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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.

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 precooled 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 (Vacuubrand, 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 × 4.6 mm, 5 μm particle size (Part no. 993967-902) with an Agilent ZORBAX Eclipse XDB guard column, 1.0 mm × 17 mm (Part no. 5185-5921) and Polaris 5 C18-ether column, 250 mm × 4.6 mm, 5 μm particle size (Part no. A2020250X046) with an Agilent MetaGuard 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 ((SD × 100)/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