Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 1761–1764
Food Chemistry
www.elsevier.com/locate/foodchem

HPLC screening of anti-cancer sulforaphane from important European *Brassica* species

G. Sivakumar [a], A. Aliboni [b], L. Bacchetta [a,*]

[a] *BIOTEC-GEN, ENEA, Casaccia, Rome, Italy*
[b] *BIOTEC-AGRO, ENEA, Casaccia, Rome, Italy*

Received 31 March 2006; received in revised form 23 August 2006; accepted 14 November 2006

Abstract

Natural sulforaphane has been of increasing interest for nutraceutical and pharmaceutical industries due to its anti-cancer effect. The sulforaphane contents of 14 different major European *Brassica oleracea* L. varieties (cauliflower Alverda, cauliflower di Jesi, cauliflower Minaret, broccoli Ramoso calabrese, white cabbage, palm cabbage Cuor di Bue, broccoli Primor, savoy cabbage riccio d'Asti, broccoli Eolo, Delicato, cauliflower Nuvola, cauliflower Palla di neve, San martino, Velox) and four different *Brassica rapa* L. varieties (turnip Senza Testa, cima di rapa 40, cima di rapa 90, cima di rapa 120) which are cultivated in Italy, were analysed during two different developmental stages (3rd and 9th day after germination) of the seedlings. A quantitative determination of sulforaphane in *Brassica* species seedlings was established by HPLC. The highest amount of sulforaphane (2.21 mg/g d w) was found in San martino 3-day old seedlings. Seedlings of this European *Brassica* variety may be considered as a potential new source of natural sulforaphane.

Keywords: Anti-cancer; Brassica; Glucosinolate; Sulforaphane

1. Introduction

Brassicaceae species are one of the most important European crops, including nutritionally important human and animal foodstuffs such as *Brassica oleracea* and *Brassica rapa* varieties. All these varieties contain a group of secondary metabolites called glucosinolates (Fahey, Zhang, & Talalay, 1997). Upon mechanical damage, infection or pest attack, cellular breakdown exposes the stored glucosinolates to endogenous degradative enzymes called myrosinases (Grubb & Abel, 2006). Products derived from glucosinolate breakdown exert a variety of biological activities in plants, animals, and humans, which range from the participation in plant defence against pathogens and herbivores to the prevention of cancers (Nestle, 1997). Myrosinase-catalysed hydrolysis of glucosinolates initially involves cleavage of the thioglucoside linkage, yielding D-glucose and an unstable thiohydroximate-O-sulfonate that spontaneously rearranges, resulting in the production of sulfate and one of a wide range of possible reaction products (Fahey, Wade, Stephenson, & Chou, 2003). The products are generally a thiocyanate, isothiocyanate and nitrile depending on such factors as substrate pH (Vaughn & Berhow, 2005), or the availability of ferrous ions (Halkier & Du, 1997). Isothiocyanates are usually produced at neutral pHs while nitrile production occurs at lower pHs (Mikkelsen, Petersen1, Olsen, & Halkier, 2002). The conversion of the glucosinolate glucoraphanin to sulforaphane mediated by myrosinase and the metabolism of sulforaphane to its mercapturic acid conjugate was reported by Keck, Qiao, and Jeffery (2003). Sulforaphane is one of a class of bioactive molecules called isothiocyanates. Its molecular formula is $C_6H_{11}NOS_2$, and its molecular weight is 177.29. It is also known as 4-methylsulfinylbutyl isothiocyanate and (–)-1-isothiocyanato-4(R)-(methylsulfinyl) butane. Sulforaphane, which is predominantly found in broccoli, may help to reduce the risk of developing several types of cancers (Stoner & Morese,

* Corresponding author. Tel.: +39 06 3048 3405; fax: +39 06 3048 6044.
E-mail address: loretta.bacchetta@casaccia.enea.it (L. Bacchetta).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.11.040

1997). It is an indirect antioxidant; it does not neutralize free radicals directly, but rather boosts Phase 2 enzymes that trigger ongoing and long-lasting antioxidant activity (Basten, Bao, & Williamson, 2002; Gao, Dinkova-Kostova, & Talalay, 2001). A large number of cohort and case-control studies have established that a high consumption of broccoli and cauliflower sprouts leads to a decreased risk of carcinomas of the lung, stomach, colon and rectum (Johnson, 2002). More recently, sulforaphane has been shown to have anticarcinogenic properties, unlike sulforaphane nitrile (Matusheski et al., 2006). Currently, various *Brassica* products are available, however, their sulforaphane concentrations are not ordinarily specified (Nakagawa et al., 2006). Therefore, the objective of this investigation is to examine 18 different European *Brassica* varieties, and measure the sulforaphane fraction of the glucosinolate present in the young seedlings.

2. Material and methods

2.1. Plant material

Seeds of 14 different European *Brassica oleracea* L. varieties (cauliflower Alverda (var. *botrytis*), cauliflower di Jesi (var. *botrytis*), cauliflower Minaret (var. *botrytis*), broccoli Ramoso calabrese (var. *botrytis*), white cabbage (var. *capitata*), palm cabbage Cuor di Bue (*acephala*), broccoli Primor (var. *italica*), savoy cabbage riccio d'Asti (var. *sabauda*), broccoli Eolo (var. *botrytis*), Delicato, cauliflower Nuvola (var. *botrytis*), cauliflower Palla di neve, San martino, Velox) and four different *Brassica rapa* L. varieties (turnip Senza Testa, cima di rapa 40, cima di rapa 90, cima di rapa 120) were obtained from Olter Seeds (Piedmont, Italy). In the greenhouse, seeds were germinated in trays of soil-less potting mix (1:1:1; peat, sand and vermiculite). The controlled environment chamber had a diurnal cycle of 16 h light at 24 °C and 8 h dark at 18 °C. This experiment was performed three times with different batches of seed, each replicate containing six samples. Seedlings were grown without any added nutrients, only spray watered two times per day until harvest. Seedlings were rapidly and gently collected from the surface of the pots on the 3rd and 9th days after germination. After harvesting, seedlings were washed toughly with running tap water and excess surface water was blotted away using tissue paper.

2.2. Sulforaphane extraction and purification

Sulforaphane was extracted from *Brassica oleracea* and *Brassica rapa* varieties by the methods of Aliboni, Gatti, Scarpone, and Scortichini (2006), Bertelli, Plessi, Braghiroli, and Monzani (1998) and Nakagawa et al. (2006) with slight modification. Briefly, after harvest, plant material was immediately frozen in liquid nitrogen, lyophilised to dryness, and ground to a fine powder with a Waring blender. The lyophilised *Brassica* sample (1 g) was suspended in 20 ml of 0.1 M HCl and incubated at 42 °C for 2 h in a shaking water bath (GFL 1083, Germany) in order to release sulforaphane from the glucosinolates. After that, the incubation mixture was three time extracted with 40 ml of dichloromethane and filtered through filter paper (Whatman No. 1) with anhydrous sodium sulfate. The extract was evaporated to dryness under vacuum at 35 °C and the residue was redissolved in 2 ml of dichloromethane (Chromasolv®, Sigma–Aldrich) into vials.

The organic extract was purified by Supelclean™ LC-Si SPE 3 ml disposable columns. The extraction cartridge was inserted on an Supelco vacuum manifold column processor (Bellefonte, PA, USA). The flow rate was adjusted to 1.0 ml/min by means of a vacuum pump (Millipore, USA). Before loading the extract onto the silica tube, the cartridge was rinsed with 3 ml of dichloromethane. After the extract was loaded onto the cartridge, sulforaphane was purified by passing through 3 ml of ethyl acetate, and the eluent was discarded. Sulforaphane was recovered by passing 3 ml of methanol (Chromasolv). The methanol extract was filtered through a 0.45 μm millipore PTFE membrane filter (Advantec MFS, Inc., USA).

2.3. High-performance liquid chromatography (HPLC)

Purified extracts were analysed to determine the amount of sulforaphane, using reversed phase HPLC. The HPLC system consisted of a Perkin Elmer series 200 quaternary pump and a Perkin Elmer series 200 UV–VIS detector. Data acquisition was carried out on a PC equipped with TotalChrom software (v 6.2.0.0.1) and interfaced to the HPLC system through a Perkin Elmer Series 600 link interface. Analysis were carried out with a 70% acetonitrile/30% water isocratic elution on a Supelco Supelcosil™ LC-18 (25 cm × 4.6 mm, 5 μm) column, with a flow of 1.00 ml/min. Detector wavelength was fixed at 205 nm. Under these conditions, retention time of sulforaphane was found to be 5.68 min. Sulforaphane peak identity was assessed by comparing the retention time of the peak in the specimen with the retention time of a standard. Quantitative analysis was carried out with a calibration curve prepared over the expected analytical concentration range.

2.4. Statistical analysis

Three replicates were performed for each sample. Every analysis was repeated six times. Statistical analysis was performed using the SAS System (Version 6.21, SAS Institute Inc. Cary, NC 27513, USA). Statistical significance of the differences observed among mean values was assessed using a Duncan's multiple range test. A probability of $P \leqslant 0.05$ was considered significant.

3. Results and discussion

The differences in glucosinolate profiles between young seedlings and mature broccoli are of considerable nutraceu-

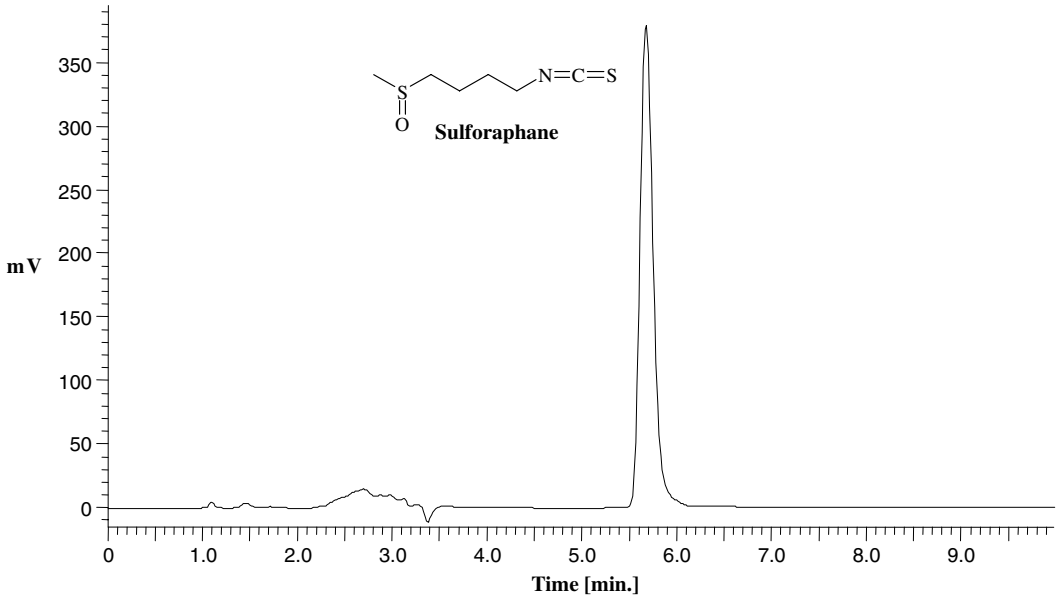

Fig. 1. HPLC chromatogram of sulforaphane from *Brassica* varieties.

tical interest and potential importance in devising chemoprotective strategies in humans. 3-day-old broccoli and cauliflower seedlings contain 20 times more glucosinolates (glucoraphanin and glucoerucin) compared with mature broccoli (Fahey et al., 1997). Large quantities of inducers of enzymes that protect against carcinogens can be delivered in the diet by small quantities of young *Brassica* seedlings (e.g., 3-day-old broccoli sprouts) that contain as much inducer activity as up to 100 times larger quantities of mature *Brassica oleracea* and *Brassica rapa* (Fahey et al., 1997). Sulforaphane could only be detected in trace amounts in mature broccoli (Nakagawa et al., 2006). Sulforaphane from chemical synthesis requires several highly toxic substances, and final products from these reactions still contain toxic residues and require further purification. This disadvantage limits the use of synthesised sulforaphane as a food additive (Liang, Yuan, & Xiao, 2005).

To separate the sulforaphane fraction, the freeze-dried samples was treated with 0.1 M HCl and incubated at 42 °C for 2 h in a shaking water bath to hydrolysis the glucosinolate into sulforaphane. Acid hydrolysis performed before extraction consistently enhances the yield of sulforaphane (Bertelli et al., 1998). This method is much faster than any other hydrolysis procedure with better recovery of sulforaphane. From the dichloromethane extract, the sulforaphane fraction was purified by silica column. This method, evaluated through a repetitive analysis of *Brassica* sps. extracts, shows that the sulforaphane extraction, purification and quantification can be considered accurate (Fig. 1). Our purification method can be used to isolate naturally-occurring sulforaphane from *Brassica* seedlings with the advantages inherent to this technique such as a speed and reproducibility.

Table 1 shows the contents (mg/g dry weight) for sulforaphane in some important European *Brassica oleracea* and *Brassica rapa* seedlings. Three-day old San martino seedlings showed the highest sulforaphane value (2.21), whereas turnip Senza Testa, cauliflower Alverda, cauliflower Nuvola, broccoli Eolo and cauliflower di Jesi (0.93; 0.92; 0.91; 0.87; and 0.81, respectively) recorded the lowest. Broccoli Primor, broccoli Ramoso calabrese, palm

Table 1
Sulforaphane content from some important European *Brassica* varieties *in vivo*

Brassica varieties	Sulforaphane content (mg/g d w)	
	3-day-old seedlings	9-day-old seedlings
Brassica oleracea L.		
Cauliflower Alverda (var. *botrytis*)	0.92 g[A]	0.14 f
Cauliflower di Jesi (var. *botrytis*)	0.81 g	0.54 e
Cauliflower Minaret (var. *botrytis*)	1.20 f	0.66 e
Broccoli Ramoso Calabrese (var. *botrytis*)	1.74 b	1.60 a
White cabbage (var. *capitata*)	1.67 c	0.84 d
Palm cabbage Cuor di Bue (*acephala*)	1.71 b	1.11 c
Broccoli Primor (var. *italica*)	1.82 b	1.31 b
Savoy cabbage riccio d'Asti (var. *sabauda*)	1.35 e	0.85 d
Broccoli Eolo (var. *botrytis*)	0.87 g	0.24 f
Delicato	1.32 e	0.79 d
Cauliflower Nuvola (var. *botrytis*)	0.91 g	0.17 f
Cauliflower Palla di neve	1.50 d	0.93 c
San martino	2.21 a	1.53 a
Velox	1.39 e	0.76 d
Brassica rapa L.		
Turnip Senza Testa	0.93 g	0.61 e
Cima di rapa 40	1.15 f	0.92 c
Cima di rapa 90	1.62 c	1.22 b
Cima di rapa 120	1.05 f	1.01 c

[A] Mean separation within columns by Duncan's multiple range test, $P \leqslant 0.05$.

cabbage Cuor di Bue, white cabbage, cima di rapa 90, Palla di neve, Velox, savoy cabbage riccio d'Asti, Delicato, cauliflower Minaret, cima di rapa 40, and cima di rapa 120 (1.82; 1.74; 1.71; 1.67; 1.62; 1.50; 1.39; 1.35; 1.32; 1.20; 1.15 and 1.05, respectively) sulforaphane contents were significantly less when compared to San martino but higher than turnip Senza Testa, cauliflower Alverda, cauliflower Nuvola, broccoli Eolo and cauliflower di Jesi. Nine-day-old seedlings recorded significantly lower sulforaphane contents compared to 3-day old seedlings (Table 1). Sulforaphane concentration tended to decrease during the growth of the seedlings. This was also noted by other workers (Jeffery et al., 2003; Nakagawa et al., 2006). Young seedlings, may be undergoing greater stress or the associated genes might be highly expressed at such a young stage, and so develop greater sulforaphane levels. To date there are no reports giving a reason for higher accumulation of sulforaphane in young seedling and decrease during the growth of the seedlings.

In conclusion, our HPLC screening data from major European *Brassica* varieties could allow natural manipulation of ruminant diets to increase uptake of natural sulforaphane by humans. Furthermore, the selection of *Brassica* varieties could result in new natural sources high in sulforaphane. The present data may be applicable to the use of sulforaphane-rich *Brassica* varieties as functional foods.

Acknowledgments

The authors acknowledge the CNR-MIUR for the funding of this work through the INAGRIMED Project, ENEA, Rome, Italy. We also thank Olter Seeds Company Piedmont, Italy, for providing seed materials and G. Zappa for solid phase extraction facilities.

References

Aliboni, A., Gatti, R., Scarpone, R., Scortichini, G., & Zappa G. (2006). Improved method for reverse-phase HPLC determination of sulforaphane *in Brassica. Private Communication*.

Basten, G. P., Bao, Y., & Williamson, G. (2002). Sulforaphane and its glutathione conjugate but not sulforaphane nitrile induce UDP-glucuronosyl transferase (UGT1A1) and glutathione transferase (GSTA1) in cultured cells. *Carcinogenesis, 23*(8), 1399–1404.

Bertelli, D., Plessi, M., Braghiroli, D., & Monzani, A. (1998). Separation by solid-phase extraction and quantification by reverse phase HPLC of sulforaphane in broccoli. *Food Chemistry, 63*, 417–421.

Fahey, J. W., Zhang, Y., & Talalay, P. (1997). Broccoli sprouts: An exceptionally rich source of inducers or enzymes that protect against chemical carcinogens. *Proceedings of the National Academy Science, 94*, 10367–10372.

Fahey, J. W., Wade, K. L., Stephenson, K. K., & Chou, F. E. (2003). Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography. *Journal of Chromatography A, 996*, 85–93.

Gao, X., Dinkova-Kostova, A. T., & Talalay, P. (2001). Powerful and prolonged protection of human retinal pigment epithelial cells, keratinocytes, and mouse leukemia cells against oxidative damage: The indirect antioxidant effects of sulforaphane. *Proceedings of the National Academy Science, 98*, 15221–15226.

Grubb, C. D., & Abel, S. (2006). Glucosinolate metabolism and its control. *Trends Plant Science, 11*(2), 89–100.

Halkier, B. A., & Du, L. (1997). The biosynthesis of glucosinolates. *Trends Plant Science, 2*(11), 425–431.

Jeffery, E. H., Brown, A. F., Kurilich, A. C., Keck, A. S., Matusheski, N., Klein, B. P., et al. (2003). Variation in content of bioactive components in broccoli. *Journal of Food Composition Analysis, 16*, 23–330.

Johnson, I. T. (2002). Glucosinolates in the human diet: Bioavailability and implications for health. *Phytochemistry Reviews, 1*, 183–188.

Keck, A. S., Qiao, Q., & Jeffery, E. H. (2003). Food matrix effects on bioactivity of broccoli-derived sulforaphane in liver and colon of F344 rats. *Journal of Agricultural Food Chemistry, 51*, 3320–3327.

Liang, H., Yuan, Q., & Xiao, Q. (2005). Purification of sulforaphane from Brassica oleraceaseed meal using low-pressure column chromatography. *Journal of Chromatography B, 828*, 91–96.

Matusheski, N. V., Swarup, R., Juvik, J. A., Mithen, R., Bennett, M., & Jeffery, E. H. (2006). Epithiospecifier protein from broccoli (Brassica oleracea L. ssp. italica) inhibits formation of the anticancer agent sulforaphane. *Journal of Agricultural Food Chemistry, 54*, 2069–2076.

Mikkelsen, M. D., Petersen1, B. L., Olsen, C. E., & Halkier, B. A. (2002). Biosynthesis and metabolic engineering of glucosinolates. *Amino Acids, 22*, 279–295.

Nakagawa, K., Umeda, T., Higuchi, O., Tsuzuki, T., Suzuki, T., & Miyazawa, T. (2006). Evaporative light-scattering analysis of sulforaphane in broccoli samples: Quality of broccoli products regarding sulforaphane contents. *Journal of Agricultural Food Chemistry, 54*, 2479–2483.

Nestle, M. (1997). Broccoli sprouts as inducers of carcinogen-detoxifying enzyme system: Clinical, dietary and policy implications. *Proceedings of National Academy Science, 94*, 11149–11151.

Stoner, G. D., & Morese, M. A. (1997). Isothiocyanates and plant polyphenols as inhibitors of lung and espophageal cancer. *Cancer Letter, 114*, 113–119.

Vaughn, S. F., & Berhow, M. A. (2005). Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Industrial Crops and Products, 21*, 193–202.