behavior has been expected, since it is well known that volatile compounds from plants serve as insect attractants (25); thus, the distribution of the different classes of compounds in the different parts of the plants may reflect the different biological roles of the compounds identified.

In our samples, isothiocyanates, nitriles and thiocyanates, closely related to GLS, were also identified. GLS are glucose and sulfur-containing organic anions whose decomposition products are produced when

Table 1. Composition[a] as volatile constituents and classes of substances for leaves and roots of *Brassica fruticulosa* and *Brassica incana*.

Compounds	LRI[b] CP-Wax	LRI Mega 5 MS	Standard injection	*B. fruticulosa* Leaf	*B. fruticulosa* Root	*B. incana* Leaf	*B. incana* Root
Esters							
Allyl formate	918	–		856	87	–[c]	–
Methyl butanoate	959	723	x	–	–	–	203
Methyl-2-methyl butanoate	1014	674		890	786	–	–
(*Z*)-4-Hexenyl acetate	1317	1009	x	–	455	–	286
(*Z*)-2-Hexenyl acetate	1334	–	x	–	–	119	–
Ethyl benzoate	1642	1185	x	–	281	–	–
Ethyl decanoate	1649	1398	x	–	106	289	–
Ethyl salicylate	1792	1436	x	–	779	–	–
Methyl dodecanoate	1797	1509	x	–	56	87	141
All				*1746*	*2550*	*495*	*630*
Alcohols							
3-Butanol	1061	–		1462	708	–	–
1-Butanol	1141	668	x	396	–	684	–
1-Penten-3-ol	1161	686	x	1198	557	2276	–
1-Pentanol	1246	761	x	308	–	–	96
1-Hexanol	1349	851	x	754	260	9207	88
(*Z*)-3-Hexenol	1366	858	x	131	141	943	–
(*E*)-3-Hexenol	1385	878	x	3150	311	7272	–
3-Octanol	1391	981	x	95	–	201	571
(*E*)-2-Hexenol	1407	853	x	631	–	522	–
1-Octen-3-ol	1446	980	x	6704	844	213	–
1-Heptanol	1454	966	x	–	–	1002	–
2-Ethyl-1-hexanol	1488	1032	x	574	233	166	200
1-Octanol	1552	1066	x	–	–	193	177
2-Butyl-1-octanol	1712	–	x	65	114	–	61
β-Phenylethyl	1923	1118	x	487	863	–	–
All				*15955*	*4031*	*22679*	*1193*
Acids							
2-Methyl butanoic	1691	873		69	188	–	–
Hexanoic	1841	1019	x	141	–	–	–
All				*210*	*188*	–	–
Ketones							
2-Pentanone	974	683	x	628	111	255	–
1-Penten-3-one	1020	650	x	–	–	824	–
3-Octanone	1258	965	x	992	–	–	–
6-Methyl-5-hepten-2-one	1339	984	x	465	467	370	267
Acetophenone	1676	1065	x	–	–	56	187
All				*2085*	*578*	*1505*	*454*
Aldehydes							
Hexanal	1081	770	x	14451	1068	28166	–
(*Z*)-2-Pentenal	1112	703		93	35	–	–
(*E*)-2-Pentanal	1132	724	x	1060	100	456	–
Heptanal	1182	864	x	164	125	141	117
(*Z*)-2-Hexenal	1204	801		2167	231	2597	–
(*E*)-2-Hexenal	1223	821	x	66482	606	88646	–
(*E*)-2-Heptenal	1328	925	x	104	102	335	11
Nonanal	1395	1080	x	–	622	211	719
(*Z*,*E*)-2,4-Hexadienal	1405	853		1493	–	–	–
(*E*,*E*)-2,4-Hexadienal	1410	879	x	2961	–	2424	–
(*E*)-2-Octenal	1436	1025	x	358	388	–	–
Furfural	1469	891	x	–	–	609	338
(*E*,*Z*)-2,4-Heptadienal	1472	980		195	118	–	–
(*E*,*E*)-2,4-Heptadienal	1500	1011	x	–	–	999	410
Decanal	1502	1179	x	187	1783	–	–
Benzaldehyde	1533	968	x	203	341	390	406
5-Methyl furfural	1580	963		–	–	–	127
Undecanal	1610	1281	x	–	178	–	–

(*Continued*)

Table 1. (*Continued*).

Compounds	LRI[b] CP-Wax	LRI Mega 5 MS	Standard injection	*B. fruticulosa* Leaf	*B. fruticulosa* Root	*B. incana* Leaf	*B. incana* Root
Phenylacetaldehyde	1671	–	x	481	695	–	–
Dodecanal	1728	1380	x	38	73	452	168
4-Ethyl-benzaldehyde	1733	1171		58	45	558	134
Tetradecanal	1922	1581	x	–	–	31	62
All				*90495*	*6510*	*126015*	*2492*
Terpenes and C_{13}-norisoprenoides							
Limonene	1192	1021	x	77	174	–	–
β-Caryophyllene	1614	1467	x	109	–	–	–
β-Cyclocitral	1639	1223	x	215	–	990	–
Geranyl acetone	1860	1454	x	80	724	276	323
(*E*)-β-Ionone	1948	1485	x	–	–	773	–
5,6-Epoxy-β-ionone	1981	1610		–	–	617	–
All				*481*	*898*	*2656*	*323*
Thiocyanates and isothiocyanates							
Methyl thiocyanate	1277	661		–	–	–	2262
Allyl isothiocyanate	1363	787		–	482	–	–
3-Butenyl isothiocyanate	1460	985		55476	248614	28770	71982
2-Butenyl isothiocyanate	1480	–		–	154	–	–
4-Pentenyl isothiocyanate	1544	1112		–	–	–	2039
3-Methylthiopropyl isothiocyanate	1597	1260		136	3433	–	360
Hexyl isothiocyanate	1642	1241		–	599	–	181
Benzyl thiocyanate	1809	–		–	374	–	–
Benzyl isothiocyanate	2068	1317		–	291	–	–
All				*55612*	*253947*	*28770*	*76824*
Sulfuric compounds							
2-Methyl thiophene	1107	759		–	–	–	1193
2-Ethyl thiophene	1170	871		–	–	489	–
Benzothiazole	1970	1224	x	2485	8949	2433	8827
All				*2485*	*8949*	*2922*	*10020*
Nitriles							
4-Pentenenitrile	1275	742	x	3736	834	2333	428
2-Phenyl acetonitrile	1941	1068		–	190	–	–
5-(Methylthio)-pentenenitrile	1942			–	–	–	1362
Decanenitrile	1956		x	141	172	–	–
All				3877	1196	2333	1790

Notes: [a] Peak area; [b]linear retention index; [c]not detected.

plant cells are ruptured and the GLS, present in vacuoles, are hydrolyzed by the enzyme myrosinase (26). The hydrolysis products, many of them with biological activity, include substituted isothiocyanates, nitriles, thiocyanates, epithionitriles and oxazolidinethiones, which vary depending on the plant species studied, side-chain substitution, cell pH and cell iron concentration (27–31). Corresponding aliphatic or aromatic volatiles formed by known degradation of GLS, constituted 15.4–32.2% of the isolated volatiles in leaves and 82.0–91.1% in roots for *B. incana* Ten. and *B. fruticulosa* Cyr., respectively. The amounts of these substances (μg/g) are given in Table 2. 3-Butenyl isothiocyanate prevailed in all the samples with the highest amount in the roots of *B. incana* and *B. fruticolosa*, respectively. Moreover, the roots showed a large number of isothyocyanates, aliphatic and aromatic. There are different types of GSL commonly found in Brassicaceae, which vary in their structure depending on the type of organic side chain (aliphatic, aromatic or indolyl) on the molecule. Profile, concentration and distribution of these GSL vary within and among *Brassica* species, and in different plant tissues and consequently also the concentration and type of biocidal hydrolysis products vary (32). The higher amount of isothyocyantes in the roots than in the leaves was in agreement with different authors who studied the glcosinolate degradation products of other *Brassica* species, such as *Raphanus sativus* L. (33). Regarding 3-butenyl isothiocynates, this was also the main isothyocyante in other wild Brassicaceae, for instance *Brassica campestris* Oed. (34) and *Isatis tinctoria* L. (35, 36). Recently, it has been demonstrated that 3-butenyl isothiocyanate inhibits the proliferation of human colorectal carcinoma cells by blocking the cell cycle (37).

From our results, the revaluation of these plants, especially *B. fruticulosa* Cyr., widely appreciated as a wild vegetable, will be interesting for consumer health

Table 2. Content (μg/g) of the isothiocyanates identified in the leaves and roots of *Brassica fruticulosa* and *Brassica incana*.

		B. fruticulosa		*B. incana*	
Isothiocyanates	Glucosinolates	leaf	root	leaf	root
Butyl isothiocyanate	Glucococochicarin	–	–	–	tr
Allyl isothiocyanate	Sinigrin	–	0.002	–	–
3-butenyl isothiocyanate	Gluconapin	0.263	0.932	0.134	1.151
2-butenyl isothiocyanate	Gluconapin	–	0.001	–	–
4-pentyl isothiocyanate	–	–	–	–	–
3-methylthiopropyl isothiocyanate	Glucoiberverin	tr	0.013	–	0.033
Hexyl isothiocyanate	–	–	0.002	–	0.006
Benzyl thiocyanate	Glucotropaeolin	–	0.001	–	0.003
Benzyl isothiocyanate	Glucotropaeolin	–	0.001	–	–
All		0.263	0.952	0.134	1.193

Note: Tr, trace.

since they yield biologically active GLS products. Regarding the roots, they could be considered for the extraction of substances for pharmaceutical applications.

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