



Short communication

Comparative study between extraction techniques and column separation for the quantification of sinigrin and total isothiocyanates in mustard seed

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ABSTRACT

Glucosinolates are β -thioglycosides which are found naturally in Cruciferae including the genus *Brassica*. When enzymatically hydrolysed, glucosinolates yield isothiocyanates and give a pungent taste. Both glucosinolates and isothiocyanates have been linked with anticancer activity as well as antifungal and antibacterial properties and therefore the quantification of these compounds is scientifically important. A wide range of literature exists on glucosinolates, however the extraction and quantification procedures differ greatly resulting in discrepancies between studies. The aim of this study was therefore to compare the most popular extraction procedures to identify the most efficacious method and whether each extraction can also be used for the quantification of total isothiocyanates. Four extraction techniques were compared for the quantification of sinigrin from mustard cv. Centennial (*Brassica juncea* L.) seed; boiling water, boiling 50% (v/v) aqueous acetonitrile, boiling 100% methanol and 70% (v/v) aqueous methanol at 70 °C. Prior to injection into the HPLC, the extractions which involved solvents (acetonitrile or methanol) were freeze-dried and resuspended in water. To identify whether the same extract could be used to measure total isothiocyanates, a dichloromethane extraction was carried out on the sinigrin extracts. For the quantification of sinigrin alone, boiling 50% (v/v) acetonitrile was found to be the most efficacious extraction solvent of the four tested yielding 15% more sinigrin than the water extraction. However, the removal of the acetonitrile by freeze-drying had a negative impact on the isothiocyanate content. Quantification of both sinigrin and total isothiocyanates was possible when the sinigrin was extracted using boiling water. Two columns were compared for the quantification of sinigrin revealing the Zorbax Eclipse to be the best column using this particular method.

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1. Introduction

Glucosinolates are β -thioglycosides and can be found in cruciferous vegetables including the genus *Brassica*. The enzyme-catalysed hydrolysis of glucosinolates involves the cleavage of the thioglucoside linkage, yielding D-glucose and an unstable thiohydroximate-O-sulphonate. This unstable compound spontaneously rearranges, resulting in the production of sulphate and either a thiocyanate, isothiocyanate or nitrile, depending on substrate, pH or availability of ferrous ions [1]. The main glucosinolate in mustard (*Brassica juncea* L.) is sinigrin and when hydrolysed by the enzyme myrosinase (following tissue damage), yields allyl isothiocyanate which is important for flavour and pungency [2,3].

Glucosinolates are important bioactives as they have suggested health promoting properties. An increase in the consumption of cruciferous vegetables has been associated with a reduced risk of

lung, stomach, colorectal, breast, bladder and prostate cancer as well as a reduced risk of myocardial infarction [4,5]. Although other potentially important compounds are present in cruciferous vegetables, many cell culture and animal model studies have supported the link between these health benefiting properties and glucosinolates and their degradation products [4].

Several extraction techniques for the quantification of sinigrin exist in the literature; Tsao et al. [6] compared five extraction techniques including Soxhlet extraction, boiling 50% (v/v) acetonitrile followed by reflux, boiling or room temperature 50% (v/v) acetonitrile and room temperature water. The authors found that different methods were more efficacious depending on whether the mustard was in seed or bran form. During sinigrin quantification, it is important that the enzyme, myrosinase, responsible for hydrolysis, is denatured prior to extraction. Myrosinase activity can be reduced at high temperatures, this said, heating prior to extraction may not completely inhibit the enzyme when the moisture level of the seed is above 8%. Also, heating to 100 °C for 1 h does not completely destroy all enzymes and depending on the pH, sinigrin can be degraded into allyl isothiocyanate in the absence of myrosinase in an aqueous solution [6].

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Isothiocyanates and nitriles are often extracted using hydrodistillation using a Clevenger-style system and analysed using gas chromatography. This process can be laborious and is carried out at high temperatures which can cause thermal degradation of glucosinolates into volatile aglucones and also requires a high volume of plant material [7]. Simpler techniques exist for the quantification of isothiocyanates, the simplest of which involves HPLC quantification of total isothiocyanates by spectrophotometrically measuring the reaction product (1,3-benzodithiole-2-thione) of the isothiocyanates with 1,2-benzenedithiol [8]. Although this method is simple and rapid, the reaction is fully quantitative with nearly all isothiocyanates except for those in which the linking carbon of the R-group ($R-N=C=S$) is tertiary [9]. Many techniques for the extraction and quantification of glucosinolates in *Brassica* species have been published making it difficult to compare results between authors. The aim of this study was therefore to compare the efficacy of the most popular extraction techniques for the quantification of sinigrin and investigate whether total isothiocyanates can also be extracted effectively using the same method. Furthermore, column types (stationary phase) were compared.

2. Materials and methods

2.1. Sinigrin extraction

Canadian brown mustard cv. Centennial seed harvested in 2010 was crushed using a hand held pestle and mortar in liquid nitrogen to prevent myrosinase activity following cell damage. The crushed seed was then weighed (150 mg) out into brown glass vials pre-cooled in liquid nitrogen to again prevent enzyme activity and stored at -40°C until extraction. Three replicate samples were analysed for each of the four extractions giving a total of 12 samples. For each extraction method, 3 mL of solvent was added to each vial. Extraction Method 1 (EM1) was carried out according to Kaushik and Agnihotri [3] where 3 mL of boiling water was added to each of the three frozen replicate samples and placed in boiling water for a further 15 min. Extraction Method 2 (EM2) was carried out according to Gerendás et al. [10] where 3 mL of 70% (v/v) aqueous methanol heated to 70°C was added to the replicate samples and incubated for a further 10 min at 70°C in a shaking water bath. Tsao et al. [6] compared five different extraction methods however only the extraction using boiling 50% (v/v) aqueous acetonitrile for 30 min was adopted for Extraction Method 3 (EM3). According to Tsao et al. [6], the extraction method with 50% (v/v) acetonitrile was more efficacious when followed by reflux however, reflux is relatively laborious and would have a deleterious effect on isothiocyanate content therefore 3 mL of boiling 50% (v/v) acetonitrile was added to the sample and incubated in a sealed glass vial for 30 min. The final extraction method (EM4) was carried out according to Song et al. [11]. Macerated mustard was mixed with 3 mL of boiling methanol for 5 min. Each sample was cooled at -20°C before the lids were removed to avoid isothiocyanate loss. For Extraction Methods 2, 3 and 4, solvents were evaporated to dryness by freeze-drying overnight at -100°C and 0.0004 mbar (Vacubrand, Germany) before being reconstituted in 3 mL of water. Following extraction, the samples were filtered using 0.2 μm Millex-GV syringe driven filter units.

2.2. HPLC quantification of sinigrin

Two columns were tested for the quantification of sinigrin; Agilent ZORBAX Eclipse XDB-C18 column (Agilent, Berks), 250 mm \times 4.6 mm, 5 μm particle size (Part no. 993967-902) with an Agilent ZORBAX Eclipse XDB guard column, 1.0 mm \times 17 mm (Part no. 5185-5921) and Polaris 5 C18-ether column, 250 mm \times 4.6 mm,

5 μm particle size (Part no. A2020250X046) with an Agilent Meta-Guard 4.6 mm Polaris C18-ether 5 μm V-Polaris guard column (Part no. A2020MG). The samples (10 μL) were injected into an Agilent 1200 series HPLC system. The mobile phase consisted of isocratic HPLC grade water with 0.2 M ammonium sulphate [3] at a flow rate of 1.5 mL min^{-1} and a column temperature of 30°C . An Agilent 1200 DA G1315B/G1365B photodiode array detected eluted sinigrin at a wavelength of 229 nm. The data was presented in Agilent ChemStation Rev. B.02.01 software and sinigrin concentration calculated against an authentic sinigrin calibration standard (Extrasynthese, France).

2.3. HPLC quantification of total isothiocyanates

To assess whether the same extract could be used to quantify total isothiocyanates, following resuspension in water and prior to filtration, the extracts were mixed with 500 μL dichloromethane (DCM) to remove the total isothiocyanates. Total isothiocyanates were quantified according to Zhang et al. [8]. The DCM extract (100 μL) was added to 1 mL 4 mM benzene 1,2-dithiol in methanol and 1 mL 10 mM potassium phosphate (pH 8.5) then incubated at 65°C for 2 h. In the place of the samples, 100 μL of five known concentrations of allyl isothiocyanate (Sigma, Dorset, UK); 2.5, 5.0, 7.5, 10 and 15 mM were added to five separate reaction mixtures as known calibration standards. A blank mixture was also created whereby 100 μL of pure DCM was added to a vial of reaction mixture. The reaction between the benzene 1,2-dithiol and the isothiocyanates yields the compound 1,3-benzodithiole-2-thione in a total isothiocyanate concentration dependent manner [8]. The concentration of 1,3-benzodithiole-2-thione was analysed by HPLC. Undiluted samples were injected (20 μL) into an Agilent ZORBAX eclipse XDB-C18 column. The mobile phase consisted of 80% (v/v) methanol at an isocratic flow rate of 1 mL min^{-1} and column temperature of 30°C . The compounds were detected at a wavelength of 365 nm and background wavelength of 600 nm using a photodiode array detector. Concentration of 1,3-benzodithiole-2-thione was calculated against the reactions containing known quantities of allyl isothiocyanate. The standard chromatogram was compared against the blank chromatogram to identify the retention time of 1,3-benzodithiole-2-thione.

2.4. Statistical analysis

Statistical analyses were conducted using Genstat for Windows Version 12.1.0.3278 (VSN International Ltd., Herts., UK). Analysis of variance (ANOVA) was used to identify significant differences in sinigrin and total isothiocyanate concentrations measured using each extraction method and each column. Standard errors for the differences of the means were calculated from each analysis. Limit of detection (LOD) was calculated as three times the standard deviation (SD) and limit of quantification (LOQ) as ten times the SD and relative standard deviation (RSD %) was calculated as the $((\text{SD} \times 100)/\text{mean})$ [12]. The height equivalent to a theoretical plate (HETP) was calculated according to Medina and Magan [13].

3. Results and discussion

Sinigrin concentrations were determined using four extraction methods and two different C18 column types to ascertain the most efficacious assay. In addition to this, total isothiocyanates were removed from the sinigrin extract to investigate whether the same method and therefore the same sample could also be used for the quantification of isothiocyanates. The concentration of sinigrin in the seed samples cv. Centennial were highest when extracted using 70% (v/v) methanol at 70°C or boiling 50% (v/v) acetonitrile at approximately 32 mg g^{-1} seed. The water extraction removed

Table 1

Limit of detection (LOD; $\mu\text{g mL}^{-1}$), limit of quantification (LOQ; $\mu\text{g mL}^{-1}$) and RSD (relative standard deviation, %) of ten repeat injections of four different sinigrin extracts using three different columns.

	Method	LOD ($\mu\text{g mL}^{-1}$)	LOQ ($\mu\text{g mL}^{-1}$)	RSD (%)
Zorbax Eclipse C18 column	Boiling water	3.9	13.1	9.9
	70% methanol at 70 °C	8.3	27.7	15.9
	Boiling 50% acetonitrile	2.8	9.4	6.3
	Boiling methanol	10.5	35.0	36.7
Polaris C18-ether column	Boiling water	2.9	9.9	7.4
	70% methanol at 70 °C	6.0	20.0	12.6
	Boiling 50% acetonitrile	3.9	13.0	8.1
	Boiling methanol	3.6	12.2	10.7
Kinetex C18 UPLC	Boiling water	4.4	14.7	11.1
	70% methanol at 70 °C	7.7	25.8	16.8
	Boiling 50% acetonitrile	2.4	8.1	5.2
	Boiling methanol	2.3	7.7	6.8

slightly less sinigrin at approximately 27 mg g^{-1} seed and the lowest concentration of sinigrin (20 mg g^{-1} seed) was extracted using boiling methanol (Fig. 1). This data alone suggests that the 70% (v/v) methanol at 70 °C and the boiling 50% (v/v) acetonitrile method are more efficacious at extracting sinigrin from mustard seed. This said, the LOD, LOQ and RSD (Table 1) suggest that boiling 50% (v/v) acetonitrile and water extraction resulted in more stable extractions hence the variability between multiple injections was reduced. For the quantification of sinigrin alone, extraction using boiling 50% (v/v) acetonitrile was therefore superior.

Isothiocyanates were extracted from the sinigrin extractions using DCM which is not miscible with water and therefore can be easily removed. For this reason, the DCM could only be added to the solvent extractions (EM 2, 3 and 4) once they had been freeze-dried and re-extracted in water since DCM is miscible with methanol and acetonitrile. The results in Fig. 2 show that the water extraction method was the most efficacious method at extracting the isothiocyanates compared to the other methods. During the water extraction, the lids of the vials were sealed tightly to avoid loss of the volatile isothiocyanates. The extractions were then allowed to cool to 4 °C before the vials were opened to allow for any volatile compounds to condense. The same procedure was carried out for EM 2, 3 and 4; however, the solvents were removed before being redissolved in water. It is apparent from the results of Fig. 2, that

although the freeze-drying procedure is carried out at -100°C , the procedure was detrimental to the isothiocyanate concentrations. These results suggest that the water extraction is superior for the rapid extraction of both sinigrin and isothiocyanates from the same sample. The extraction of both sinigrin and isothiocyanates simultaneously results in data which is directly comparable in contrast to separate extractions from different plant material. Other advantages of the boiling water extraction method include cost savings. Also, since the freeze-drying step was not required when using boiling water extraction, there was no need for overnight drying resulting in a rapid extraction process.

There was little difference between the columns based on the calibration curves (each column $r^2 = 1$), LOD, LOQ and RSD (Table 1). Therefore from this data alone; both columns could be used to accurately quantify sinigrin in mustard. This said, a further parameter; height equivalent to a theoretical plate (HETP), was calculated to obtain data on the efficiency of the columns. The number of theoretical plates is a measurement for the efficiency of a column by the amount of solute spreading as it travels through the column. An efficient system is one where the number of theoretical plates (N) is high [14] and takes into account the elution volume (the amount of solvent eluted prior to the peak of interest and is therefore calculated using the flow rate and retention time) and the width of the peak. The N is used to calculate the HETP which is a

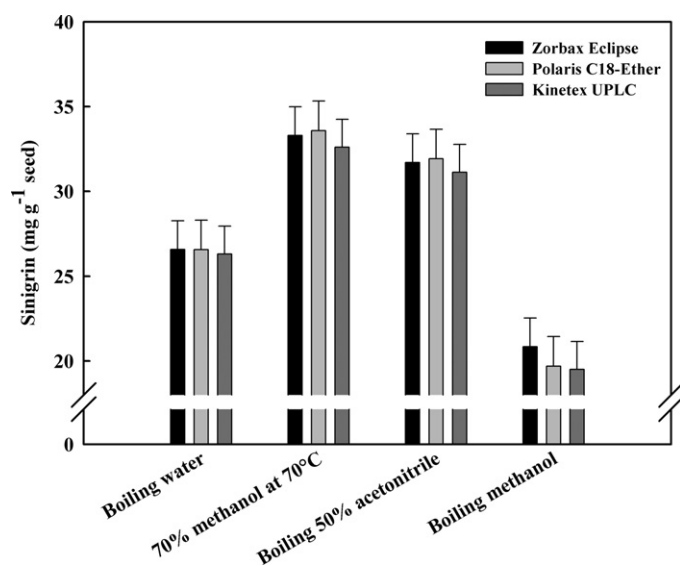


Fig. 1. Concentration of sinigrin (mg g^{-1} seed) extracted from mustard seed cv. Centennial using four different methods of extraction and analysed using three separate columns. Standard errors of the differences of means are shown by the error bars.

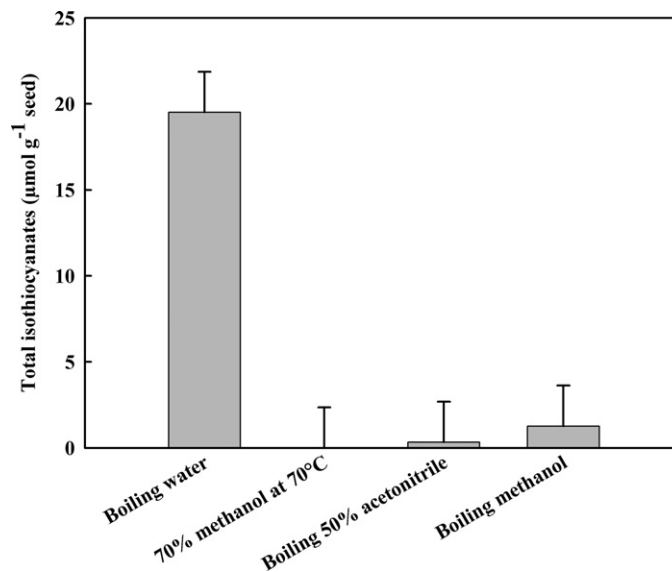


Fig. 2. Concentrations of total isothiocyanates ($\mu\text{mol g}^{-1}$ seed) extracted using DCM from each sinigrin extraction method. Standard errors of the differences of means are represented by the error bars.

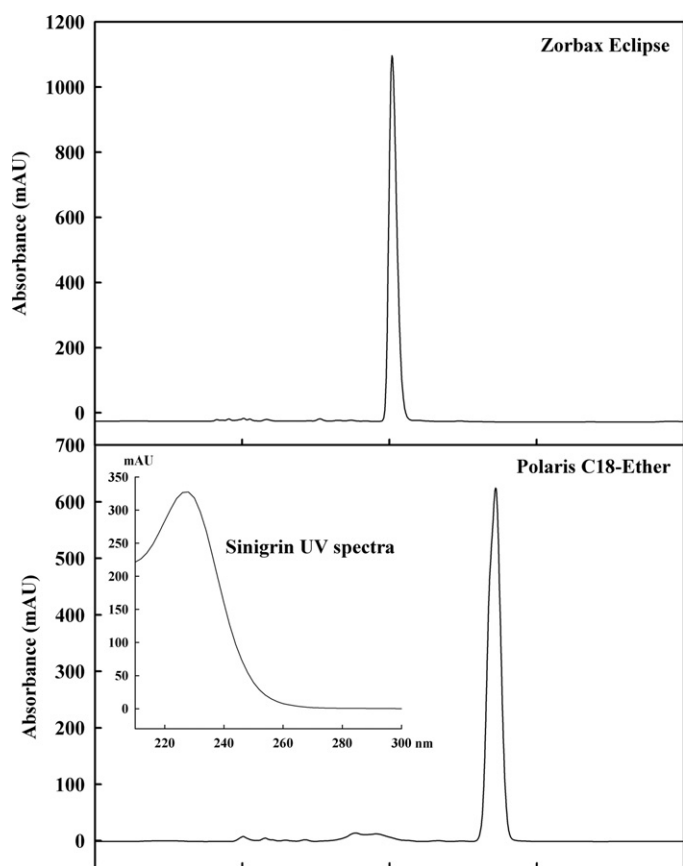


Fig. 3. Chromatograms of sinigrin measured at 229 nm separated using a Zorbax Eclipse, Polaris C18-ether or Kinetex C18 UPLC and sinigrin UV spectra.

measurement of the length of the theoretical plate therefore the shorter the plates, the more plates can be theoretically contained in the length of the column. The HETP of the Zorbax Eclipse column was $5.3 \pm 0.07 \mu\text{m}$ whereas the Polaris C18-ether column had a HETP of $11.2 \pm 2.59 \mu\text{m}$ suggesting the Zorbax Eclipse had the highest efficiency. The long HETP found using the Polaris C18-ether column was due to the wide peak shape indicating solute spread (Fig. 3). Gerendás et al. [10] used the Polaris C18-ether column

yet, they used a gradient mobile phase of acidified water and acetonitrile instead of the isocratic 0.2 M ammonium sulphate. This method improved the peak shape of the sinigrin peak using the C18-ether column (data not shown) however the run time of this gradient method was in excess of 1 h.

In conclusion, for the quantification of sinigrin alone from mustard seed, extraction using boiling 50% (v/v) acetonitrile was superior based on sinigrin concentrations where approximately 15% more sinigrin was yielded compared to the water extraction. Moreover, for the extraction of both sinigrin and isothiocyanates, extraction using boiling water was superior since the use of solvents required freeze-drying which resulted in reduced isothiocyanate content. There was no difference between the columns in terms of sinigrin quantity, LOD, LOQ and RSD values. Once column efficiency was calculated, the Zorbax Eclipse C18 was preferred using this particular method. The simultaneous extraction of sinigrin and isothiocyanates could be extended to other sinigrin containing *Brassica* species.

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