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Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography

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Abstract

Glucosinolates are anionic, hydrophilic plant secondary metabolites which are of particular interest due to their role in the prevention of cancer and other chronic and degenerative diseases. The separation and purification of glucosinolates from a variety of plant sources (e.g. seeds of broccoli, arugula and the horseradish tree), was achieved using high-speed counter-current chromatography (HSCCC). A high-salt, highly polar system containing 1-propanol–acetonitrile–saturated aqueous ammonium sulfate–water (1:0.5:1.2:1), was run on a semi-preparative scale and then transferred directly to preparative scale. Up to 7 g of a concentrated methanolic syrup containing about 10% glucosinolates was loaded on an 850-ml HSCCC column, and good separation and recovery were demonstrated for 4-methylsulfinylbutyl, 3-methylsulfinylpropyl, 4-methylthiobutyl, 2-propenyl and 4-(rhamnopyranosyloxy)benzyl glucosinolates. Multiple injections (5 to 6 times) were performed with well-preserved liquid stationary phase under centrifugal force. Pooled sequential runs with broccoli seed extract yielded about 20 g of its predominant glucosinolate, glucoraphanin, which was produced at >95% purity and reduced to powdered form.

Keywords: Counter-current chromatography; Vegetables; Plant materials; *Brassica* spp.; Glucosinolates; Glucoraphanin

1. Introduction

Glucosinolates are abundant plant secondary metabolites that are found in cruciferous plants (e.g. the *Brassica* spp. vegetables: broccoli, Brussels sprouts, cabbage, cauliflower and kale), but also occur in 15 other plant families [1]. More than 120 glucosinolates (β-thioglucoside N-hydroxysulfates) (Fig. 1), have been isolated and they are biosynthesized from alkyl, thioalkyl (methionine), aromatic, and indole amino acids. Along with their cognate isothiocyanates, glucosinolates such as glucoraphanin (GR) (Fig. 2) and sulforaphane, have become the subject of intense scrutiny as a result of their cancer chemoprotective, antioxidant, and antibiotic activities [1–5] and for their potential value as phytochemical components of healthy diets that could be added to functional foods. Isothiocyanates in plants occur almost exclusively as their cognate glucosinolates. Conversion to isothiocyanates results from rapid

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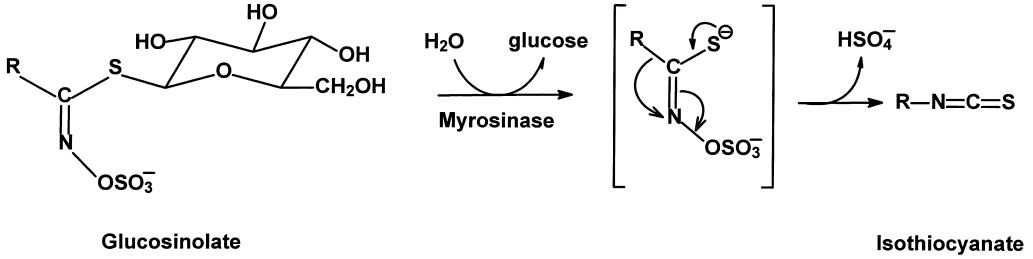

Fig. 1. Glucosinolates (β-thioglucoside N-hydroxysulfates), and the myrosinase-catalyzed production of an unstable intermediate, which rearranges to form isothiocyanates (and can form other degradation products such as nitriles, thiocyanates, epithionitriles, and oxazolidonethiones depending upon reaction conditions).

enzymic hydrolysis by a thioglucosidase, myrosinase (E.C. 3.2.3.1) (Fig. 1), that coexists within the plant cells and is released upon tissue damage when food is prepared or chewed [6]. Purification and separation of many of the more common glucosinolates (e.g. those found in the *Brassica* vegetables), however, is exceedingly difficult. Glucosinolates are water-soluble compounds and their physicochemical properties are dominated by the ionized sulfate and hydrophilic carbohydrate moieties. Separations between them are difficult because of minor differences in the R groups. For the isolation and separation of glucosinolates, most previous work has utilized flash chromatographic and/or preparative HPLC techniques so that substantial (gram) quantities of only a few glucosinolates have so far been available [7,8]. We have attempted to use these techniques to scale-up production of large quantities of certain of these compounds, but have had minimal success. We therefore evaluated a method (high-speed counter-current chromatography; HSCCC), for rapid chromatographic purification employing highly efficient fractionation by a hybrid technique of liquid–liquid counter-current distribution and liquid chromatography, in conjunction with the use of centrifugal force. The centrifugal force field generated from both rotational and synchronous planetary motion of coiled columns containing two immiscible liquid phases provides vigorous mixing between stationary and mobile phases, as well as retention of a very large fraction of the stationary phase [9]. HSCCC has several distinctive properties: (a) it depends for its efficiency solely upon the partition coefficient of the solute between the stationary and mobile phases, (b) it accomplishes excellent separation by taking advantage of the high volume of stationary phase (50% to 90% of total volume), with a resolution of about 1000 theoretical plates, (c) it eliminates the

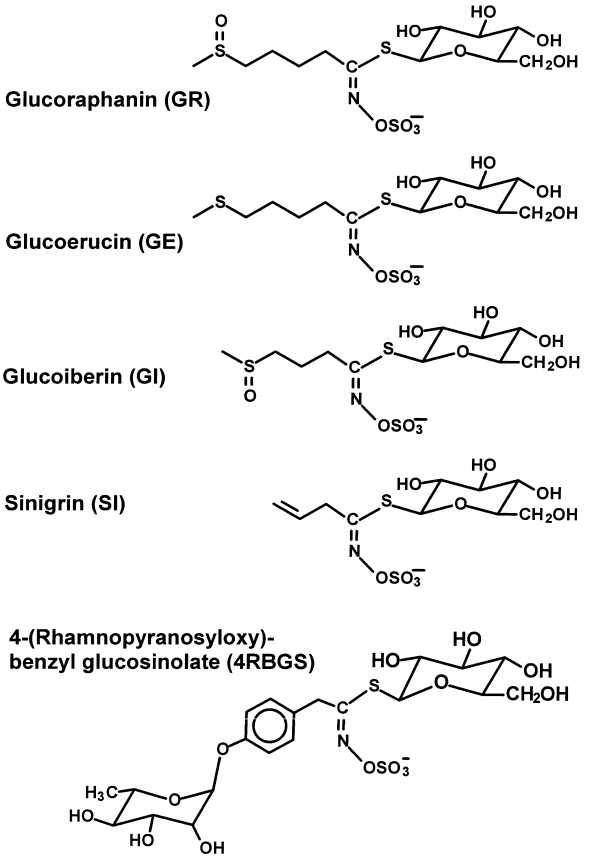

Fig. 2. The five glucosinolates, representing some of the diversity of naturally occurring side chains, that were resolved by HSCCC in this study.

irreversible adsorptive loss of samples onto the solid support matrices used in conventional column chromatography and HPLC, (d) it uses remarkably small amounts of expensive solvents, (e) it permits quantitative recovery of applied sample regardless of the resolution of components (i.e. a failed separation can be easily and rapidly re-run on an appropriately modified solvent system without loss of precious starting material), and (f) it can thus be scaled-up from analytical- to preparative-scale separation in a completely straightforward manner.

HSCCC has recently been used to effectively separate a large variety of natural products [9–14]. We have utilized this technique to separate five glucosinolates containing both very similar side chains, [e.g. 4-methylsulfinylbutyl glucosinolate (glucoraphanin; GR) and 3-methylsulfinylpropyl glucosinolate (glucoiberin; GI)] and dissimilar side chains [e.g. 4-methylthiobutyl glucosinolate (glucoerucin; GE), 2-propenyl glucosinolate (allyl glucosinolate; sinigrin; SI) and 4-(rhamnopyranosyloxy) benzyl glucosinolate (4RBGS), and to purify four of these compounds from concentrated crude plant extracts. As a demonstration of the scalability of this technique, we also produced 20 g of glucoraphanin and 5 g of glucoiberin from continuous multiple preparative runs made using an aqueous extract of broccoli seed as the sample source. The two glucosinolates were separated completely, even though their side chains differed by the presence of only one internal methylene group.

2. Experimental

2.1. Reagents and materials

Sinigrin was purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents used for HPLC were chromatographic grade and were filtered and degassed prior to use. HSCCC reagents were reagent grade or higher. Myrosinase was purified from daikon seedlings as described by Shikita et al. [15]. Broccoli (*Brassica oleracea* var. *italica*) seeds were obtained from Caudill Seed (Louisville, KY, USA), arugula (*Eruca sativa*) seeds from Johnny's Selected Seeds (Albion, ME, USA), and horseradish tree (*Moringa oleifera*) seeds were from Dr. Manuel Palada (University of the Virgin Islands, Kingshill, St. Croix, US Virgin Islands).

2.2. Apparatus

2.2.1. HSCCC

A Model CCC-1000 HSCCC (Pharma-Tech Research, Baltimore, MD, USA) was used for development of separation protocols on both semi-preparative columns (325 ml, wound with 1.6 mm I.D. PTFE tubing) and preparative columns (850 ml, wound with 2.6 mm I.D. PTFE tubing). A Series 2, piston-style, medium-pressure pump (Scientific Systems, State College, PA, USA), was used to achieve a flow-rate of 1 ml/min (semi-preparative) or 5 ml/min (preparative) and the centrifugal speed for both was 1000 rev./min. Peak elution was monitored at 235 nm using a Model SPD-6A variable-wavelength detector (Shimadzu, Columbia, MD, USA).

2.2.2. HPLC

The HPLC system used employed a 3 μm, 100× 4.6 mm polyhydroxyethylaspartamide column (PolyLC, Columbia, MD, USA) connected to a Model 616 pump, with a Model 717^{plus} autosampler and a Model 996 photodiode array detector (Waters, Milford, MA, USA). Detection was at 235 nm and data were processed using $Millennium^{32}$ software (ver. 3.0; Waters).

2.3. Preparation of crude plant extracts

A crude broccoli seed extract was used as the source of GR and GI for both semi-preparative and preparative scale separations. In brief, broccoli seeds were boiled and stirred for 2 h in a 6.3-fold (w/v) excess of water. After cooling to 60 °C, seeds and supernatant were separated by filtration through a stainless steel screen and the resultant cooled supernatant was then agitated for 2 h in the presence of 10 g/l activated charcoal. It was then filtered through Whatman No. 4 filter paper with added Celite (W.R. Grace, Columbia, MD, USA), concentrated, and spray-dried with the addition of 1% SiO_2 (Syloid Silica grade 63FP, W.R. Grace) and 10% maltodextrin (A.E. Staley, Decatur, IL, USA) as drying and anti-caking agents. Each gram of resulting powder, containing ca. 76 mg GR, was then extracted with

10 ml of boiling methanol and the methanolic extract was concentrated to a syrupy consistency by rotary evaporation under vacuum, and frozen at −20 °C until used for HSCCC. Aqueous extracts were prepared from seeds of arugula and the horseradish tree in a similar fashion to those described above, but they were subject to initial purification by flash chromatography on acidic alumina, followed by flash chromatography on a C_{18} reversed-phase column [7,8,16,17]. Briefly, the seeds or dry leaf powder of these plants were homogenized in a five-fold (w/v) excess of boiling 80% methanol. These extracts were concentrated under vacuum, and for initial clean-up, 200–250 ml of concentrated extract were applied to a 30×5.0 cm acidic alumina column (activity grade I, Type WA-1: Acid; A-8753; Sigma–Aldrich), washed exhaustively with water, eluted with 57 m*M* K_2SO_4 and monitored at 224 nm (Dual Path Monitor UV-2, Pharmacia, Uppsala, Sweden). Glucosinolate-rich fractions were concentrated, applied to a 40×4 cm column of 40 μm C_{18} (Bakerbond No. 7025-00; J.T. Baker, Phillipsburg, NJ, USA), and eluted successively with 287 m*M* K_2SO_4, water, and methanol. When dilution was performed in this manner with a crude extract from arugula seeds, for example, GR was eluted first, GE second, and indole glucosinolates (primarily glucobrassicin and 4-hydroxyglucobrassicin) last. Final yield from 3 kg of seeds was 7.3 g of GE and 1.0 g of GR. This approach also yielded 4RBGS from seeds of *Moringa oleifera*. The plant extracts applied to HSCCC for recovery of GE and 4RBGS were thus highly enriched, containing about 15% GE (arugula preparation) and 84% 4RBGS (horseradish tree seed preparation). Each of these powders contained significant quantities of K_2SO_4 and other unidentified contaminants. The crude horseradish tree leaf preparation was obtained by re-hydrating 500 mg of dry, powdered horseradish tree leaves (collected in April, 2001 in Kingshill, St. Croix, US Virgin Islands), homogenizing them in 10 ml of mixed stationary and mobile phase prepared as described in the following section, and filtering them prior to introduction into the HSCCC coils.

2.4. HSCCC separation

The biphasic solvent system ultimately utilized was made by mixing four different solvents (1-propanol–acetonitrile–saturated aqueous ammonium sulfate–water) at the ratio of 1:0.5:1.2:1. The organic phase was the mobile phase and the aqueous phase was the stationary phase. The saturated ammonium sulfate was prepared at 78 °C and then promptly equilibrated with the other solvents in a separatory funnel at room temperature prior to use in the HSCCC.

2.5. Identification of fractionated compounds

Analytical detection of glucosinolates was by HPLC on a 3 μm, 100×4.6 mm polyhydroxyethylaspartamide column (PolyLC) run under isocratic conditions at 2 ml/min using ammonium formate at a final concentration of 30 m*M*, at pH 5.4, in acetonitrile–water (85:15) [18], with the detector set at 235 nm. Retention times for the elution of GE, SI, GR, 4RBGS, and GI under these conditions was about 2.5, 3.9, 12, 12.6 and 13 min, respectively, with a response factor of 170,000 $\mu V \cdot s \cdot nmol^{-1}$, based on a crystalline sinigrin standard (Sigma–Aldrich). Confirmation of identity was made by comparison with standards whose identity was verified by HPLC and spectrophotometry [18,19], and by mass spectrometry [19]. The m/z for the molecular ions of GR, GI, GE, and 4RBGS was determined by electrospray mass spectrometry on a Model API 150EX mass spectrometer (Applied Biosystems, Foster City, CA, USA) to be 436, 422, 420, and 570 amu, respectively, in accordance with expectations [19]. Glucosinolate purity was estimated based upon quantitative HPLC, and cyclocondensation of their myrosinase hydrolyzates (glucosinolates liberate molar equivalents of glucose and of their cognate isothiocyanates (see Fig. 1) when treated with purified myrosinase at neutral pH [1,15]). Purified myrosinase and ascorbic acid (0.0003 U/ml and 0.5 μmol/ml, respectively), were added to aqueous solutions of purified glucosinolate. The hydrolyzates were then split into two aliquots for separate analysis of isothiocyanate and glucose yield: isothiocyanate yield was determined by treatment with 1,2-benzenedithiol [20] as described by Zhang et al. [21]. Release of equimolar levels of glucose was verified with an Infinity Glucose Reagent test kit (Sigma–

Aldrich), according to the manufacturer's instructions (Procedure No. 18-UV).

3. Results and discussion

Method development required selection of a highly polar solvent system for this very hydrophilic class of phytochemicals. Many systems were evaluated with the primary criterion that manipulation of solvent composition should achieve a partition coefficient (K) for glucoraphanin (GR) of between 0.3 and 0.7. Table 1 shows the systematic modification of the original method to optimize the system for glucosinolates, in particular for the resolution of the structurally similar GR and GI. The selected system was derived from a high-salt, high-polarity solvent system that has been used for highly water soluble compounds such as hydrophilic betalains, betanin and isobetanin from red beet juice [22]. Once identified, the chosen solvent system (1-propanol–acetonitrile–saturated aqueous ammonium sulfate–water; 1:0.5:1.2:1), was run on a small scale and then transferred directly, without any modifications, to preparative scale.

Crude, concentrated methanolic syrups, containing between about 0.5 and 1 g of glucoraphanin (6–7 g total mass) were dissolved in 10 ml of a mixture of equilibrated mobile and stationary phases (6 ml stationary phase and 4 ml mobile phase) and loaded in a 20-ml injection loop. Forty-five to 50 fractions, each 18 ml, were collected after each injection, following a void volume of about 200 ml. Glucoraphanin eluted in one broad peak starting at a retention time of about 97 min, followed by return to baseline at about 153 min, and elution of GI between 153 and 189 min (Fig. 3). Retention times were remarkably consistent between chromatographic runs of the same plant extract and product yield was close to 100% (Table 2). Separation was completed within about 3 h such that at least two chromatographic runs could be performed in 1 day. Both the mean and median yield from 26 preparative runs was about 500 mg. The stationary phase required renewal (change-out) every five or six runs. Both operating time, and total organic solvent usage was only about one tenth of that which would have been required if separation had been performed by preparative HPLC. Over 20 g of GR and 5 g of GI were thus produced, at an aggregate purity of over 95%, in 26 runs as described above, requiring about 100 h of run time. These two compounds have historically been difficult to separate in isocratic HPLC systems [18,19] due to their structural similarity (they differ by only one methylene group in the side chain). Thus the excellent separation between GR and GI was surprisingly efficient with this relatively simple HSCCC protocol. Fig. 3 demonstrates the complete separation using HPLC analyses to verify the purity of successive fractions from the HSCCC. In Fig. 3, HPLC chromatogram A shows the crude preparation prior to HSCCC fractionation while chromatograms B–F represent consecutive 18-ml fractions. Note that chromatogram D, representing the return almost to baseline of the HSCCC separation, is the only fraction containing both GR and GI. Chromatograms B and C on the other hand, contain only GR, and chromatograms E and F contain exclusively GI.

Table 1
Partition coefficients for GR and GI in various solvent systems

Solvent system	Ratio	Partition coefficient[a]	
		GR	GI
EtOH–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	1:0.5:1:1	0.05	0.20
EtOH–2-butanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	0.5:0.5:0.5:1.2:1	0.50	0.81
EtOH–1-propanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	0.5:0.5:0.5:1.2:1	0.35	0.58
2-Butanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	1:0.5:1.2:1	2.99	4.21
2-Propanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	1:0.5:1.2:1	0.41	0.68
1-Propanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water	0.75:0.5:1.2:1	0.99	1.43
1-Propanol–ACN–$(NH_4)_2SO_4$ (satd. soln)–water[b]	1:0.5:1.2:1	0.63	1.03

[a] Expressed as: $\text{concentration}_{\text{stationary}}/\text{concentration}_{\text{mobile}}$.
[b] Solvent system ultimately selected for use with glucosinolates.

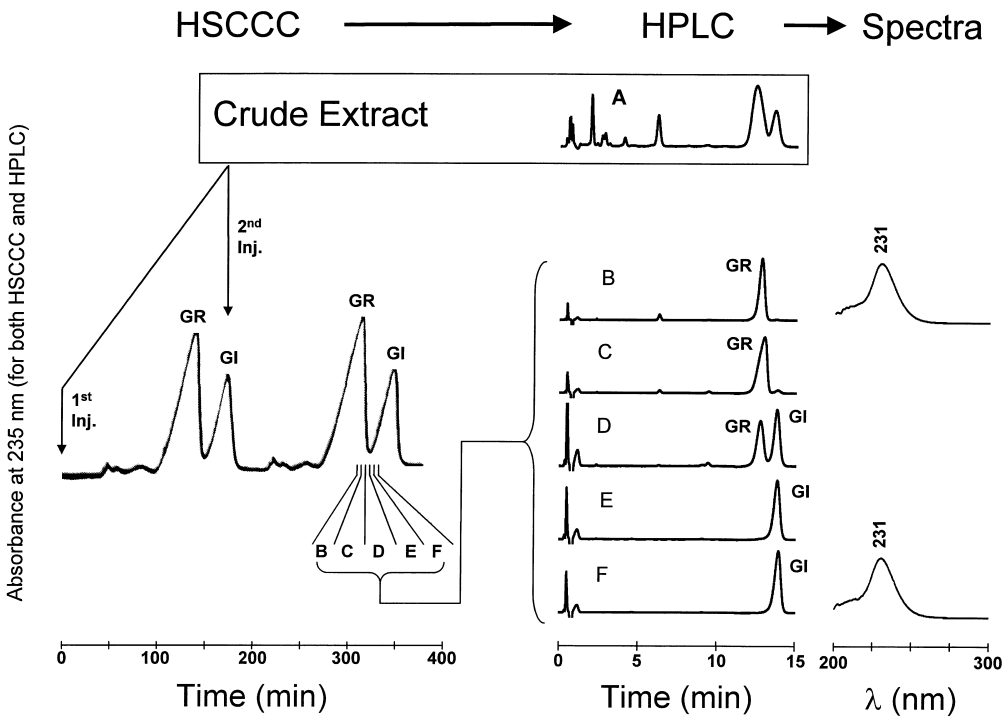

Fig. 3. HSCCC: representative chromatograms for 2 glucosinolates, on a Model CCC-1000 HSCCC (Pharma-Tech) equipped with an 850-ml column wound with 2.6 mm I.D. PTFE tubing. The solvent system consisted of 1-propanol–acetonitrile–saturated aqueous ammonium sulfate–water (1:0.5:1.2:1), using the organic phase as the mobile phase and the aqueous phase as the stationary phase. A piston-style, medium-pressure pump was used to achieve a flow-rate of 5 ml/min in the tail to head orientation and the centrifugal speed was 1000 rev./min. Stationary phase retention averaged ~80%, but ranged from 72% to 94% and was influenced by the purity and viscosity of the applied sample. Eighteen-ml fractions were collected. Peak elution was monitored at 235 nm using a variable wavelength detector. HPLC: aliquots sampled from the HSCCC injection loop (labeled A) and from consecutive 18-ml HSCCC fractions (labeled B–F) were injected on a 3 μm, 100×4.6 mm polyhydroxyethylaspartamide column run under isocratic conditions at 2 ml/min using 30 m*M* ammonium formate, pH 5.4, in acetonitrile–water (85:15) as the mobile phase [18]. Detection was performed with a photodiode array detector set at 235 nm. All runs were for 20 min, but no peaks eluted after 15 min, thus the chromatograms are truncated at 15 min for simplicity of presentation. Spectra: spectra from 200 to 400 nm were collected from the photodiode array detector. All peaks occurred at <300 nm, at which point spectra are truncated in this figure. Repetitive separation of GR and GI by HSCCC. First and second injections are denoted by arrows.

Table 2
Recovery and purity of glucosinolates from HSCCC

Glucosinolate (GS)	Crude preparation applied (mg)	GS in crude (mg)	GS recovered (mg)	% GS recovery
GR[a]	6394	519.6	512.3	98
GI[a]	6394	210.7	205.2	99
GE	52.4	≈35	31.3	≈90
SI	112.2	112.2	99.2	88
	45.3	45.3	43.4	96
4RBGS	21.2	17.0	15.8	93
	500.8	8.94	7.83	88

[a] Yield from a representative run; pooled run purity was >95% based upon direct HPLC analyses, UV–visible spectroscopy, and glucose and isothiocyanate analyses of a myrosinase-hydrolyzed purified glucosinolate.

With the successful separation of two very similar glucosinolates, GR and GI, attention was focused on other glucosinolates, at various stages of purity, to determine how broadly applicable this solvent system was. Additional glucosinolates that were separated on a small scale using the same preparative rotor and solvent system, include sinigrin (SI), glucoerucin (GE), and 4-rhamnopyranosyloxybenzyl glucosinolate (4RBGS) (Fig. 4). Sinigrin is present in mustard seed, wasabi, horseradish and other crucifers and it is the only glucosinolate commercially available as a crystalline standard. 4RBGS is a member of a very small group of glucosinolates that contain a second sugar moiety located in the side chain (the glucose moiety common to all glucosinolates is attached to the central carbon by a thioglucoside linkage). In the case of 4RBGS, the second sugar moiety is a rhamnose, which is esterified to a benzyl group, and these benzyl glucosinolate derivatives are much more hydrophilic than the parent benzyl glucosinolate [1]. The separations shown in Fig. 4A and B result from highly purified GE or partially purified 4RBGS plant extracts, spiked with high-purity SI. Differences in stationary phase retention and high salt concentrations in the GE and 4RBGS powders used in Fig. 4A and B, respectively, account for a very slight shift in retention time of SI. The amount of stationary phase displaced by mobile phase (the void volume) differs between the two runs presented, because the samples applied were of different consistencies and purities, and thus retention times differed slightly. When one calculates the partition coefficient, K, for sinigrin in each run, one obtains virtually identical values (e.g. 0.37 and 0.36 for the runs depicted in Fig. 4A and B, respectively) [$K = (V_{RT} - V_W)/(V_{TV} - V_W)$, where V_{RT} is the retention volume, V_W is the void volume, and V_{TV} is the total column volume].

Furthermore, HSCCC proved to be an ideal technique with which to separate glucosinolates (e.g.

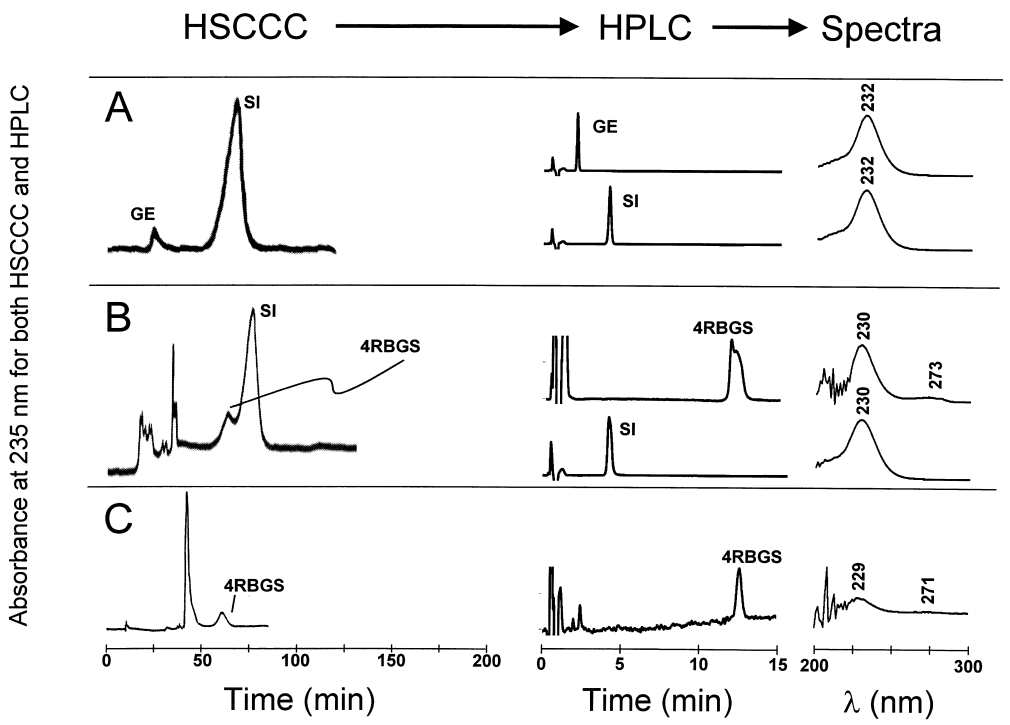

Fig. 4. HSCCC, HPLC, and UV spectra were obtained as described in Fig. 3 and Table 2. (A) Separation of GE (isolated from arugula seeds by flash chromatography), and crystalline SI. (B) Separation of 4RBGS (isolated from horseradish tree seeds by flash chromatography), and crystalline SI. (C) Separation of 4RBGS from horseradish tree leaf powder. The large unidentified peak was bright green and the 4RBGS peak was chromatographically free of other glucosinolates.

4RBGS) from the plant matrix, when a crude, aqueous plant homogenate was chromatographed directly (Fig. 4C). The elution time of 4RBGS was very similar to that presented in Fig. 4B, but of greater interest is that fact that there was a tremendously efficient separation of the glucosinolate from contaminating co-extractives. The large peak eluting at ca. 50 min was highly colored (green) whereas the 4RBGS peak produced a single peak upon HPLC and as such was ready for a final clean-up step. Of 8.9 mg 4RBGS applied to the column (in 500 mg of leaf powder), recovery was about 90% in a peak eluting between 62 and 75 min (Fig. 4C, Table 2).

A λ_{max} of 227 nm, and a molar extinction coefficient (ε) of 6780 M^{-1} cm^{-1} for SI is commonly used in the literature based in part upon the widespread availability of this glucosinolate in pure form, and its long time use in an enzymatic assay for myrosinase (the enzyme responsible for glucosinolate degradation in vivo) [15,23]. Values for λ_{max} and ε, and UV spectra for other glucosinolates are much less widely available. Some of the early glucosinolate literature provides representative UV spectra, and estimates of ε for other glucosinolates that are in the range of 6800 M^{-1} cm^{-1} [23–25], but their UV spectra are not available. UV spectra in water, for the five purified glucosinolates whose separations we have presented in the preceding discussion (Fig. 2) gave calculated molar extinction coefficients ε_{235} of 6234, 6531, 7408, and 6872 M^{-1} cm^{-1} for GI, GE, 4RBGS, and GR, respectively. The ε_{237} of sinigrin was 7369, in our hands. For sinigrin, $\lambda_{max} = 227$ nm, and for the other four glucosinolates whose spectra were determined, $\lambda_{max} = 225$ nm. 4RBGS has a broad, minor peak in the range 260–280 nm, attributed to absorbance by the benzyl ring. (The λ_{max} presented in Figs. 3 and 4 were obtained from chromatographic peaks in HPLC mobile phase, and are thus slightly different from those just discussed.) Knowledge of both ε and λ_{max} are of considerable importance in quantifying glucosinolates following their separation by HPLC or other chromatographic means. Most of these methods monitor a single wavelength, typically 235 nm, which is not the λ_{max} for any of the glucosinolates, but rather, is a compromise wavelength that avoids some of the interference in the shorter wavelength region. The steepest portion of the absorption spectrum is thus being used for sensitive quantitative comparisons. Such quantitative comparison of glucosinolates has historically been based primarily upon the internal standards benzyl glucosinolate [26], or sinigrin [18,19]. Thorough knowledge of the spectral properties of these compounds is a prerequisite to accurate quantification from HPLC chromatograms. We provide both chromatograms and UV spectra in Figs. 3 and 4 for each of the glucosinolate peaks of interest.

As more is learned about the importance of glucosinolates in protection against cancer and other chronic and degenerative diseases brought on by oxidative stress, more investigators are using these compounds in animal and clinical studies. These studies will require multi-gram quantities of glucosinolates, and the techniques presented herein provide an efficient and economical method of producing them.

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