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Food Chemistry 98 (2006) 179–187
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

The determination of glucoraphanin in broccoli seeds and florets by solid phase extraction and micellar electrokinetic capillary chromatography

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Received 16 May 2005; accepted 10 July 2005

Abstract

A robust method for the determination of glucoraphanin in broccoli (*brassica oleracea* ssp. *italica* 'Marathon') seeds and florets has been developed using solid phase extraction (SPE) and micellar electrokinetic capillary chromatography (MECC) as the determinative step. Glucosinolates were extracted from the broccoli seeds and florets with hot water. Unwanted impurities were removed by passing the extracts through C18 and protonated amino propyl SPE cartridges connected in series. The glucosinolate fraction was removed from the protonated amino propyl cartridge with 2% v/v ammonia solution in methanol. The solvent was removed with a stream of nitrogen, the residue dissolved in water and the level of glucoraphanin determined by MECC using a 77 cm × 75-μm id bare fused silica capillary column (effective length 69.4 cm) and a buffer consisting of 18 mM sodium tetraborate, 30 mM sodium dihydrogen orthophosphate and 30 mM cetyltrimethylammonium bromide, pH 7. MECC parameters and capillary conditioning procedures were optimised with respect to reducing the analysis time without compromising peak integrity.

The level of glucoraphanin in broccoli seeds and florets compared favorably with the levels determined by a validated high performance liquid chromatography (HPLC) literature procedure; broccoli seeds MECC 2.1 gm/100 g, HPLC 2.0 gm/100 g; broccoli florets, MECC 71 mg/100 g, HPLC 70 mg/100 g. The MECC instrument reproducibility data ($n = 7$) for glucoraphanin in broccoli seed and floret extracts for migration time (CV; seeds 1.2%, florets 2%) and area calculation (CV; seeds 3.7%, florets 7%) relative to the internal standard were suitable.

Keywords: Glucoraphanin; Broccoli seeds; Broccoli florets; Solid phase extraction; Micellar electrokinetic capillary chromatography; High performance liquid chromatography

1. Introduction

Many cancer prevention approaches recommend the need for lifestyle changes accentuating exercise, the cessation of smoking and the increased consumption of plant foods such as fruits and vegetables (Nestle, 1998). Fruit and vegetables contain not only nutrients, fibre, vitamins and minerals, but also phytochemicals. Phytochemicals are biologically active non-nutrients that play an important role in cancer prevention (Pfannhauser, Fenwick, & Khokhar, 2001). Glucosinolates, a class of phytochemical found in the Brassica and Cruciferae plant species including broccoli, cabbage and Brussels sprouts (Botting, Davidson, Griffiths, Bennett, & Botting, 2002) have been of scientific interest in recent years due to the implicated health benefits associated

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0308-8146/$ - see front matter.
doi:10.1016/j.foodchem.2005.07.002

with their dietary intake. Glucosinolates appear to have little biological impact themselves, but release biologically active isothiocyanates upon enzymatic degradation by myrosinase in the presence of water (Fahey, Zalcmann, & Talalay, 2001). Epidemiological studies have shown positive health aspects associated with the intake of Brassica vegetables in terms of a decreased risk of cancer in the lung, stomach, colon and rectum (Matusheski & Jeffery, 2001). In vitro studies have demonstrated that sulforaphane, the isothiocyanate derived from glucoraphanin, inhibit Phase I enzymes, responsible for activation of carcinogens, and induce Phase II detoxification enzyme systems, thereby increasing the body's cancer defence mechanisms (Zhang, Talalay, Cho, & Posner, 1992).

Glucosinolates are found in differing levels throughout the plant (Karcher & El Rassi, 1999). Concentrations differ depending on variety, tissue type (i.e. floret, stem, seed), physiological age, season, growing climate and plant health (Rangkadilok et al., 2002; Rosa & Rodrigues, 2001). Generally, levels in the seed are high (up to 10% of the dry weight), whereas the levels in the leaf, stem and root are ≈10 times lower. Glucosinolates are responsible for the unique taste of many foods and in the case of broccoli, enhanced levels of glucoraphanin impart a strong bitter taste (Songsak & Lockwood, 2002). The analysis of glucosinolates, in particular glucoraphanin (Fig. 1) in Brassica and Cruciferae plant species, and their metabolites forms an integral part of the health and nutritional studies associated with these compounds. This information allows society to make informed choices relating to the possible benefits in incorporating phytochemical containing foods as a greater part of their diet to attain the associated health benefits. There is also a need to ensure that levels of phytochemicals consumed do not reach levels where the benefits may become detrimental.

Currently, the major method of analysis for glucosinolates is high performance liquid chromatography (HPLC) (Rangkadilok et al., 2002; Songsak & Lockwood, 2002), although gas chromatography (GC) can also be used provided the glucosinolate of interest is volatile. The separation of glucosinolates and the related desuloglucosinolates, has also been achieved by Micellar Electrokinetic Capillary Chromatography (MECC) (Feldl, Moller, Otte, & Sorensen, 1994; Karcher & El Rassi, 1999; Michaelsen, Moller, & Sorensen, 1992; Morin, Villard, Qunisac, & Dreux, 1992). MECC is a modern, highly efficient separation technique that is complimentary to HPLC and GC and is especially suited to the analysis of low to medium molecular weight ionic compounds. MECC separations are often faster and more cost effective than the corresponding HPLC or GC procedures (Trenerry, 1998).

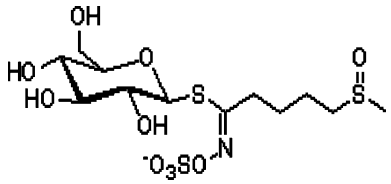

Fig. 1. Glucoraphanin.

MECC was introduced by Terabe in 1984 and has become one of the most widely used capillary electrophoretic (CE) methods due to the ability to separate both charged and neutral compounds (Terabe, Otsuka, Ichikama, Tsuchiya, & Ando, 1984). MECC separates compounds by using a surfactant as part of the separation buffer. The surfactant forms a micelle and neutral analytes separate based on their affinity for the micelle and their hydrophobicity. The amount of the surfactant present in the system needs to be above that of the concentration of the critical micelle level to allow for the micelles to form (Heiger, 1992). Most MECC systems for glucosinolate analysis are based on the cationic surfactant cetyltrimethylammonium bromide (CTAB) (Karcher & El Rassi, 1999; Michaelsen et al., 1992; Morin et al., 1992). Bjergegaard, Michaelsen, Moller, and Sorensen (1995) also separated a number of desulfoglucosinolates using the cationic surfactant sodium cholate. Paugman, Menard, Larue, and Thouvenot (1999) used sodium dodecyl sulfate (SDS) as the surfactant to separate glucobrassicin and methoxyglucobrassicin, with tetramethylammonium hydroxide (TMAH) as the ion-pair reagent and methanol as an organic modifier.

Prior to the analysis of glucosinolates in plants either by MECC or HPLC, there is a requirement to extract the glucosinolate from the plant material. For MECC analysis, it is particularly important to provide a clean extract in a solvent that is compatible with the running buffer. Clean extracts prevent column fouling and compatible solutions decrease the risk of bubble formation which can result in the loss of current and overall failure of the separation.

Extraction methods include high speed blending of the sample with boiling water (Matusheski & Jeffery, 2001; Rangkadilok et al., 2002; Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001) or with organic solvents such as a 30% aqueous methanol (Bjergegaard et al., 1995; Karcher & El Rassi, 1999), methylene chloride or hexane (Matusheski & Jeffery, 2001). Pretreatment with boiling water or liquid nitrogen (Bjergegaard et al., 1995; Karcher & El Rassi, 1999), is used to deactivate myrosinase and prevent enzymatic hydrolysis.

Solid phase extraction (SPE) is widely used in general food and environmental analysis as a method of sample clean up. C18 SPE was used by Botting et al. (2002) to isolate glucoraphaninn from broccoli seeds after a 30% aqueous methanol extraction. Anion exchange chromatography with Sephadex A-25 was used by Thies (1997) to isolate the glucosinolates from rapeseed seeds and

anion exchange membranes were used to isolate glucosinolates from canola seeds (Szmigielska & Schoenau, 2000).

SPE also allows for the introduction of a concentration step into the analysis which compensates for the inherent lack of sensitivity in the MECC analyses. The probability of acquiring reproducible results by MECC increases significantly once the sample extract is cleaned by SPE, as column integrity is maintained at an optimal level (Ward, Trenerry, & Pant, 1997).

This paper describes the determination of gluocraphanin in broccoli seeds and florets using SPE and MECC as the determinative procedure. C18 coupled to protonated weak anion exchange SPE was used to provide clean extracts for broccoli seeds and broccoli florets prior to MECC analysis. For method verification, the extracts were also analysed by an HPLC procedure described by Rangkadilok et al. (2002). Broccoli seed, which has a high level of glucoraphanin (Rangkadilok et al., 2002) is a potential source of a pure reference standard material via preparative high performance liquid chromatography.

2. Materials and methods

2.1. Reagents

Glucoraphanin, potassium salt was obtained from the Royal Veterinary and Agricultural University (Copenhagen, Denmark). Sodium tetraborate · $10H_2O$ and sodium dihydrogen orthophosphate · H_2O were obtained from BDH chemicals, Kilsyth, Australia. Cetyl trimethyl ammonium bromide was obtained from Sigma Chemical Company, Sydney, Australia, and sorbic acid was sourced from Asia Pacific Specialty Chemicals, Seven Hills, Australia. All other chemicals and reagents were of AR or HPLC grade. C18 Sep-pak (500 mg) and amino propyl (500 mg) cartridges were obtained from Waters Corporation, USA and Varian Inc., USA, respectively.

2.2. Preparation of standards and samples

2.2.1. Standards

Stock and working standards of glucoraphanin were prepared by dissolving glucoraphanin, potassium salt in deionised water. The solutions were stable for at least 3 months when stored at 4 °C. Sorbic acid was added as the internal standard for MECC assays at a final concentration of 12 μg/ml. The detector response was linear to at least 300 μg/ml for MECC and for HPLC.

2.3. Sampling and sample preparation

A local grower provided samples of broccoli seeds and fresh broccoli florets were purchased from a commercial outlet in Werribee, Victoria. The seeds were stored in a sealed container at 4 °C until used. The florets were removed from the stalk and extracted as soon as practical after purchase.

2.3.1. Broccoli seeds

To ≈1 g of broccoli seeds was added 30 ml of boiling water and the mixture boiled for 5 min. The bulk of the water was decanted and the seeds transferred to a mortar with ≈5 ml of water and the seeds ground to a paste. The resultant slurry was transferred to a 100-ml volumetric flask with deionised water, made to the mark with deionised water and sonicated for 5 min. The extract was filtered through Whatman No. 4 filter paper.

A C18 Sep-pak cartridge (500 mg) was activated with methanol/water and a Bond Elut amino cartridge (500 mg) was activated with methanol followed by 1% v/v acetic acid in water. The cartridges were connected in series (C18 on top) and 5 ml of the extract loaded onto the C18 Sep-pak cartridge. The cartridges were washed with 5 ml of deionised water, the C18 Sep-pak cartridge discarded and the protonated amino cartridge washed with 5 ml of methanol. The glucosinolate fraction was removed from the protonated amino cartridge with 8 ml of freshly prepared 2% v/v solution of concentrated ammonium hydroxide in methanol. The solvent was evaporated to dryness under a stream of nitrogen at room temperature. The residue was dissolved in 5 ml of deionised water. The solution was split into two portions, one for HPLC analysis and the other for MECC analysis. For MECC analysis, sorbic acid was added at a final concentration of 12 μg/ml. The solutions were filtered through 0.45-μm nylon filter discs before analysis.

2.3.2. Broccoli florets

To ≈10 gm of broccoli florets was added 70 ml of boiling water and the mixture boiled for 5 min. The mixture was homogenised with a Bamix blender for 5 min, and the resultant slurry transferred to a 100-ml volumetric flask with deionised water, made to the mark with deionised water and sonicated for 5 min. The extract was filtered through Whatman No. 4 filter paper and 10 ml purified by SPE as described for broccoli seeds. However, for broccoli florets, the residue was made to 2 ml with deionised water. The solution was split into two portions, one for HPLC analysis and the other for MECC analysis. For MECC analysis, sorbic acid was added to produce a final concentration of 12 μg/ml. The solutions were filtered through 0.45-μm nylon filter discs before analysis.

2.4. MECC buffer preparation

Stock solutions of sodium tetraborate (100 mM), sodium dihydrogen orthophosphate (150 mM) and

cetyltrimethylammonium bromide (100 mM) were prepared by dissolving appropriate amounts of the chemicals in deionised water. The running buffer was prepared by mixing appropriate quantities of each solution. The pH of the final solution was adjusted to 7 with 0.1 M NaOH. The solution was filtered through a 0.45-μm nylon filter and then degassed by sonication prior to use.

2.5. Apparatus

2.5.1. MECC

The extracts were analysed using a 77 cm × 75 μm uncoated fused silica capillary column with an effective length of 69.4 cm to the detector (SGE, Ringwood, Australia), fitted to a Beckman P/ACE model 5010 Capillary Electropherograph (Beckman Instruments, Inc., CA, USA). The separations were performed at −15 kV and at 30 °C using a buffer consisting of 50 mM CTAB, 18 mM sodium borate and 30 mM sodium phosphate, pH 7. The capillary was washed with 1 M NaOH (1 min), deionised water (1 min) followed by buffer (3 min) between analyses. The electropherograms were monitored at 230 nm. Data processing was carried out by P/ACE system software 5000 series on IBM personal computer 340. Solutions were loaded onto the column under "high pressure" for 2 s. The detector response for glucoraphanin was linear to at least 300 μg/ml using these conditions. Peak areas were used in the calculations.

2.5.2. HPLC

The analyses were performed with an Agilent series 1100 HPLC equipped with a quaternary gradient pump, autosampler and diode array detector (Melbourne, Australia). The compounds were separated on a 3.9 × 300 mm C18 μBondapak HPLC column (Waters Corporation, Milford, MA, USA) fitted with a C18 guard column and a mobile phase consisted of 0.005 M tetramethylammonium bromide (TMAB) dissolved in 2% v/v methanol/water. The chromatograms were monitored concurrently at 230 and 270 nm. Chemstation software was used to process the chromatographic data.

3. Results and discussion

MECC with CTAB as the cationic surfactant was successfully employed to separate a wide range of glucosoinolates, including glucoraphanin, in broccoli, cauliflower and kale (Michaelsen et al., 1992). The running buffer consisted of 50 mM CTAB in a 18 mM phosphate/30 mM borate solution, pH 7. The separations were achieved using a 72 cm × 50-μm id fused-silica capillary with an operating voltage of −20 kV and at a temperature of 30 °C. The compounds were loaded onto the column under positive pressure and detected by UV at 235 nm.

Initial attempts to repeat the separation of glucoraphanin using similar conditions were encouraging. Pure glucoraphanin had a migration time of ≈16 min when assayed using a 77 cm × 75-μm id bare fused silica capillary column (effective length, 69.4 cm). However, the migration time varied considerably when aqueous extracts of broccoli seeds and broccoli florets were analysed. More consistent migration times were realised when the capillary column was washed sequentially with 1 M NaOH followed by deionised water and refilling with running buffer between analyses (1 min 1 M NaOH, 1 min deionised water wash and a 3 min buffer replenishment). Optimal separation was achieved by reducing the sample injection time, however, this also resulted in a loss of sensitivity as less material was loaded onto the column. Shortening the column length by 10% resulted in a decreased run time; however, this also reduced the separation efficiency resulting in the partial comigration of glucoraphanin with another compound of similar migration time. A reduction of the operating voltage from −20 to −15 kV gave baseline separation of glucoraphanin from neighbouring peaks (Fig. 2). Sorbic acid was added to the extract as an internal standard to compensate for peculiarities in the injection procedure and for the gradual change in migration times that are often seen due to buffer degradation and column fouling (Weinberger, 1993). Ideally, a glucosinolate not present in the sample extract would be the first choice for an internal standard. However, pure glucosinolates are not readily available, and so alternative compounds were trialed. Sorbic acid, which has a longer migration time than glucoraphanin was chosen, as it did not co-migrate with any naturally occurring compounds present in the extracts.

MECC analysis of the broccoli floret extract produced a more complex electropherogram, however, sorbic acid was still suitable as the internal standard as it separated well from other naturally occurring compounds in the extract (Fig. 3).

SPE has been used successfully to provide cleaner and more concentrated extracts for MECC determinations (Ward et al., 1997). The procedure itself consumes minimal amounts of time and solvents and can be automated to improve analytical efficiency. For this work, both C18 (reverse phase) and anion exchange packing materials were trialed.

3.1. C18

C18 SPE was used by Botting et al. (2002) to isolate glucoraphanin from broccoli seeds after a 30% v/v aqueous methanol extraction. In our hands, when a 5 ml aliquot of an aqueous sample extract was loaded onto an activated C18 SPE cartridge, the glucoraphanin passed straight through. However, non-polar compounds,

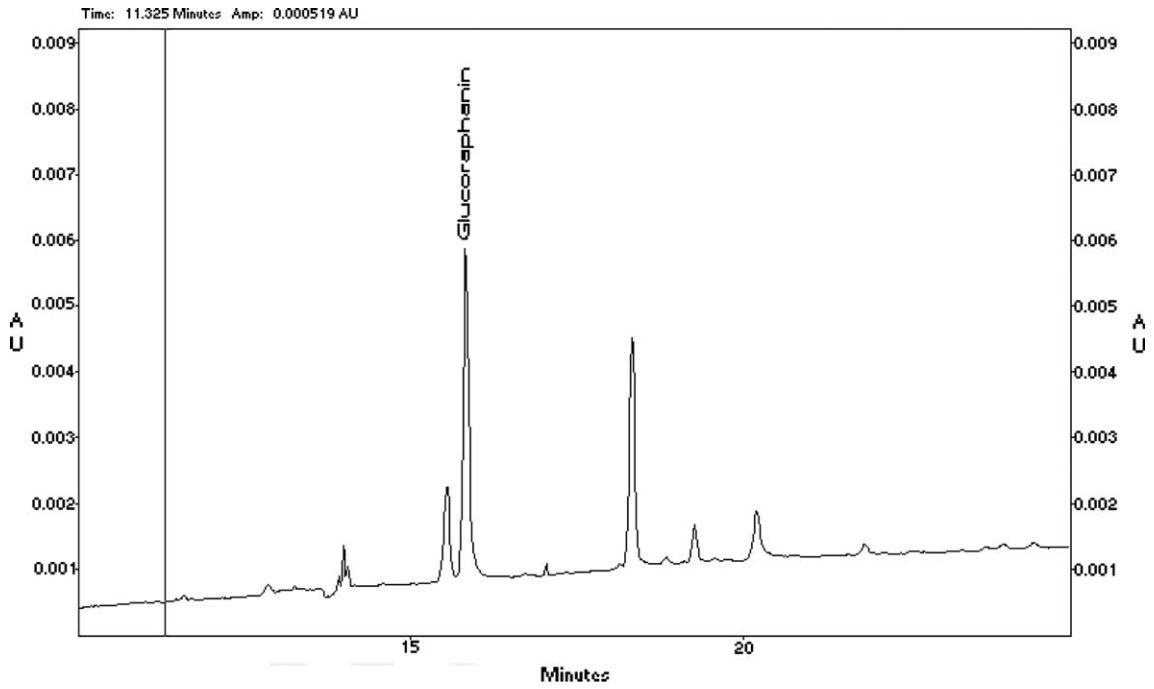

Fig. 2. Partial electropherogram showing the presence of glucoraphanin in an aqueous extract of broccoli seeds using the MECC conditions described in Section 2.

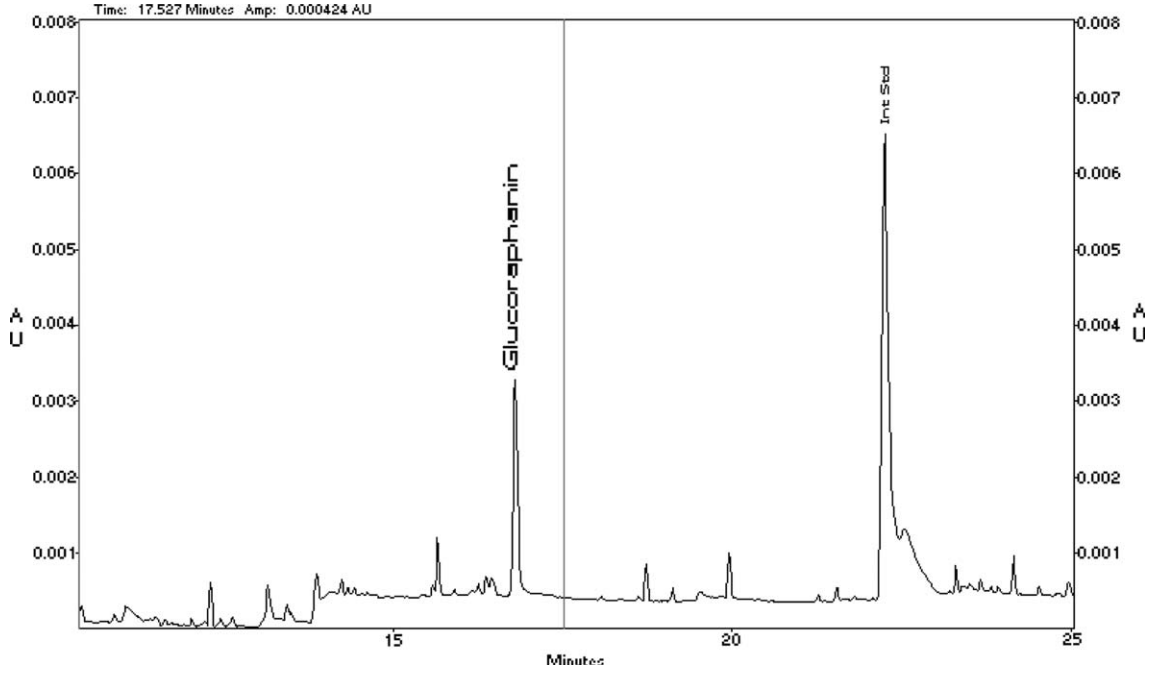

Fig. 3. Partial electropherogram showing the separation of glucoraphanin and the internal standard (sorbic acid) in an aqueous extract of broccoli florets using the MECC conditions described in Section 2.

which could potentially interfere with the separation were trapped on the column. Glucoraphanin was quantitatively removed from the C18 column with a further 5 ml wash with deionised water. This procedure, even though it separated glucoraphanin from potential interfering compounds, did not allow for the inclusion of a concentration step.

3.2. Anion exchange

3.2.1. Strong anion exchange

Strong anion exchange (SAX) SPE was not suitable to isolate and concentrate glucoraphanin using the conditions described in Section 2 as glucoraphanin was too tightly bound to the packing material.

3.2.2. Weak anion exchange

Glucoraphanin was quantitatively recovered when the experiment was repeated with a protonated 500 mg amino propyl cation exchange cartridge as described in Section 2. The residue, after Weak anion exchange (WAX) treatment, was dissolved in deionised water (5 ml for broccoli seed extract and 2 ml for broccoli floret extract) and assayed by MECC. Both electropherograms were cleaner than those recorded for the original extracts.

3.2.3. Combined C18 and WAX cleanup

C18 and WAX cleanup provided solutions that were cleaner than the original extracts. C18 and ion exchange cartridges in series was previously used successfully to produce clean solutions for the determination of niacin in a variety of foods by CE (Ward et al., 1997). This process was trialed with C18 and WAX cartridges in series to prepare the broccoli extracts for analysis.

Five millilitre aliquots of broccoli seed or broccoli floret extracts were passed through activated C18 cartridges attached to the top of protonated WAX cartridges. The cartridges were washed with water, the C18 cartridges removed and the WAX cartridges washed with methanol followed by 2% v/v of concentrated ammonium hydroxide in methanol. The residues that remained after solvent removal were dissolved in deionised water (5 ml for broccoli seed extract and 2 ml of broccoli floret extract) and assayed by MECC. Fig. 4 shows the partial electropherogram of a broccoli seed extract after SPE cleanup. Figs. 5 and 6 show the partial electropherograms of broccoli seed and broccoli floret extracts with added internal standard after SPE cleanup.

Seven consecutive injections of pure standard solution, purified broccoli seed extract and purified broccoli floret extracts were assayed to gauge migration time and area count reproducibility. The data are displayed in Table 1. Overall, the method reproducibility was acceptable.

The %CVs for migration time and peak area repeatabilities for the glucoraphanin standard were lower than the values obtained for the broccoli seeds and florets. This was most likely due to other plant material remaining in the sample extracts affecting the overall separation. The %CVs for the normalised peak areas improved when compared to the absolute values in all cases. Also, using an internal standard for normalised ratios showed a significant improvement in variability.

3.3. Quantitation

The levels of glucoraphanin in broccoli seeds and broccoli florets were determined concurrently by MECC and by an HPLC procedure based on that described by Rangkadilok et al. (2002). The identity of the glucoraphanin peak in the HPLC trace was confirmed by UV spectral data from the photodiode array detector and by spiking the extract with pure glucoraphanin standard material. Similar levels were obtained by both proce-

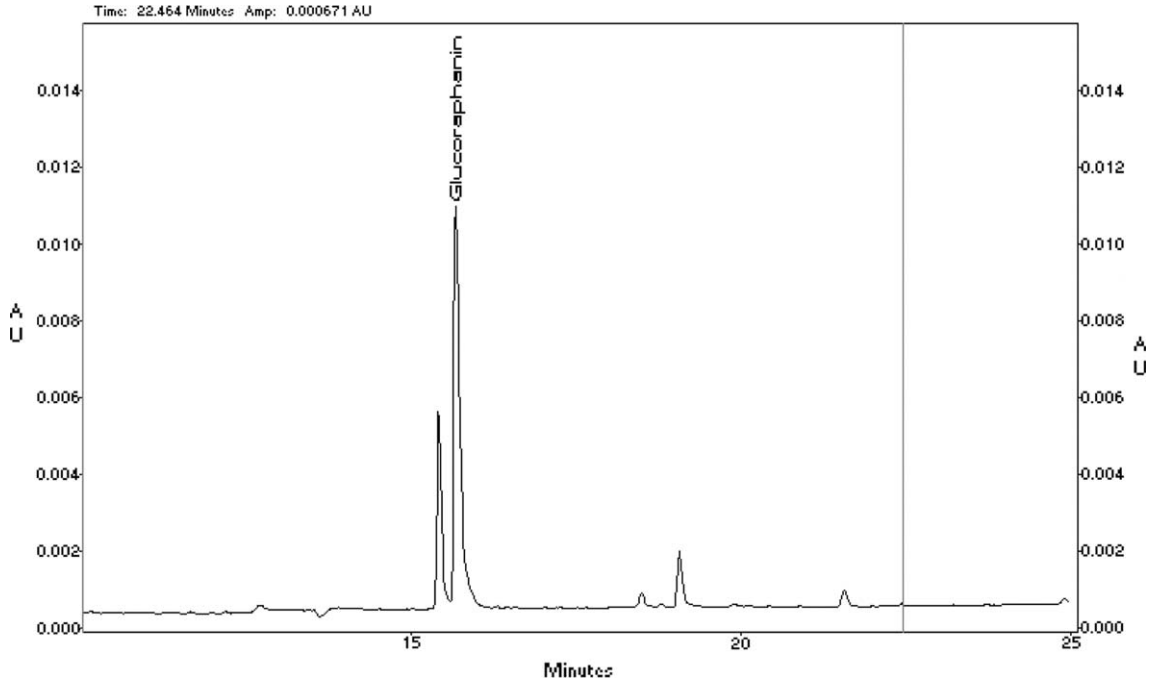

Fig. 4. Partial electropherogram showing the presence of glucoraphanin in an aqueous extract of broccoli seeds after SPE cleanup using the MECC conditions described in Section 2.

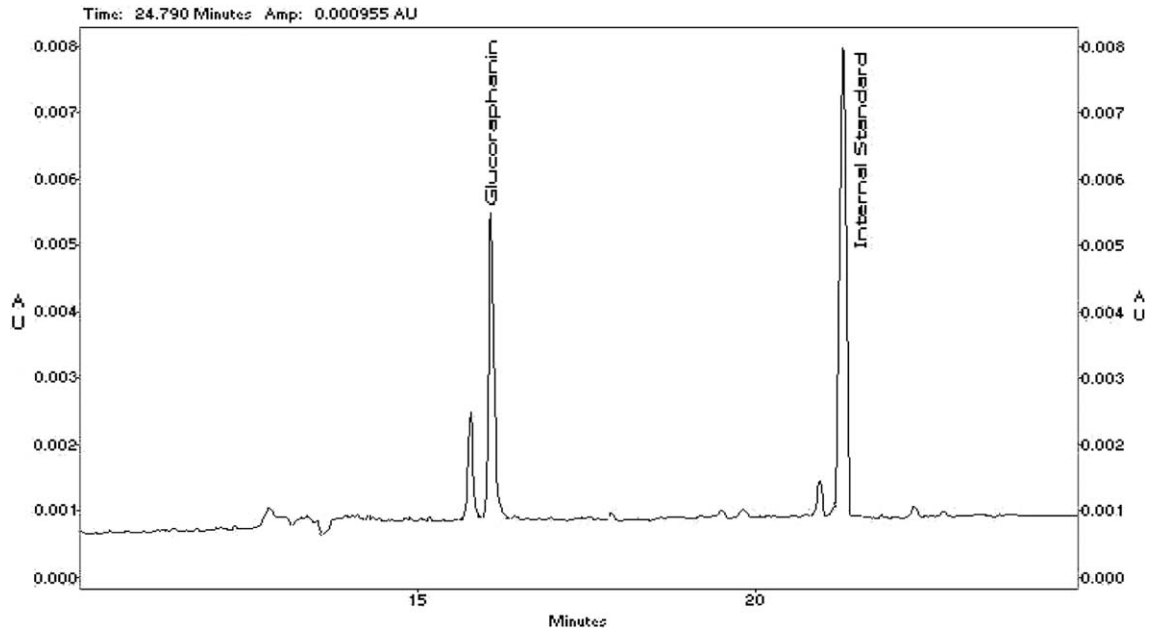

Fig. 5. Partial electropherogram showing the separation of glucoraphanin and the internal standard (sorbic acid) for an aqueous extract of broccoli seeds after SPE cleanup using the MECC conditions described in Section 2.

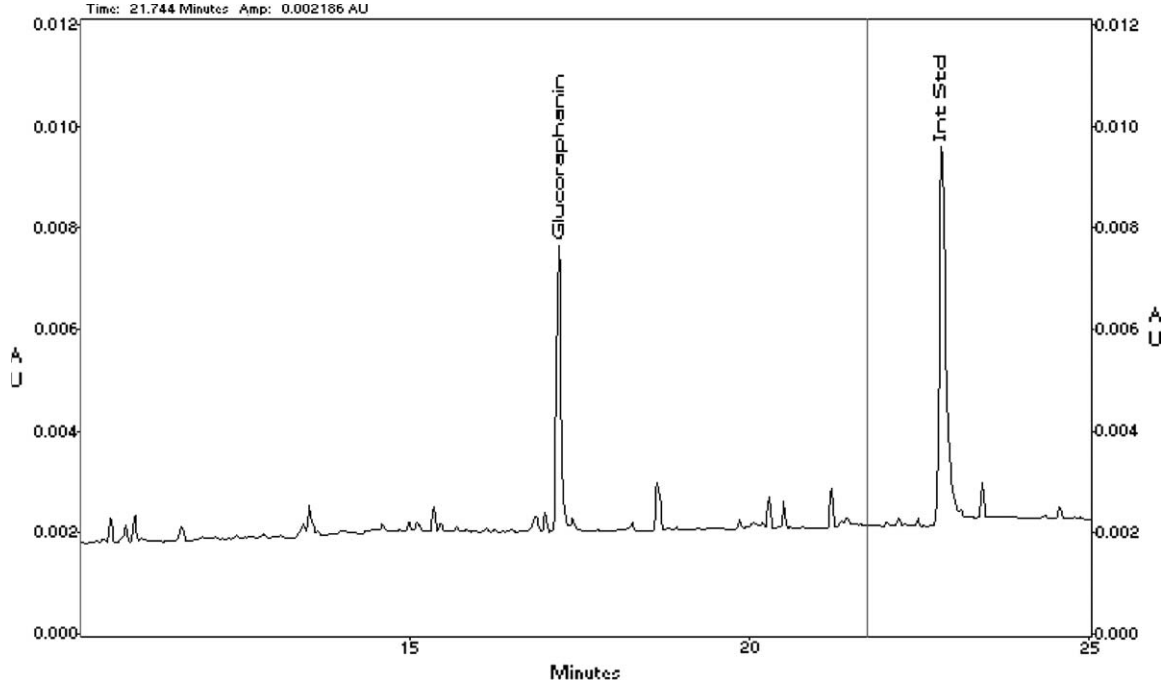

Fig. 6. Partial electropherogram showing the separation of glucoraphanin and the internal standard (sorbic acid) for an aqueous extract of broccoli florets after SPE cleanup using the MECC conditions described in Section 2.

dures, e.g., broccoli seeds (MECC 2.1 g/100 g, HPLC 2.0 g/100 g) and broccoli florets (MECC 71 mg/100 g, HPLC 70 mg/100 g). Glucoraphanin, which was added to a sample of broccoli florets before extraction, SPE cleanup and MECC analysis, was recovered at a level of 95%. Typical HPLC chromatograms of broccoli seed and broccoli floret extracts after SPE cleanup are seen in Figs. 7(a) and (b). SPE cleanup, although not necessary for the broccoli seed extract, simplified the chromatography of the broccoli floret extract by removing a compound that had a similar retention time to glucoraphanin (Fig. 8).

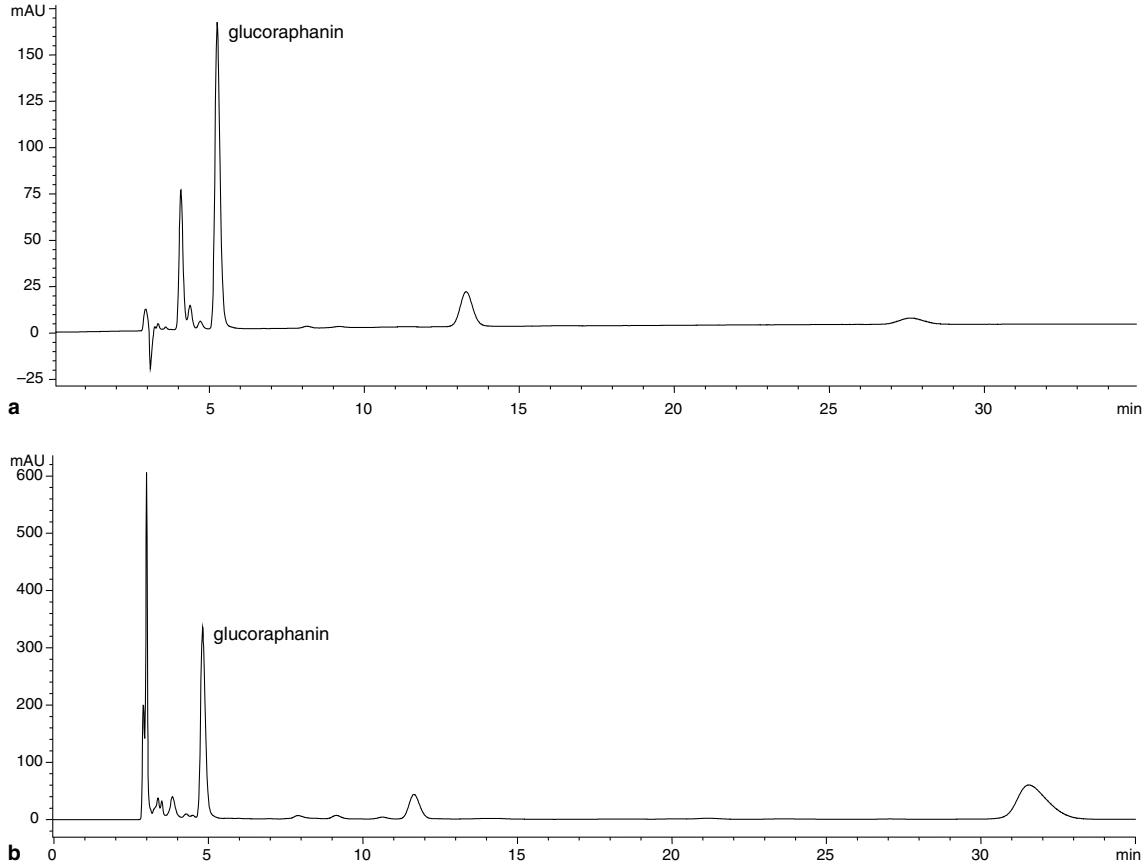

Fig. 7. Chromatograms of aqueous extracts of (a) broccoli seeds and (b) broccoli florets after SPE cleanup showing the presence of glucoraphanin using the HPLC conditions described in Section 2.

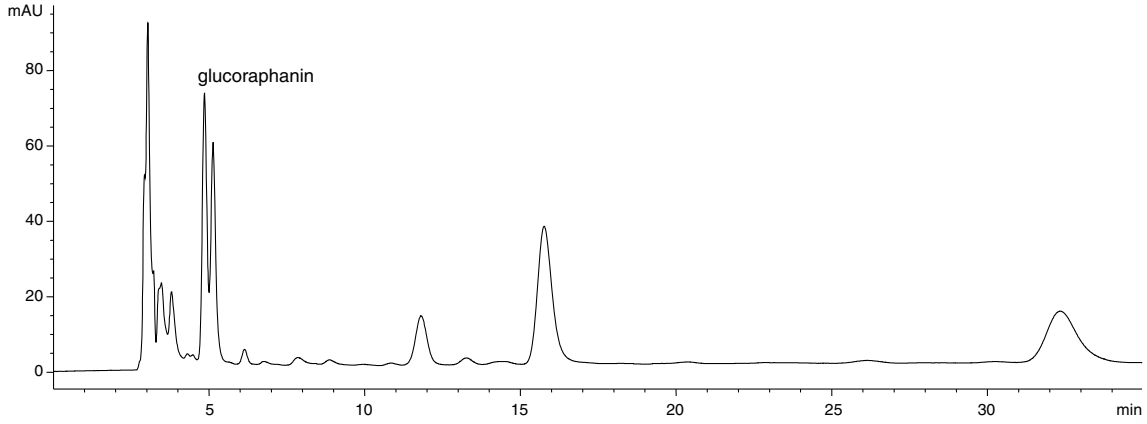

Fig. 8. Chromatogram of an aqueous extract of broccoli florets before SPE cleanup showing the presence of glucoraphanin using the HPLC conditions described in Section 2.

Table 1
MECC migration time and area count repeatability (%CV, $n = 7$) for glucoraphanin in glucoraphanin standard solution (300 μg/ml), broccoli seed extract and broccoli floret extract

Sample	Migration time repeatability %CV, $n = 7$		Peak area repeatability %CV, $n = 7$	
	With internal standard	Without internal standard	With internal standard	Without internal standard
Glucoraphanin standard solution (300 μg/ml)	0.1	0.3	0.9	2.3
Broccoli seed extract	1.2	1.8	3.3	4.7
Broccoli floret extract	2	6	7	12

Small amounts of progoitrin and glucoiberin were also present in the extracts and were well separated from glucoraphanin by HPLC and MECC.

4. Conclusion

A robust MECC method for the determination of glucoraphanin in broccoli seeds and broccoli florets has been developed and validated against a literature HPLC procedure. SPE cleanup with C18 and WAX cartridges in series provided clean extracts that were suitable for repetitive MECC assays. MECC parameters including capillary length, applied voltage and capillary conditioning were all investigated to optimise the procedure with respect to analysis time without compromising the quality of separation.

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