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# Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography

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**Abstract**

While methods for the identification and quantification of total plant glucosinolate content typically utilize desulfation of glucosinolates followed by reversed-phase chromatography, the analysis of intact glucosinolates has been problematic. Hydrophilic interaction chromatography offers a novel method for analyzing intact glucosinolates and when performed along with ion-pair reversed-phase chromatography offers a powerful and complementary method for glucosinolate analysis.


*Keywords:* Vegetables; Food analysis; Glucosinolates

## 1. Introduction

Glucosinolates are plant secondary products (β-thioglucoside *N*-hydroxysulfates) that are found in over 500 different species, in 16 families of dicotyledonous (Magnoliophyta) higher plants [1]. They are most prominently represented in the family Cruciferae (Brassicaceae) which is one of the 10 most economically important plant families in the world and includes such vegetables as broccoli, Brussels sprouts, cabbage, cauliflower, bok choy, kale, radish, rutabaga and turnip. Glucosinolates may contain at least 120 different aglycones that can be grouped into at least 10 structural classes [1]. Average per person glucosinolate intake has been estimated at about 16 mg/day in Canada [2], 30 mg/day in the UK [3] and 112 mg/day in Japan [4], with US consumption estimated to be at the low end of this range (J.W. Fahey, unpublished data). There are efforts underway to develop vegetables with higher levels of certain of these chemoprotective glucosinolates [5–10]. Since both beneficial and toxic effects have been attributed to glucosinolates and their breakdown products, knowledge of both the types of glucosinolates and the quantities ingested by both humans and livestock is of considerable importance [11–16].

This paper describes an efficient, novel method for the chromatographic separation of intact glucosinolates. It is a powerful complement to ion-pair reversed-phase chromatographic techniques in that the hydrophobic interaction liquid chromatography (HILIC) system described herein, readily separates the most polar glucosinolates – those that are not

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well resolved in ion-pair systems without the use of multiple chromatographies, different ion pairs and mobile phases. High-performance liquid chromatography (HPLC) separation of sulfohydrolase desulfation products is now the most widely used method for glucosinolate analysis, but it suffers from inherent difficulties in interpretation as a result of pH, time and enzyme activity effects on the desulfation products [17–19]. Furthermore, the resultant desulfoglucosinolates cannot be enzymatically hydrolyzed with myrosinase in order to yield their cognate isothiocyanates which could subsequently be assayed chemically or for biological activity – key tools in the study of the pharmacokinetics, pharmacodynamics and bioactivity of these compounds. A reversed-phase $C_{18}$ ion-pair chromatography (IPC) technique was recently developed for the separation of glucosinolates [20–22]. The ion pairing agents neutralize the high charge conferred by the sulfate groups (which are ionized in the physiological pH range), while allowing for subtle differences in the non-polar side chains (R) to have a predominant effect on separation. This technique, while being effective and quantitative, does not, however, permit effective separation of all of the important glucosinolates in cruciferous vegetables (e.g., glucoraphanin, GR, and glucoiberin, GI) in a single chromatographic step ([22]; K. Stephenson, unpublished data). The chromatographic procedure reported herein complements the IPC method for the comparison of polar and non-polar glucosinolates in that it: (a) does not require desulfation for separation, (b) is rapid and inexpensive, (c) provides excellent separation of the glucosinolates most poorly resolved by IPC, and (d) it is a robust method that can also be used with larger, preparative columns.

## 2. Experimental

### *2.1. Glucosinolate standards*

1-Methoxyindol-3-ylmethyl-GS (neoglucobrassicin), 4-hydroxyindol-3-ylmethyl-GS (4-hydroxyglucobrassicin), 4-methylthiobutyl-GS (glucoerucin), 3-methylsulfonylpropyl-GS (glucocheirolin) and 3-methylsulfinylpropyl-GS (glucoiberin) (see structures in Fig. 1) were gifts from Robert K. Heaney (Insti-

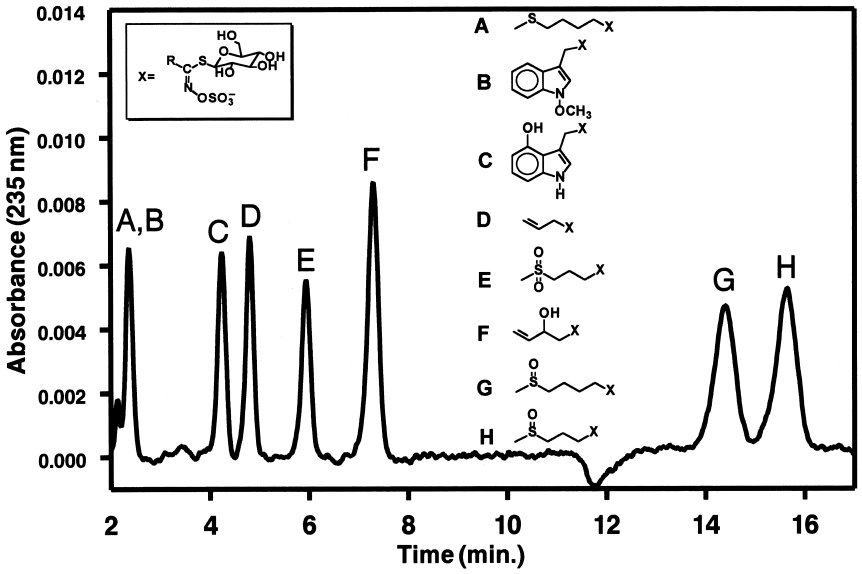


Fig. 1. HILIC (polyhydroxyethyl aspartamide column, 100×4.6 mm, 3 μm, 100 Å column; 2 ml/min isocratic run, 85% acetonitrile in water with 30 m*M* ammonium formate overall, pH 5.4) of glucosinolate standards showing the separation of a mixture of glucoerucin (A) & neoglucobrassicin (B); 4-hydroxyglucobrassicin (C); sinigrin (D); glucocheirolin (E); progoitrin (F); glucoraphanin (G), and glucoiberin (H). Note that more polar glucosinolates are easily separated while non-polar glucosinolates are not.

tute of Food Research, Norwich, UK). 4-Methylsulfinylbutyl-GS (glucoraphanin) was purified in our laboratory from broccoli seeds. Allyl glucosinolate (sinigrin) was obtained from Aldrich (Milwaukee, WI, USA). The identity and purity of all standards used were verified by electrospray mass spectrometry. All other chemicals and solvents were obtained from Sigma (St. Louis, MO, USA), Aldrich or J.T. Baker (Phillipsburg, NJ, USA).

### 2.2. Preparation of standards and plant samples

The purified, crystalline glucosinolate standards were resuspended in water, then diluted with acetonitrile in order to bring the sample organic concentration to within 3% of that of the mobile phase, then centrifuged prior to chromatography.

Broccoli seeds were homogenized with a Brinkmann Polytron Homogenizer (Kenimatica, Luzer, Switzerland) with 10 volumes of triple solvent (equal volumes of dimethylsulfoxide, dimethylformamide and acetonitrile) maintained at about −50°C in a dry-ice–ethanol bath [23]. The homogenates were centrifuged prior to chromatography, the organic solvent was removed by evaporation and the pellets were resuspended in the HPLC mobile phase buffer.

### 2.3. HPLC apparatus and columns

HPLC of both plant extracts and purified glucosinolates was performed: (a) by ion-pair chromatography as previously described [22], with a Genesis $C_{18}$ column (50×4.6 mm, 3 μm particle diameter), Jones Chromatography, Lakewood, CO, USA and (b) by HILIC on both analytical polyhydroxyethyl aspartamide columns (200×4.6 mm, 100 Å pore diameter, 5 μm particle diameter and 100×4.6 mm, 100 Å pore diameter, 3 μm particle diameter), and a preparative column (250×21 mm, 100 Å pore diameter, 12 μm particle diameter), PolyLC, Columbia, MD, USA. Columns were extensively equilibrated with a minimum of 60 column void volumes of solvents and conditioned as per manufacturer's instructions. Two identical Waters HPLC systems were used, each equipped with a Model 996 photodiode array detector and a 717 WISP automated injector. Data were processed with Waters Millennium software. The isocratic mobile phases used for HILIC ranged from 82.5 to 87% (v/v) acetonitrile in water with 30 m*M* ammonium formate, apparent pH 5.4–8.0 (measured in final solvent) at flow-rates of 2 ml/min (analytical) and 20 ml/min (preparative). All solvents were filtered and degassed prior to use.

## 3. Results and discussion

Excellent separation of a mixture of glucosinolate standards, possessing a wide range of side chain moieties was achieved using a mobile phase consisting of 85% acetonitrile (aqueous) with 30 m*M* ammonium formate, pH 5.4 (Fig. 1). The more polar glucosinolates such as glucoiberin and glucoraphanin were well retained by the column while hydrophobic glucosinolates, such as neoglucobrassicin and glucoerucin were weakly retained and only partially resolved. The identity of all standards was confirmed by electrospray mass spectroscopy (data not shown). In order to successfully separate the wide spectrum of side chain moieties that are present in glucosinolates of natural origin (reviewed by Fahey et al. [1]), the mobile phase can be modified in two different ways. First, the organic concentration can be adjusted to change the partition coefficient and, second, the pH can be adjusted to alter selectivity [24]. For example, at pH<3.5 and pH>8.0 there is no separation or selectivity of glucosinolates by this stationary phase (data not shown), presumably because the *N*-hydroxysulfate group of the glucosinolates is fully protonated at low pH, and fully ionized at the higher pH. At pH≈5.4 this moiety is expected to be partially protonated and therefore contributes to separation based upon hydrophilic interactions. The polarity of the side chain thus also influences separation in this pH range.

We have previously demonstrated the separation of intact glucosinolates using ion-pair chromatography on a non-polar stationary phase ($C_{18}$ reversed-phase chromatography) [22]. The result of a typical separation of a broccoli seed extract using this method is shown in Fig. 2. In contrast, Fig. 3 shows a HILIC chromatogram from extracts of broccoli seeds (Fig. 3a) 4-day-old sprouts (Fig. 3b) and florets from a market stage head (Fig. 3c), all from the most

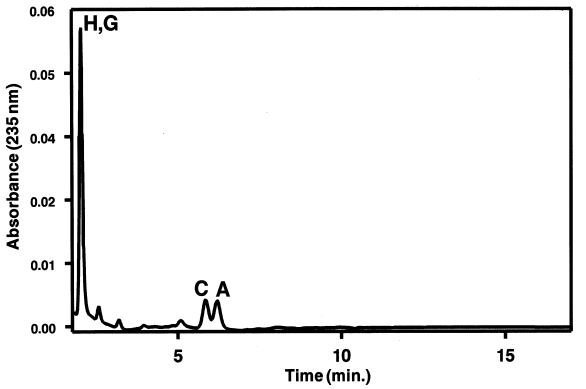


Fig. 2. IPC of a broccoli seed extract showing good separation of the relatively non-polar indole glucosinolates 4-hydroxyglucobrassicin (C), and glucoerucin (A), and no separation of the more polar glucosinolates glucoiberin (H), and glucoraphanin (G). [Genesis $C_{18}$ column (50×4.6 mm, 3 μm); 2 ml/min isocratic run – mobile phase 5 m*M* tetradecylammonium bromide in 53% acetonitrile].

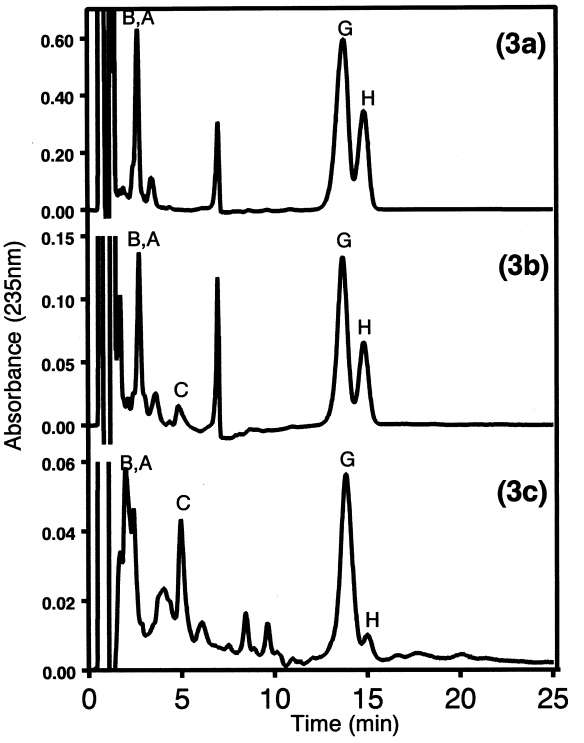


Fig. 3. HILIC of broccoli (*Brassica oleracea* var. *italica*) cultivar Marathon, which is the most widely grown commercial cultivar in the US. Major glucosinolate peaks are identified with letters. Peaks without letters are not glucosinolates based upon their failure to disappear after myrosinase digestion and re-chromatography of the hydrolysate. A broccoli seed extract (a), demonstrates good separation of the polar glucosinolates glucoraphanin (G), and glucoiberin (H). Further developmental stages of the same cultivar – 4-day-old sprouts (b), and florets from market stage broccoli heads (c), have progressively less glucoiberin (H), and more 4-hydroxyglucobrassicin (C). As a metric for comparison between (a), (b) and (c), the total glucoraphanin (G) content of each, was 36, 6.8 and 0.88 μmol/g fresh mass for seeds, sprouts and florets, respectively. [polyhydroxyethyl aspartamide column (100×4.6 mm, 3 μm, 100 Å); 2 ml/min isocratic run – mobile phase 85% acetonitrile in water with 30 m*M* ammonium formate overall, pH 5.4].

widely grown broccoli cultivar used in frozen and fresh broccoli production for United States markets (*Brassica oleracea* var. *italica* cv. Marathon). The elution pattern for HILIC is essentially the converse of that from reversed-phase IPC, and glucoraphanin and glucoiberin were resolved using HILIC whereas they co-elute using the IPC method of Prestera et al. [22].

The $\lambda_{max}$=224 nm for the *N*-hydroxysulfate moiety of glucosinolates, with a molar extinction coefficient of ca. 7000 $M^{-1}$ $cm^{-1}$. Indole, aryl and alkenyl side chains contribute absorbance at $\lambda_{max}$=250–280 nm with a lower molar extinction coefficient. The limit of quantitation of sinigrin at 228.5 nm (its $\lambda_{max}$) based on a minimum reproducible peak area of 10 000 μV s is approximately 58 pmol/injection. The $\lambda_{max}$ for a number of other glucosinolates is 224 nm, but at this wavelength, there is significant absorbancy of the formate ion. There is thus an elevated baseline even with relatively clean samples, and this wavelength is not suitable for mixed natural samples (e.g., plant extracts). We therefore routinely monitor absorbance at three wavelengths (224, 235 and 280 nm) using a photodiode array detector. All quantitation was based upon peak area at 235 nm in order to balance the interference caused by other UV absorbing substances in crude plant extracts with the $\lambda_{max}$ of the compounds being analyzed – a compromise wavelength adopted by many others for the spectrophotometric detection of glucosinolates. Ultimate confirmation of glucosinolate identity from crude natural samples can then be easily performed by repeat injection of a companion sample that has been treated by myrosinase and by mass spectroscopy, which can be performed directly upon the collected peak [1,22]. The linear range of the peak area

calibration curve of sinigrin using a 200×4.6 mm polyhydroxyethyl aspartamide column (2 ml/min 82.5% acetonitrile in water, 30 m*M* ammonium formate, pH 5.4), was between 58 pmol/injection and 580 nmol/injection. The regression line has an $r^2$ correlation coefficient of 0.994. Individual glucosinolates in edible plants (e.g., vegetables) typically range from ca. 0.01 to 10 μ*M* (nmol/g fresh mass) [1,10,25–27], and can therefore be detected in plant extracts, either directly, or after concentration. Repeated injections ($n=10$) of 20 μmol of a sinigrin standard resulted in standard deviations of only 1.03 and 3.04% for the retention time and integrated peak area, respectively, and are thus highly reproducible.

We have also used a preparative HILIC column for the rapid clean-up of large quantities of partially purified glucosinolates. Using an identical running buffer (30 m*M* ammonium formate in 85% acetonitrile, pH 5.4), we have been able to obtain 100 mg at a time of highly purified glucosinolates from a plant extract that had been partially purified by ion-exchange chromatography.

## 4. Conclusion

Whereas reversed-phase liquid chromatography on octadecyl silane ($C_{18}$) supports has become an indispensable method for separating and purifying many classes of compounds from complex mixtures such as plant extracts, fermentation broth, serum, plasma and urine, certain polar compounds cannot be separated effectively on such matrices. These polar compounds either do not associate with the stationary phase or are only weakly retained. HILIC utilizes a polar stationary phase and a mobile phase sufficiently organic to lead to partitioning between the mobile phase and the aqueous layer. It has previously been used for the separation of peptides, nucleic acids, phosphorylated peptides, and other polar compounds [24,28–35]. Compounds elute in order of increasing hydrophilicity with decreasing organic gradients. This results in an elution pattern that is typically in the opposite order compared to that from traditional non-polar stationary phases such as octadecyl silane ($C_{18}$). A stationary phase coated with polyhydroxyethyl aspartamide retains solutes predominantly through hydrophilic interactions when using mobile phase organic concentrations in the range of 60–95% acetonitrile [24]. Some mixed-mode interactions are possible below 70% acetonitrile. Highly charged molecules, such as kanamycin or ATP, require salt (e.g., 10 m*M*) for ion suppression and a gradient of perchlorate, acetate or chloride in a high organic solvent concentration to effect desorption [24].

The most widely used technique for the analysis of plant glucosinolates relies upon hydrolytic removal of the sulfate group of intact glucosinolates followed by reversed-phase chromatography. Intact glucosinolates can be analyzed using both a reversed-phase ion-pair method and a complementary, normal-phase hydrophilic interaction (HILIC) method which allow for the separation of both polar and non-polar glucosinolates. Combined, these two methods are a powerful tool for the evaluation of total plant glucosinolates. The HILIC method permits further direct use of the eluted compounds (e.g., in a bioassay as described by Prochaska et al. [36] as modified by Fahey et al. [23]) and their enzymatic conversion to isothiocyanates prior to such assay or to use in a cyclocondensation assay for the rapid quantitation of isothiocyanates [37,38]. Since ammonium formate is easily volatilized, peaks can be directly introduced into mass spectrometers and nuclear magnetic resonance (NMR) spectrometers for confirmation of molecular mass and structure. The ability to resolve recalcitrant, structurally similar glucosinolates like glucoraphanin and glucoiberin represents a significant achievement in separation methodology which should also be of value to those studying the medicinal and dietary applications of plant glucosinolates.

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