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High performance liquid chromatography protocol for glucosinolate profiling in *Brassica* L. species

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Isothiocyanates, the degradation products of glucosinolates reported specifically in the members of Brassicaceae family are potential alternative compounds to currently used fumigants. In the present study, six *Brassica* species, viz. *Brassica alba* (L.) Rabenh, *B. nigra* (L.) W. D. J. Koch (cv. 'Banarasi rai'), *B. napus* L. (cv. 'PPNS-1'), *B. rapa* L. (cv. 'RESBR-240'), *B. juncea* (L.) Czern. (cv. 'Kranti') and *B. carinata* A. Braun (cv. 'Kiran') were evaluated for Sinigrin (one of the most important glucosinolates found in *Brassica*) content using High Performance Liquid Chromatography. Sinigrin concentration in different *Brassica* species ranged between 3.65 µM/g in *B. napus* L. to 16.42 µM/g in *B. juncea* (L.) Czern. (cv. 'Kranti'). Therefore, *B. juncea* (L.) Czern. (cv. 'Kranti') was found as the most appropriate *Brassica* species to be used as biofumigant followed by *B. nigra* (L.) W. D. J. Koch (cv. 'Banarasi rai') and *B. alba* (L.) Rabenh.

Keywords: Biofumigation, *Brassica* sp., Glucosinolate, HPLC.
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Introduction

Over the last few decades, the importance of nitrogen and sulfur containing plant secondary metabolites has increased following the discovery of their potential as cancer-prevention agents and crop-protection compounds in agriculture[1]. For such a widely studied group of plant compounds, glucosinolates (GSLs) are known from only a few Angiosperm families i.e. Brassicaceae, Capparaceae, and Caricaceae[2]. Curiously, GSLs are also known from *Drypetes* Vahl (Euphorbiaceae), a genus completely unrelated to the other GSLs containing families[1]. GSLs, earlier known as mustard oil glucosides are part of human life for more than thousand years because of the strong flavours and tastes they bring forth in cabbage, broccoli and other *Brassica* vegetables.

Approximately 120 described GSLs reported till date, share a chemical structure consisting of a β-D-glucopyranose residue linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester, plus a variable R group derived from one of the eight amino acids[3]. GSLs themselves have very limited or no biological activity. When plant tissue is disturbed by crushing, cutting, or other physical damage, GSLs are brought into contact with the endogenously present enzyme myrosinase (thioglucoside glucohydrolase), which rapidly hydrolyzes them in to several compounds like isothiocyanates (ITCs), organic cyanides, oxazolidinethiones, nitriles and ionic thiocyanates[4,5]. Among the degradation products of GSLs, the ITCs have been generally reported as the most biologically active, being recognized since early in the twentieth century as broad-spectrum biocides[6]. The type of isothiocyanate generated corresponds to the type of GSLs substrate, as characterized by its side-chain. Sinigrin (2-Propenyl GSL; MW-359.03), with hydrolysis products ITCs and nitriles, is known to be one of the major active members in GSLs group. Characteristics of ITCs i.e. their occurrence in nature conveniently and biocidal activity have made them to be recognized worldwide for their pest suppression potential when released from crop residues. ITCs and nitriles have been demonstrated to control several fungi[7,8], bacteria[9], nematodes[10], insects[11] and some weed seeds in laboratory experiments[8].

The antimicrobial activity of any of the *Brassica* or other genus/species is directly correlated to the amount of ITCs present, which is further decided by the corresponding GSLs in it. Therefore, utilization of

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any *Brassica* sp. for pest suppression depends upon its GSLs profile. Various methods reported for the estimation of GSLs include spectroscopic, enzyme immobilization, gravimetric, X-ray fluorescence, gas liquid chromatographic and high performance liquid chromatographic methods. Of these, High performance liquid chromatography (HPLC) has many advantages over the others. Because of anionic nature of GSLs they can be separated by reversed-phase HPLC (RP-HPLC) as desulfated compounds i.e. desulfoglucosinaltes. Therefore the present study aimed to determine the glucosinolate (Sinigrin) content in six different species of *Brassica* namely *Brassica alba* (L.) Rabenh, *B. nigra* (L.) W. D. J. Koch (cv. 'Banarasi rai'), *B. napus* L. (cv. 'PPNS-1'), *B. rapa* L. (cv. 'RESBR-240'), *B. juncea* (L.) Czern. (cv. 'Kranti') and *B. carinata* A. Braun (cv. 'Kiran'), grown in Tarai region of Uttarakhand, India using RP-HPLC.

Materials and Methods

Sample preparation

Six *Brassica* species viz. *B. alba* (L.) Rabenh, *B. nigra* (L.) W. D. J. Koch (cv. 'Banarasi rai'), *B. napus* L. (cv. 'PPNS-1'), *B. rapa* L. (cv. 'RESBR-240'), *B. juncea* (L.) Czern. (cv. 'Kranti') and *B. carinata* A. Braun (cv. 'Kiran') grown at Norman E. Borlaug–Crop Research Centre, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India were uprooted at 50 % flowering stage and taken to laboratory where they were separated into root, stem and leaves, freeze dried and stored at -20 ^{o}C till sample preparation. Samples for HPLC analysis were prepared by a new protocol, modified from the previously described methodology[12]. 500 mg of freeze dried *Brassica* leaves were crushed into fine powder using pestle and mortar in liquid nitrogen. This powder was then added to 50 mL plastic centrifuge tube having 10 mL hot 70 % methanol. The tubes were caped, shaken vigorously and placed in water bath for 20 min at 70 ^{o}C. After cooling to room temperature, the tubes were centrifuged at 3000 rpm for 6 min. Three mL of the supernatant was then applied to a prepared 0.5 cm plug of Sephadex A-25 poly-prep columns (Biorad Laboratories, CA, USA).

Column preparation and desulfation

One gram of Sephadex A-25 (Sigma Aldrich Co. USA) was added to 20 mL of milli-Q water and left overnight. Next day, Sephadex was added up to the thickness of 0.5 cm into the poly-prep columns fitted with the stand. The Sephadex was then washed with 1 mL milli-Q water followed by 1 mL 0.2 M sodium acetate (pH-5). After discarding water and buffer, 100 µL aliquot of prepared sulfatase (Sigma Aldrich Co. USA) was added to the column. After 1 drop of buffer was displaced off, 3 mL of supernatant prepared earlier was added to each column for desulfation of GSLs and the column was end capped and left overnight. Next day 1 mL milli-Q water was added to the column and the effluent was collected in HPLC vial. The samples were stored frozen until analysis.

Standard dilution and desulfation

Sinigrin (2-Propenyl GSL), as glucosinolate standard, purchased from ChromaDex Inc. (Irvine CA, USA) was used in the study. One mg of the standard was added to 1 ml of milli-Q water to prepare stock solution of 1000 ppm, which was further diluted to 500, 400, 300, 200 and 100 ppm solutions for calibration purpose. The desulfation of the standards was also carried out by adopting the same protocol used for the samples.

HPLC analysis

HPLC experiments were performed on a Dionex UltiMate 3000 intelligent LC series HPLC instrument with an UltiMate 3000 VWD Series Variable Wavelength Detectors and UltiMate 3000 x 2 Dual LC systems with Dual-Gradient Pump. A reversed-phase Acclaim 120, C18 column (2.1 x 50 mm i.d., 3 µm Analytical) was used for separation of the desulfoglucosinolates in six *Brassica* sp. Ten µL of the aqueous sample extract was injected into the HPLC system by 1000 µL WPS-3000 and ACC-3000 Series Syringe. Individual desulfoglucosinolates were detected by the VWD Series Variable Wavelength Detectors at a UV wavelength of 229 nm. Chromeleon version 6.80 was used to control the operation of the system. A gradient program was used for sufficient retention and baseline separation of the desulfoglucosinolates, in which the mobile phase consisting of a mixture of water (A) and acetonitrile (B) was operated at a constant flow rate of 0.8 mL/min. During the initial time period (8 min) the eluent contained 0 % B, and from 8 to 24.5 min a linear eluent gradient to yield 20 % B was adjusted. From 24.5 to 28 min a further increase of B up to 25 % was set. From 28 to 33 min percent of B in the eluent was reduced to 0 % through a linear gradient. Finally, after a period of 33 min, the column was equilibrated for 10 min at the end of each run[13].

Chromatograms were obtained by UV-detection at 229 nm and the peaks were quantified.

The desulfoglucosinolate standard was separated completely under these conditions and its corresponding retention time (5.7 min) was recorded. By comparing the retention times of the desulfoglucosinolate standard with those of the sample extracts, the presence of the desulfoglucosinolate in the sample extracts was identified.

Determination of desulfoglucosinolates concentration

The peak areas of the identified desulfoglucosinolates in the sample extracts were recorded and from this data calibration curve was prepared in Microsoft Excel. The correlation equation obtained from the curve was used for quantitative analysis of Sinigrin in plant extracts by comparing the peak areas of Sinigrin in sample with the peak area of Sinigrin in standard. Peaks other than that of Sinigrin were removed using Chromeleon programme itself. Each sample was replicated thrice to reduce chances of error. The experimental data was analyzed using software test procedure-3, as per the procedure of completely randomized design, critical difference at 1 % and Standard error of mean were calculated.

Results

The most popular glucosinolate, Sinigrin in six *Brassica* species was analyzed using HPLC by comparing the retention times (5.7 min) of the sample extracts with that of the glucosinolate standard. However, the retention times of the GSLs in the sample extracts varied a little, which may be due to the complicated matrices in the sample extracts. By comparing the peak areas of the corresponding retention times in the chromatogram of original sample extract with that of the standard the glucosinolate was quantified.

A calibration curve for standard was obtained by plotting the peak areas against concentrations of the standard. The graph of the calibration curve for Sinigrin and the summary of the equation and its corresponding correlation coefficients (R^2 values) for Sinigrin are shown in Fig. 1. According to the R^2 value (0.9998) of the calibration curve, the linearity of calibration curve was found to be acceptable. Equation of the calibration curve was found as Y = 4.402 X, where Y and X are peak area of Sinigrin from *Brassica* samples and concentration of Sinigrin in samples, respectively. The method of quantification of Sinigrin in *B. alba* (L.) Rabenh is described below.

Sinigrin in *B. alba* (L.) Rabenh extract was quantified as follow:

X = Y/4.402

Y = Peak area of Sinigrin in *B. alba* (L.) Rabenh extract (1082.26)
X = 1082.265/4.402

By substituting the peak area of Sinigrin in *B. alba* (L.) Rabenh extract into the equation of the calibration curve for Sinigrin, the concentration of Sinigrin in 10 mL sample extract was determined.

Therefore, X = 245.858 ppm (mg/L)

Thus, weight of Sinigrin in 0.5g *B. alba* (L.) Rabenh extract

= Concentration of Sinigrin x diluted factor
= 245.858 ppm x 10 x 10^{-3} L
= 2.46 mg

Concentration of Sinigrin per gram of dry weight in *B. alba* (L.) Rabenh = Weight of Sinigrin/weight of *B. alba* (L.) Rabenh

= 2.46 mg/0.5g
= 4.92 mg/g tissue
=13.70 µM/g (Molecular weight of Sinigrin 359.03)

Therefore, the concentration of Sinigrin in *B. alba* (L.) Rabenh was found to be 13.70 µM/g of dry weight. Similarly, Sinigrin concentration in other samples was also calculated. Sinigrin concentration in the *Brassica* samples ranged between 3.65-16.42 µM/g of dry weight (Table 1). Maximum Sinigrin content was recorded from *B. juncea* (L.) Czern. (16.42 µM/g) followed by *B. nigra* (L.) W. D. J. Koch (14.6 µM/g) and *B. alba* (L.) Rabenh (13.69 µM/g). Sinigrin concentration was minimum in *B. napus* L. (3.65

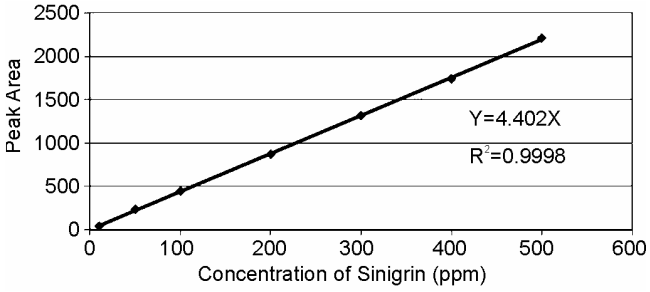

Fig. 1—Calibration curve for Sinigrin (standard). Calibration curve was prepared by plotting the peak areas against concentrations of the standard.

Table 1—Sinigrin concentrations in *Brassica* species

Glucosinolate	*Brassica* species					
	B. alba (L.) Rabenh	*B. nigra* (L.) W. D. J. Koch	*B. napus* L.	*B. rapa* L.	*B. juncea* (L.) Czern.	*B. carinata* A. Braun
Sinigrin concentration (μM/g dry weight)	13.69[a]	14.6[a]	3.65[b]	10.04[c]	16.42[d]	12.78[a]
CD at 1 %	1.61					
CV	5.46					
SEM	0.37					

Row values with the same superscript letter are not different statistically (at P≤1 %)

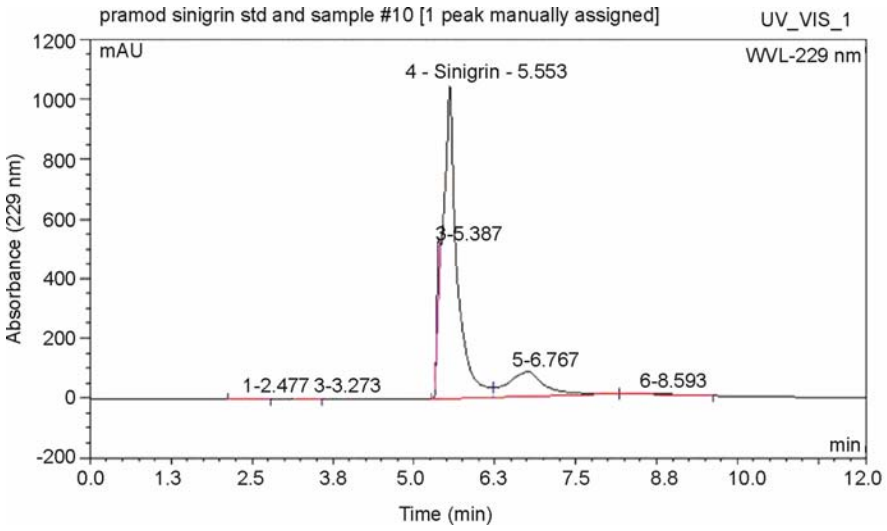

Fig. 2—Chromatogram of Sinigrin in *B. alba* (L.) Rabenh. UV detector was operated at 229 nm and the retention time was 5.7.

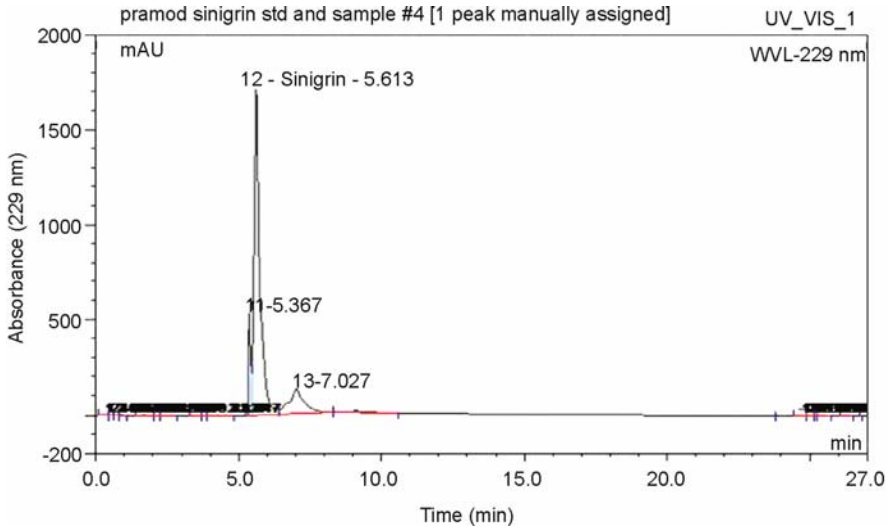

Fig 3—Chromatogram of Sinigrin in *B. nigra* (L.) W. D. J. Koch. UV detector was operated at 229 nm and the retention time was 5.7.

μM/g). Chromatograms for Sinigrin in *B. alba* (L.) Rabenh and *B. nigra* (L.) W. D. J. Koch samples are depicted in Fig. 2 and 3, respectively.

Discussion

Brassicaceous food crops contain different types and quantities of GSLs in their tissues. These compounds

co-occur with myrosinase isoenzymes, which catalyze the hydrolysis of the β-D-thioglucopyranoside bond releasing a variety of biologically active products like ITCs. GSLs and products thereof have various physiological and biological effects, including beneficial effects on human health, biocidal effect on crop pests including pathogens, insects and weeds, which have increased the interest on the presence of these compounds in *Brassica* crops.

Evidence suggests that ITCs and other volatile glucosinolate-derived compounds play a significant role in the short-term (<10 days) suppressive effects of macerated *Brassica* tissues on bacterial wilt in field studies[14]. Brassicas containing higher levels of more volatile isothiocyanate precursor GSLs are more suppressive than those with lower concentrations or less volatile types[15]. Brassicas, high in glucosinolate, would be the most suppressive biofumigants if conditions conducive to isothiocyanate release could be satisfied in the field[16]. Careful selection of Brassicas high in the most toxic ITCs that are least absorbed by soil and management of the incorporation process to maximize their release in soil could result in significant levels of biofumigation-based control in the field[17]. Sinigrin, one of the important glucosinolate in respect of pathogen suppression was quantified in six *Brassica* sp. using HPLC.

Sinigrin has already been reported as the major glucosinolate in edible cabbage and cauliflower heads[18], seeds and seedlings with values ranging from 6 mmol/g DM to 125 mmol/g DM[19] depending upon the cultivar and the tissue assessed. Sinigrin content may range from 0.1-26 µM/g of dry mass of different *Brassica* species[12]. In this study Sinigrin content in all *Brassica* species tested ranged between 3.65-16.42 µM/g of dry weight. Maximum Sinigrin content was recorded from *B. juncea* (L.) Czern. followed by *B. nigra* (L.) W. D. J. Koch and *B. alba* (L.) Rabenh. Patel *et al*, also reported high Sinigrin levels in mustard greens, *B. juncea* (L.) Czern.[20]. Whereas, using anion exchange membrane extraction and HPLC, Szmigielska and Schoenau reported low Sinigrin concentration in seeds of Indian mustard, *B. juncea* (L.) Czern.[21]. However, their results showed a high concentration of Sinigrin in brown and oriental mustard seeds. Sinigrin concentration was minimum in *B. napus* L. Similarly, Rangkadilok *et al* found Sinigrin in most of the *Brassica* species analyzed i.e. *B. nigra* (L.) W. D. J. Koch, *B. oleracea*, and *B. juncea* (L.) Czern. but it was absent in *B. rapa* L. and *B. napus* L. genotypes[19].

Conclusion

The study suggests that *B. juncea* (L.) Czern. (cv. 'Kranti') followed by *B. nigra* (L.) W. D. J. Koch (cv. 'Banarasi rai') and *B. alba* (L.) Rabenh could be good choice as biofumigants for the management of soil borne plant pathogens and insect pests whereas *B. napus* L. (cv. PPNS-1) with small Sinigrin content would be less effective.

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