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Short communication

# Single column approach for the liquid chromatographic separation of polar and non-polar glucosinolates from broccoli sprouts and seeds

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**Abstract**

Ion-pair and hydrophilic interaction chromatographies are considered to be complementary methods of choice for analyzing intact glucosinolates from broccoli. Ion-pair chromatography resolves non-polar glucosinolates, such as those containing indole moieties, while hydrophilic interaction chromatography is superior for separating polar glucosinolates, such as glucoraphanin and glucoiberin. Reversed-phase separations using hydrophilic endcapped $C_{18}$-bonded silica and a 50 m*M* ammonium acetate–methanol gradient mobile phase resolve both polar and non-polar glucosinolates negating the need for switching columns.


*Keywords:* Food analysis; Glucosinolates

## 1. Introduction

Our research required that we have a procedure for reliably measuring the cancer-chemoprotective polar glucosinolate, glucoraphanin in extracts from broccoli sprouts and seeds. A main requirement for us was the complete resolution of glucoraphanin from glucoiberin, another polar glucosinolate differing by one side chain methylene group, but with much less cancer-chemoprotective activity [1]. As reported by others, resolution of these compounds has been problematic [2]. In addition, we wanted information regarding the presence of various non-polar glucosinolates, namely those containing certain indole moieties, because some are thought to give rise to substances detrimental to health [3,4]. We also wanted to analyze extracts prepared by established procedures, specifically those using an organic triple solvent [5] or boiling water [5,6]. Finally, it was our desire to have a single chromatographic approach for detecting both polar and non-polar glucosinolates found in broccoli while maintaining the quality of the analytical measures recently reported for a polar glucosinolate separation technique [2].

The current recommendation for analyzing intact glucosinolates is to employ complementary chromatographic techniques, one based on reversed-phase ion-pair chromatography (IPC) and the other normal-phase hydrophilic interaction chromatography (HILIC) [2]. IPC was reported best for resolv-

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ing non-polar glucosinolates whereas HILIC was superior at resolving polar glucosinolates. Additional details of these techniques have been the subject of recent publications [2,3].

The separation of phenethyl glucosinolate in cabbage and watercress extracts using a hydrophilic endcapped $C_{18}$-bonded silica column [7] as well as other reports of the utility of this type of stationary phase for the chromatography of very water soluble molecules in highly aqueous mobile phases [8] suggested to us that this column chemistry might be a good candidate for combining the non-polar glucosinolate separating power of IPC with the polar glucosinolate resolving power of HILIC.

In this short communication, we present chromatographic conditions that provide superior resolution of both non-polar and polar glucosinolates, including glucoraphanin and glucoiberin, from broccoli sprouts and seeds using a single column.

## 2. Experimental

### 2.1. Reagents

Allyl glucosinolate 98% purity (sinigrin) and ammonium acetate 99.999% purity were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were either from Aldrich or Fisher Scientific (Pittsburgh, PA, USA).

### 2.2. Sample preparation

Glucosinolates were extracted from freeze–dried and pulverized broccoli sprouts (BroccoSprouts; Brassica Protection Products, Baltimore, MD, USA) by homogenizing in a mixture of equal volumes of dimethylsulfoxide, dimethylformamide and acetonitrile maintained at about −50 °C [5]. After centrifugation and filtration, the filtrate was concentrated to dryness under rotary vacuum evaporation. Broccoli seeds (cultivar Pirate; New England Seed, Hartford, CT, USA) were pulverized and defatted with hexane. Pulverized broccoli sprouts or defatted seed powder were stirred into boiling water and held for 5 min [6]. After filtering, water extracts were freeze–dried. Samples prepared by either procedure were reconstituted in water (~1 mg/ml), further diluted with water as necessary, and injected directly onto the high-performance liquid chromatography (HPLC) columns.

### 2.3. HPLC–UV analyses

The HPLC instruments were either HP1090 (Hewlett-Packard, Wilmington, DE, USA) or Alliance 2690 (Waters, Milford, MA, USA) liquid chromatographs. The columns were either a Prevail $C_{18}$, 5 μm, 250×4.6 mm (Alltech Associates, Deerfield, IL, USA) or a Waters YMC-Pack ODS-AQ, 3 μm, 120 Å, 150×2.0 mm. With the Prevail column, the mobile phase was a linear gradient from 100% 50 m*M* ammonium acetate to 50 m*M* ammonium acetate–methanol (80:20) in 40 min. The column was operated at ambient temperature with a flow-rate of 1.0 ml/min. The injection size was 10 μl. For the YMC column, the mobile phase and gradient were identical to that used with the Prevail column, except that the column was operated at 30 °C and a flow-rate of 0.2 ml/min. The injection volume was 2 μl. Columns were protected with appropriate guard columns. The wavelength for detecting glucosinolates in all instances was 235 nm and data was processed with a HP3396 Series II integrator. Quantitation was based on allyl glucosinolate; a technique used by others [5].

### 2.4. HPLC–mass spectrometric analyses

An Alliance 2690 HPLC system was series coupled to a Waters 2487 absorbance detector and a Finnigan MAT TSQ 700 (triple-stage quadrupole) mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with a Finnigan electrospray ionization (ESI) source. Flow-rates from the HPLC system were reduced after the UV flow cell using a high-pressure micro-splitter valve (Upchurch Scientific, Oak Harbor, WA, USA) to a flow-rate of 0.10 to 0.15 ml/min, which was then delivered to the ESI source. The ESI parameters were with the source operated in the negative-ion mode, heated desolvation capillary held at 270 °C, sheath gas ($N_2$) $6.89\times10^5$ Pa (100 p.s.i.), auxiliary gas ($N_2$) $1.4\times10^5$ Pa (20 p.s.i.), and needle voltage of 2.6 kV (1 p.s.i.=6894.76 Pa). Full-scan mass spectra were acquired with Q1 being repetitively scanned from $m/z$ 200 to 1000 in 2 s.

Product-ion mass spectra were acquired with a retention-time-dependent, automated LC–tandem mass spectrometry (MS–MS) procedure written in the Instrument Control Language. The collisionally induced dissociation (CID) parameters involved selecting the putative $(M-H)^-$ ions with Q1 and scanning Q3, argon collision gas at a pressure of 0.4 Pa (3 mTorr), collision energies ranging between 15 and 28 eV, and the MS–MS correction factor set to zero. All identified peaks were confirmed homogeneous and consistent with their assigned chemical structure by MS–MS [9].

## 3. Results and discussion

As presented in Figs. 1 and 2, baseline separation of intact broccoli sprout and seed glucosinolates was essentially achieved with both the Prevail and YMC columns. Although chromatograms of only two of the six possible combinations of column manufacturer matched with extraction method and plant material evaluated by us are presented, in all cases the quality of the chromatography was equally good. The only major difference between the columns was the reversal of the elution order for 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl glucosinolate) and glucoibervirin [3-(methylthio)propyl glucosinolate], with the latter eluting before the former on the Prevail column (chromatogram not shown). Glucoraphanin [4-(methylsulfinyl)butyl glucosinolate] was completely resolved from glucoiberin [4-(methylsulfinyl)propyl glucosinolate] using either column. This is the first report demon-

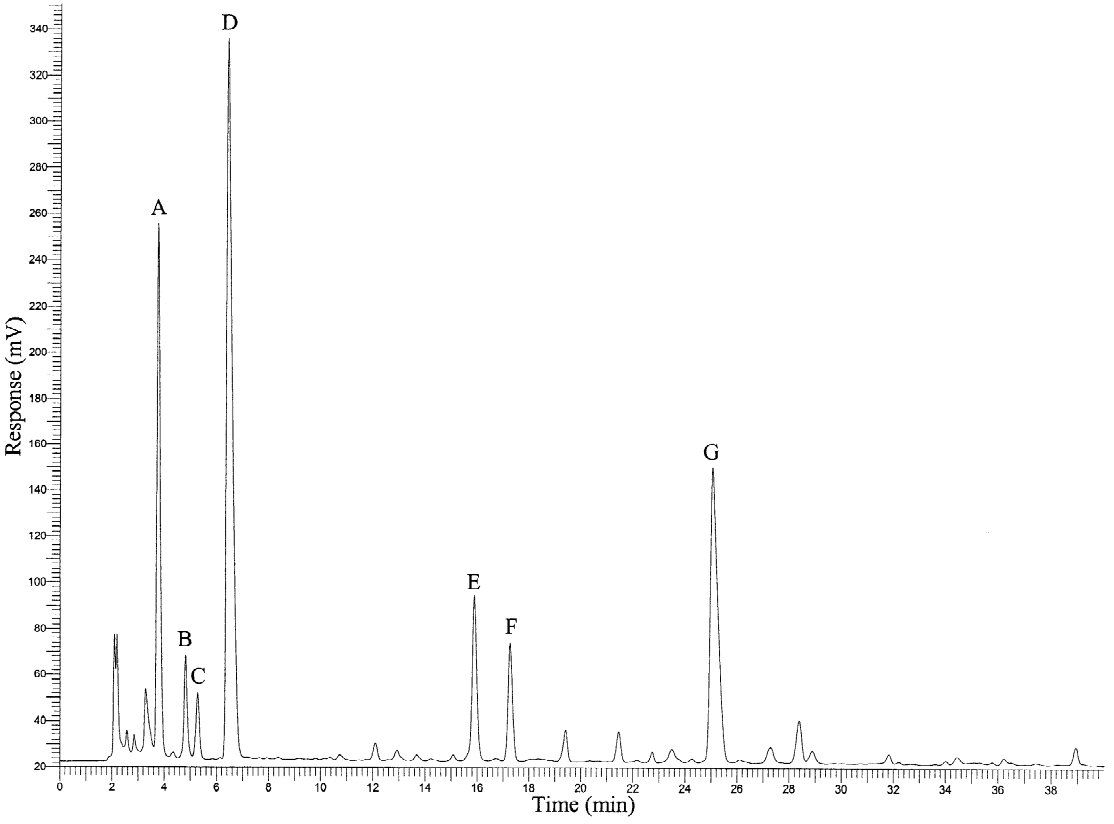



Fig. 1. HPLC–UV chromatogram of broccoli sprout glucosinolates extracted with an organic triple solvent showing the separation of (A) glucoiberin, (B) progoitrin and/or epiprogoitrin, (C) sinigrin, (D) glucoraphanin, (E) 4-hydroxyglucobrassicin, (F) glucoibervirin, and (G) glucoerucin (Waters YMC-Pack ODS-AQ column; conditions presented in Section 2.3).

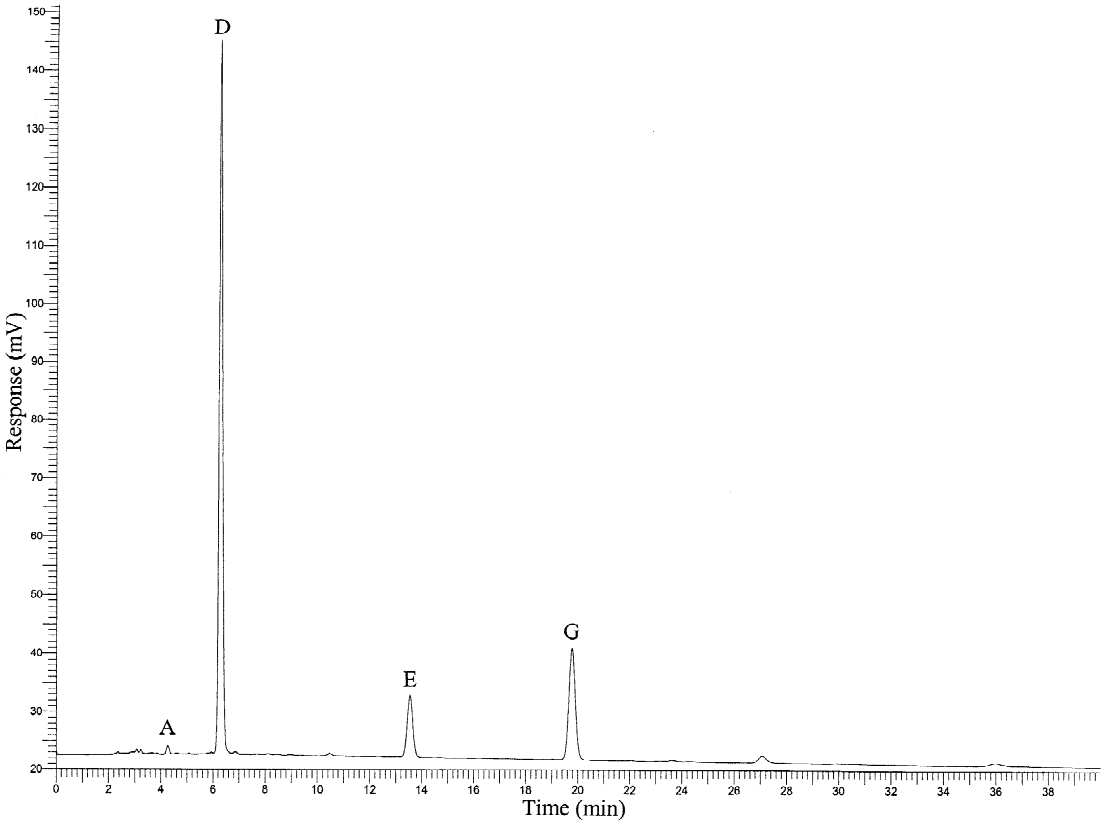


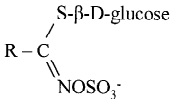

Fig. 2. HPLC–UV chromatogram of broccoli seed glucosinolates extracted with boiling water showing the separation of (A) glucoiberin, (D) glucoraphanin, (E) 4-hydroxyglucobrassicin, and (G) glucoerucin; [(B), (C), and (F) detected in sprouts and presented in Fig. 1 were not detected] (Prevail $C_{18}$ column; conditions presented in Section 2.3).

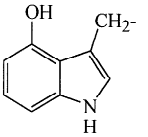
strating the baseline separation of these two compounds while simultaneously resolving other polar and non-polar broccoli glucosinolates.

Separation of progoitrin [(2(*R*)-2-hydroxy-3-butenyl glucosinolate] and epiprogoitrin [2(*S*)-2-hydroxy-3-butenyl glucosinolate] would not be expected with $C_{18}$-bonded silica and their resolution would require the use of chiral stationary phases. Since both compounds give rise to substances that have similar goitrogenic effects [3,10], measuring them together, if indeed both are present, is adequate for our purposes. Even though we detected only one indole-containing glucosinolate (4-hydroxyglucobrassicin) in any appreciable quantity in BroccoSprouts (in agreement with Fahey et al. [5]) and cultivar Pirate seeds, our chromatography makes it easy to ensure that as different varieties and forms of broccoli are evaluated to find those with the highest glucoraphanin levels, glucosinolates suspected of being harmful are not inadvertently overlooked. The structures of all identified glucosinolates are presented in Table 1.

It is worth noting that 2- to 3-day-old BroccoSprouts contained a significantly higher number of glucosinolates in addition to glucoraphanin as compared to cultivar Pirate seeds. This observation was not due to the different extracting solvents, since sprouts extracted with boiling water resulted in chromatograms essentially identical to those extracted with the dimethylsulfoxide, dimethylform-

Table 1
Chemical structures of the identified glucosinolates




| Compound | Name | R Group |
|---|---|---|
| A | Glucoiberin | $CH_3–SO–CH_2–CH_2–CH_2–$ |
| B | Progoitrin/epiprogoitrin | $CH_2=CH–C^*HOH–CH_2–$ |
| C | Sinigrin | $CH_2=CH–CH_2–$ |
| D | Glucoraphanin | $CH_3–SO–CH_2–CH_2–CH_2–CH_2–$ |
| E | 4-Hydroxyglucobrasicin |  |
| F | Glucoibervirin | $CH_3–S–CH_2–CH_2–CH_2–$ |
| G | Glucoerucin | $CH_3–S–CH_2–CH_2–CH_2–CH_2–$ |

amide and acetonitrile mixture (chromatogram not shown). Those researchers interested in isolating quantities of purified glucoraphanin will do well considering cultivar Pirate seeds as the preferred starting material. Additionally, hydrophilic end-capped $C_{18}$-bonded silica preparative columns are commercially available, and while we have not done so, our technique should be readily adaptable to preparative-scale processes.

Comparison of results for allyl glucosinolate calibration curve linearity and repeat injection peak area and retention time repeatability exceeded those published for HILIC [2]. Using the Prevail column, the previously reported 58 pmol/injection to 580 pmol/injection allyl glucosinolate standard curve linearity was duplicated ($r^2 = 0.999$ compared to 0.994) as well as the repeatability of area counts (2.3% compared to 3.0%) and retention time (0.1% compared to 1.0%) for ten 10 μl injections of a 20 μmol allyl glucosinolate standard. The limit of detection was 7 pmol/injection. The narrower bore YMC column allowed us to extend the linear range of the allyl glucosinolate calibration curve to 12–120 pmol/injection while still retaining an $r^2$ correlation coefficient of 0.999. The repeatability for area counts and retention time of ten 2 μl injections of the 20 μmol allyl glucosinolate standard was 2.7% and 0.6%, respectively. The limit of detection was 1 pmol/injection.

We have found this single column approach for readily resolving both non-polar and polar glucosinolates present in isolates obtained using proven extraction procedures extremely useful. We believe it should prove valuable to other researchers studying broccoli glucosinolates as well.

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