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ScienceDirect
Food Chemistry 107 (2008) 1687–1691
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Glucosinolates profile of *Brassica rapa* L. subsp. *Sylvestris* L. Janch. *var. esculenta* Hort

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Abstract

Glucosinolates in different ecotypes of *Brassica rapa* L. subsp. *sylvestris* L. Janch. *var. esculenta* Hort. widely distributed in Southern Italy and locally known as "friariello" and "cima di rapa", were characterized and their glucosinolate composition was compared with that of broccoli (*Brassica oleracea* L. *var. italica*).

Although these two vegetables have a similar morphologic aspect, they showed a very different glucosinolate profile. While glucoraphanin, glucobrassicin and 1-methoxyindol-3-ylmethyl were confirmed as the main compounds in broccoli, gluconapin and glucobrassicanapin were identified for the first time as the molecular markers of *friarielli* and other *Brassica rapa* plants. Broccoli can by grouped according to their glucosinolates concentration by PCA analysis.

Keywords: Glucosinolates; *Brassica rapa* L. subsp. *Sylvestris*; Broccoli; Metabolic profile

1. Introduction

Glucosinolates are an important group of phytochemicals present exclusively in 15 botanical families of the order of *Capparales* and, particularly, they are very abundant in *Brassicaceae*.

In the last years, numerous studies are been focused on this compounds. The reason of this increasing interest is due to the strong correlation between the consumption of cruciferous vegetables and the decreased risk for pancreas, lung, stomach, colon, rectal and prostate cancer (Benito et al., 1990; Cohen, Kristal, & Stanford, 2000; Le Marchand, Yoshizawa, Kolonel, Hankin, & Goodman, 1989; Olsen, Mandel, Wattenberg, & Schuman, 1989; Van Poppel, Verhoeven, Verhagen, & Goldbohm, 1999). Intact glucosinolates are biologically inactive, but, after disruption of plant cell, they are rapidly hydrolysed by a β-thioglucosidase enzyme, called myrosinase to yield glucose and instable aglycons that undergoes molecular rearrangement into different breakdown products (Bones & Rossiter, 1996). Isothiocyanates represent one of the glucosinolates breakdown products and different studies suggested that they are responsible of the cruciferous cancer protecting effects (Faulkner, Mithen, & Williamson, 1998; Tawfiq et al., 1995). Different mechanisms were proposed to explain the anticarcinogenic activity, all involving the ability of the glucosinolates-hydrolysis products to modulate the phase I and/or phase II detoxification enzymes activity (Das, Tyagi, & Kaur, 2000).

The glucosinolates pattern of Brassica vegetables varies greatly, both quantitatively and qualitatively; but it should be outlined that different isothiocyanates have different biological effects. Several information are available on glucosinolates profile of common brassica vegetables such as broccoli, cabbage, cauliflower, kale and Brussels sprouts (Kushad et al., 1999; Heaney & Fenwick, 1980; Rosa & Heaney, 1996); on the other side, few data are reported

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0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.09.054

for local *brassica* ecotypes (Branca, Li, Goyal, & Quiros, 2002).

In Southern Italy *Brassica rapa* L. subsp. *sylvestris* L. Janch. *var. esculenta* Hort. (Synonymous: *Brassica rapa* L. subsp. *campestris* (L.) A. R. Clapham, *Brassica rapa* L. var. *silvestris* (Lam.) Briggs, *Brassica rapa* L. subsp. *silvestris* (Lam.) Janch., *Brassica campestris* L., *Brassica campestris* L. subsp. *Campestris*) is widely distributed and the edible part is represented by the young shoots (sprouts plus inflorescences). In the Campania Region there are ecotypes of *Brassica rapa* L. subsp. *sylvestris* L. Janch. *var. esculenta* Hort. locally known as "friarielli", whereas in the Puglia Region there are ecotypes known as "cime di rapa". These two products represent two important commodities being part of very traditional recipes with a relevant economical impact.

Although these two vegetables present a very similar morphological aspect, the botanical classification is still a matter of dispute. Carlson et al. (1987a) report that brassicas are highly correlate with their glucosinolate pattern and then can be grouped considering this chemical characteristics. The aim of our project was to characterize different *Brassica* ecotypes and to detect molecular markers able to discriminate between them. The profile of flavonoids and phenolic acids is not significantly different (De Pascale, Maggio, Pernice, Fogliano, & Barbieri, 2007) therefore it was decided to investigate the glucosinolates profiles of different ecotypes of *friarielli* and cime di rapa that were compared, both qualitatively and quantitatively, with broccoli samples (*Brassica oleracea* L. *var. italica*) which are very well described in literature (Kushad et al., 1999; Rodrigues & Rosa, 1999; Vallejo, Tomás-Barberán, Gonzalez Benavente-García, & García-Viguera, 2003). A statistical multivariate analysis of data was used to contribute to a better understanding of the relationship between individual glucosinolates and *Brassica* species.

2. Materials and methods

2.1. Plant material and chemicals

Two commercial broccoli samples (*Brassica oleracea* L. *var. italic* cv.) named BROC1 and BROC2 and one of Cime di Rapa (*Brassica rapa* L. subsp. *sylvestris* L. Janch. *var. esculenta* Hort.) (CR) ecotypes were obtained at local market. Three *friarielli* (*Brassica rapa* L. subsp. *sylvestris* L. Janch. *var. esculenta* Hort.) ecotypes, *Lingua di Cane* (LC), *Sorrentino* (SOR) *Cinquantino* (CQ) were obtained from the Department of Agricultural Engineering and Agronomy, University of Naples "Federico II cultivated as previously described (De Pascale et al., 2007), while another variety of friariello (TG) was obtained by a local farmer.

The edible portions were cut at maturity, placed on ice and immediately transported to the laboratory where they were frozen and lyophilized.

All solvents were of HPLC grade. Sinigrin (allyl glucosinolate) was obtained from Sigma-Aldrich (USA).

2.2. Glucosinolates analyses

Glucosinolates were analysed after desulphation according with the procedure described by Kiddle et al. (2001), but with different chromatographic conditions. In particular, the desulphoglucosinolates were analysed by HPLC (Shimadzu LC 10, Shimadzu, Japan) at a flow rate of 1 ml/min, using a Prodigy column 5 μ ODS3 100A, 250 × 4.60 mm (Phenomenex, USA). The mobile phase was a mixture of ultra-pure water (A) and methanol (B). Desulphoglucosinolates elution was achieved using the following linear gradient: starting condition, 2% B; 5 min, 4% B; 20 min, 20% B; 30 min, 35% B; 35 min, 40% B; 45 min, 30% B; 50 min, 10% B; 52 min, 2% B. Chromatograms were recorded at 227 nm. 2-propenylglucosinolate (sinigrin) was used as internal standard.

LC-MS-MS analyses were performed by a LC/MS/MS System (API 3000, MDS SCIEX). The mass spectrometer is equipped with a Model 11 syringe pump (Harvard, Apparatus, Holliston, MA, USA) and with an APCI interface. The mass spectrometer was used exclusively in the triple quadrupole mode. Detection of the compounds was performed using IDA (information dependent acquisition), an artificial intelligence-based product ion scan mode, generating a survey scan, single MS spectra with molecular mass information, product ion spectra, and extracted ion fragmentograms (XIC). The APCI source was used in negative mode at temperature set at 400 °C. The collision-induced dissociation was carried out using nitrogen as collision gas. LC analysis were performed using a system consisted of a series 200 binary pump (Perkin–Elmer, USA). The analysis were performed using a Prodigy column 5 μ ODS3 100A, 250 × 4.60 mm (Phenomenex, USA) using gradient elution. Mobil phase A was H_2O, while mobile phase B consisted of MeOH. The linear solvent gradient was as follows: 0–5 min 98% A 2% B, 5–30 min 96% A 4% B, 30–35 min 60% A 40% B, 35–37 min 80% A 20% B, 37–40 min 98% A 2% B, 40–45 min 98% A 2% B returned to initial conditions. The acquisition has been carried out monitoring the transition of parent and product ions specific for each compound with a dwell time of 500 ms. To promote ionization of the precursor ion, the voltage applied was 4500 while the collision energy (CE) used 30 and collision cell exit potential (CXP) was 7.

Data acquisition and processing were performed using Analyst software 1.4.

2.3. Statistical analysis

The statistical analysis program SPSS 13.0 (LEAD TOOLS (c) 1991–2000, LEAD Technologies, Inc.) was used to calculate and plot results from Principal Component Analysis (PCA).

This analysis was applied with the purpose to determine the relationships among the glucosinolates content and the belonging to a precise Brassica group.

Factors with eigenvalues greater than 1 were selected. The varimax rotation method was applied. The outcome

of this analysis is presented as a two-dimensional (two PCs) scatter plot.

3. Results and discussion

Figs. 1 and 2 report the HPLC chromatogram obtained from one *Brassica oleracea* and two *Brassica rapa* samples, respectively. Peak identification was obtained by LC/MS/MS analysis. The molecular ion and the fragmentation patterns compared with literature information allowed the unequivocal identification of the species (Table 1).

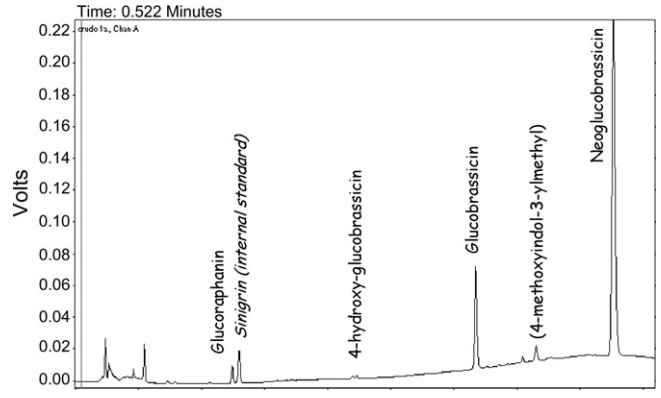

Fig. 1. Typical HPLC chromatogram of desulfoglucosinolates in broccoli.

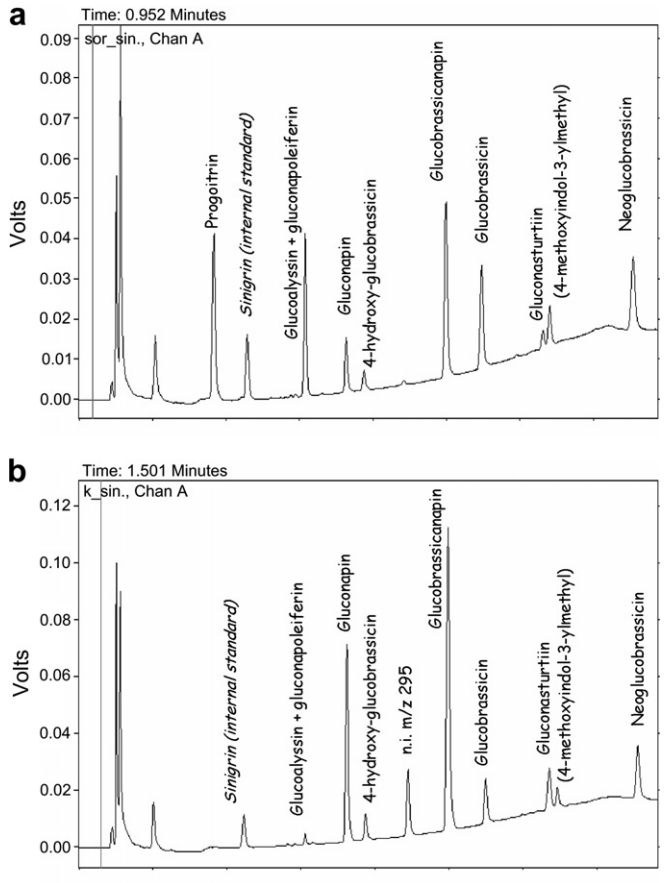

Fig. 2. Typical HPLC chromatogram of desulfoglucosinolates of two friarielli ecotypes: "Sorrentino" (panel a) and "Lingua di Cane " (panel b).

Comparing the chromatograms it is clear that "*Friarielli*" samples showed a very different chromatographic pattern respect to that detectable in broccoli sample. The sum of gluconapin and glucobrassicanapin represented 65–70% of the total glucosinolates, with the only exception of SOR variety in which they were present in lower amount (about 30% of the total); the remaining part of glucosinolates found were glucoalysin, 4-hydroxy-glucobrassicin, glucobrassicin, 4-methoxy-glucobrassicin and neoglucobrassicin. SOR also differed from the other samples for the presence of high amount of progoitrin (14% of the total glucosinolates), compound absent in the other "*friarielli*" samples. Interestingly, glucoraphanin, which is one of the most important glucosinolates in broccoli (Fahey, Zhang, & Talalay, 1997), was completely absent in "*friariello*" samples.

Table 2 summarizes the quantitative determinations of the glucosinolates in different *friarello* ecotypes in comparison with the broccoli samples.

Despite the abundance of scientific publications dealing with the glucosinolates content of broccoli a conclusive picture of their relative concentration is far to be established. It is worth to notice that the profile reported in this work is very similar to that observed by Vallejo et al. (2003), who performed many studies about the effect of agronomical parameters on broccoli glucosinolates profile. The most abundant glucosinolates found in the broccoli samples were glucobrassicin and neoglucobrassicin (1-methoxyindol-3-ylmethyl), followed by glucoraphanin; while 4-methoxy-glucobrassicin and 4-hydroxy-glucobrassicin were present in lower amount.

However previous studies (Kushad et al., 1999) reported that glucoraphanin is the main glucosinolate in broccoli, with a concentration 7-fold higher than that of glucobrassicin and also Rodrigues and Rosa (1999) report glucoraph-

Table 1
Identification data (MS/MS) of friarielli and broccoli desulphoglucosinolates

Compounds	Molecular mass	Retention time (min)
Friarielli		
Desulfosinigrin	279	11.2
Glucoalyssin + Desulfogluconapoleiferin	371 + 323	15.3
Desulfogluconapin	293	18.2
Desulfo-4-hydroxyglucobrassicin	384	19.4
Desulfoglucobrassicanapin	307	25.0
Desulfoglucobrassicin	368	27.5
Desulfogluconasturtiin	343	32.0
4-Methoxy-3-indolylmethyl dsg	421	34.0
1-Methoxy-3-indolylmethyl dsg	421	37.0
Broccoli		
Desulforaphan		12.4
Desulfosinigrin	279	13.0
Desulfoglucobrassicin	368	31.7
4-Methoxy-3-indolylmethyl dsg	421	34.0
1-Methoxy-3-indolylmethyl dsg	421	42.0

Table 2
Glucosinolate content in edible part of different *friarielli* and broccoli ecotypes

	BROC 1	BROC 2	LC	SOR	CQ	TG	CR
Progoitrin	–	–	–	2.8	–	–	–
Glucoraphanin	2.5	1.9	–	–	–	–	–
Glucoalyssin (+gluconapoleiferin)	0.1	0.1	0.5	2.2	0.7	0.5	–
Gluconapin	–	–	7.3	1.0	3.1	8.9	6.5
4-Hydroxy-glucobrassicin	0.8	0.6	0.8	0.3	0.4	0.7	0.4
Glucobrassicanapin	–	–	12.3	5.2	3.7	17.7	16.6
Glucobrassicin	26.9	10.0	1.5	4.3	0.9	3.3	4.2
4-Methoxy-glucobrassicin (+gluconasturtiin)	1.4	1.8	2.5	1.9	1.2	6.2	2.8
Neoglucobrassicin	4.6	57.7	3.9	3.1	1.5	2.5	3.3
Total glucosinolates	36.5	72.0	28.8	20.9	10.6	39.8	33.9

All values are given in μmol/g DW.

anin as the predominant glucosinolate in broccoli. Finally, Carlson, Daxenbichler, VanEtten, Kwolek, and William (1987b) found that in broccoli the concentration of glucoraphanin and glucobrassicin were quite similar. Literature works (Kushad et al., 1999; Rodrigues & Rosa, 1999; Vallejo et al., 2003) also reported that some broccoli cultivar can contain low amount of glucoiberin, glucoalyssin, gluconapin, glucobrassicanapin or progoitrin which were not present in our samples.

This variability is not surprising and can be explained considering first of all the different broccoli varieties used in these study which also interplay with other factors, such as growing conditions and agronomic practices that can affect plant glucosinolate profiles.

The data obtained for the nine glucosinolates detected were used for the Principal Component Analysis (PCA) which allow to reduce the dimensionality of a set of data. Two principal components (PC) with eigenvalues higher than one were selected by PCA. PC1 and PC2 explained 38.4% and 31.8% of the total variance, respectively.

As shown in Table 3, PC1 was highly contributed by gluconapin (0.910), glucobrassicanapin (0.927), 4-methoxy-glucobrassicin (0.676) and, but inversely correlated, by glucoraphanin (-0.797) and glucobrassicin (−0.651); PC2 was directly correlate with 4-hydroxy-glucobrassicin (0.755), strictly inversely related with glucoalyssin (−0.931) and progoitrin (−0.891).

The projection of the scatter diagram is reported in Fig. 3. The degree of overlapping between ellipses corresponding to the different samples analysed gives a good visual impression of the difference or similarity between them. The figure clearly shows that broccoli and *friarielli* can be grouped in separate ellipses, without overlapping, meanings that PCA could fully distinguish between these two species.

Broccoli samples (BROC1 and BROC2) are compactly clustered in the lower right quadrant, in the zone situated at the negative side of PC1 and the positive side of PC2; taking in account the negative loading of PC1 for glucoraphanin and glucobrassicin, it is possible to conclude that broccoli are characterized by the high presence of these two glucosinolates together with good amount of 4-hydroxy-glucobrassicin, represented by PC2.

Friarielli, instead, are more dispersed in the space of PCs. LC and the TG ecotypes together with CR samples can be grouped together as they all are located at the positive side of both PC1 and PC2. Considering the main contributing variables to PC1 and PC2, these three ecotypes are identified by high level of gluconapin, glucobrassicana-

Table 3
Rotated component matrix (Varimax with Kaiser normalization)

Glucosinolate	Component	
	PC1	PC2
Progoitrin	−0.070	**−0.891**
Glucoraphanin	**−0.797**	0.460
Glucoalyssin + gluconapoleiferin	0.065	**−0.931**
Gluconapin	**0.910**	0.362
4-Hydroxy-glucobrassicin	0.008	**0.755**
Glucobrassicanapin	**0.927**	0.193
Glucobrassicin	**−0.651**	0.331
4-Methoxy-glucobrassicin + gluconasturtiin	**0.676**	0.293
1-Methoxyindol-3-ylmethyl	**−0.499**	0.242

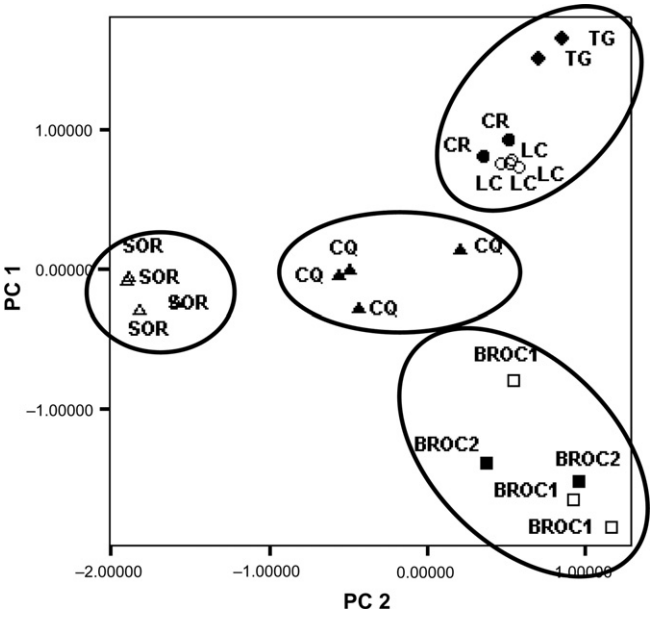

Fig. 3. Projection of the scatter diagram from a PCA, PC1 vs. PC2.

pin, 4-methoxy-glucobrassicin and 4-hydroxy-glucobrassicin. SOR samples showed a different behaviour as it was located at the very negative side of PC2; considering that this component was strongly negatively correlated with progoitrin and glucoalyssin, it can be maintained that SOR variety was actually explained by the high presence of both these glucosinolates.

CQ ecotypes was clustered in a more central zone of the figure, then it can be explained both by PC1 and PC2. This variety contained a good amount of gluconapin and glucobrassicanapin, but also of 4-methoxy-glucobrassicin and of glucoalyssin.

On the basis of the data obtained, gluconapin and glucobrassicanapin can be considered the real fingerprint of "*friarielli*" and a representative marker to differentiate them from the other Brassica vegetables and in particular from broccoli. Moreover, among the *friarielli* varieties analysed, SOR differentiates for the presence of good amount of progoitrin and glucoalysin that can be considered a further molecular marker of this ecotype.

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