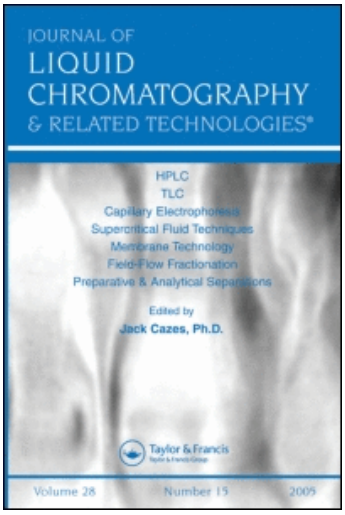

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Technology Transfer and Scale Up of a Potential Cancer-Preventive Plant Dynamic Extraction of Glucoraphanin

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Technology Transfer and Scale Up of a Potential Cancer-Preventive Plant Dynamic Extraction of Glucoraphanin

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Abstract: Glucosinolates are anionic, hydrophilic β-thioglucoside N-hydroxysulfates, which are abundant plant secondary metabolites found in cruciferous plants and are of particular interest for their chemoprotective, antioxidant and antibiotic activities. The purification of gluoraphanin (GR), the predominant glucosinolate in broccoli, was achieved using high-speed countercurrent chromatography (HSCCC) with a high salt, highly polar phase system of 1-propanol-acetonitrile-saturated ammonium sulphate-water (1:0.5:1.2:1) on a preparative scale (850 mL capacity) Pharma-Tech HSCCC instrument at ca 700 mg glucosinolates per run in about 3 hours. To scale up the production of glucosinolates the technology was transferred from Johns Hopkins to Brunel Institute for Bioengineering (BIB). Within three days a 50 × scale up was achieved with comparable target compound recovery and purity using a MIDI-dynamic extraction centrifuge (928 mL capacity) with run times of

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30 min and processing loads of 30 mL of ca 50% w/w viscous solutions (15 g injected). The 34 runs processed 589 g of extract producing a total of 52.6 g of 98% pure GR.

Keywords: Countercurrent chromatography, Dynamic extraction, Chemoprotection, Glucosinolates, Glucoraphanin

INTRODUCTION

Glucosinolates (GS) are anionic, hydrophilic β-thioglucoside N-hydroxysulfates, which are abundant plant secondary metabolites found in cruciferous plants and other plant families.[1] They are enzymatically converted to isothiocyanates by myrosinase, a thioglucosidase which coexists in the plant cells and is released upon tissue damage when food is prepared or chewed. These isothiocyanates are of considerable interest because of their cancer chemoprotective, antioxidant and antibiotic activities. Although their cancer-preventive potential was initially described as a result of their induction of Phase 2 enzymes, they have more recently been shown to possess potent antiproliferative, apotosis-promoting, redox regulatory, and phase 1 enzyme inhibitory roles,[1] as well as being directly bactericidal against the carcinogen, *Helicobacter pylori*.[2]

Glucosinolates have potential value as phytochemical components of healthy diets that could be added to functional foods.[3] To this end, the isolation of certain members of this class of molecules is of interest. Glucosinolates are water-soluble compounds whose physicochemical properties are dominated by the ionized sulphate and hydrophilic carbohydrate moieties. Recently, Fahey and colleagues[3] have purified five glucosinolates by high speed countercurrent chromatography (HSCCC) using a high salt, highly polar system of 1-propanol:acetonitrile:saturated aqueous ammonium sulphate:water (1:0.5:1.2:1) on a preparative scale, using a Pharma-Tech 850 mL capacity, 160 m length, 2.6 mm bore instrument, running at 5 mL/min in normal phase mode, with run times of ca 3 h. This phase system was developed from the ethanol:acetonitrile:saturated ammonium sulphate:water (1:0.5:1.2:1) systems used by Degenhardt and Winterhalter[4] to isolate from red beets the very hydrophilic betalains, which are also β-D-glucosides, but with nitrogen containing heterocycles as aglycones.

The dynamic extraction (DE) method is a recent development of countercurrent chromatography (CCC) introduced by the Brunel Institute for Bioengineering. It provides separations in minutes rather than hours. The DE equipment is more robust than previous HSCCC machines and scale-up to pilot scale has been shown to be both quick and easy. It is being commercialised by Dynamic Extractions Ltd (www.dynamicextractions.com). To distinguish these designs and machines from former CCC instruments, and to highlight the added advantages they provide, we have introduced the term "dynamic extraction" for CCC utilising rotating coils, which can be used

for process scale separations.[5] Three scales of DE centrifuges are currently available: MINI, MIDI, and MAXI (Table 1).

This paper describes the purification of ca 50 g of glucoraphanin (GR) (Figure 1) from broccoli seed extracts using a DE-MIDI centrifuge. It represents the first stage in the development of a large scale manufacturing process using the DE-MAXI-centrifuge.[6]

EXPERIMENTAL

Materials

1-Propanol, acetonitrile, and ammonium sulphate were all Analar grade (VWR International). Water was deionised (Purite, Thames, Oxon, UK).

Preparation of Crude Plant Extracts

Two different types of plant extract were used, both of which finally provided concentrated methanol extracts, which were loaded onto the dynamic extraction centrifuges:

Extract 1. Broccoli seeds were extracted with boiling water and spray dried and delivered as a fine powder, as described in.[3] Each gram of powder was extracted with 10 mL of boiling methanol, filtered (Whatman No 1), and concentrated to a syrupy consistency by rotary evaporation at 50°C.

Extract 2. Broccoli seeds were extracted with boiling methanol as follows. Seeds (13 kg) were first homogenised dry in a Waring blender, then boiled in methanol (ca 10 L) in a steam jacketed kettle (40 L), and the extract removed by vacuum filtration (Whatman filter paper) on a large

Table 1. DE centrifuges: comparison of characteristics

Instrument: previous designation	Milli-CCC	Brunel-CCC	Pilot-CCC
Insrument: new designation	DE-MINI	DE-MIDI	DE-MAXI
Scale of application	Analytical	Prep	Pilot
Processing rate	up to mg/h	g/h	kg/day
Main rotor radius (mm)	50	110	300
Volume of coils used (mL)[a]	5	1000	5000
Tube diameter (mm)	0.8	3.6	10
Tubing length (m)	10	90	30
Max rotor speeds (rpm)	2100	1400	850
Flow range (mL/min)	0.25–2	5–80	100–1000
Typical elution time (min) for D = 1 peak	5 min	25 min	10 min

[a]Other coils sizes can be designed.

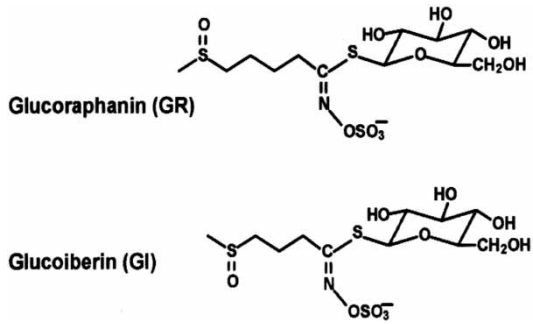

Figure 1. Structures of glucoraphanin and glucoiberin.

Buchner funnel. The residue was boiled a second time with methanol and filtered. The pooled filtrates (ca 15–18 L) were returned to the steam jacketed kettle and boiled with stirring until the volume was reduced to ca 770 mL. The viscous residue contained ca 50 g of GR. It was poured off into a pear shaped flask and residual methanol removed on a rotary evaporator by heating at 50°C to constant weight.

Preparation of the Phase System

Initially the phase system was prepared as described by Fahey et al.[3] Saturated ammonium sulphate was prepared at 78°C by preparing a saturated solution of ammonium sulphate in boiling water, letting this cool to 78°C, and decanting the supernatant. This was then mixed with water, n-propanol, and acetonitrile in the volume ratio of 1-propanol:acetonitrile:saturated ammonium sulphate: water of 1:0.5:1.2:1, equilibrated to 25°C and the phases separated. This proved too cumbersome a procedure for routine work. Since mixing a saturated ammonium sulphate solution with water is, in fact just diluting, the final ammonium sulphate concentration obtained on mixing the saturated ammonium sulphate (1.2 vol) with water (1 vol) was determined as 31% w/w by comparing the refractive index to published values.[7] This salt solution was directly prepared. The biphasic liquid system (9.2 L) was routinely prepared for the experiments by mixing 1.25 L of acetonitrile with 2.5 L of 1-propanol and 5.5 L of 31% ammonium solution. The later solution was made by dissolving 2008 g of ammonium sulphate in 4469 g of water. After equilibration at 25°C, this provided 4.3 L upper organic phase and 4.9 L of lower aqueous phase.

Dynamic Extractions: DE-MIDI Centrifuge

A "J" type coil planet centrifuge and control system (Brunel CCC machine re-branded as a DE-MIDI centrifuge) was used with a rotor radius of

110 mm as described elsewhere in detail.[8] A stainless steel coil (928 mL) was used (created by the linkage of the two bobbins, each 464 mL in volume), total length 90 m, bore 3.6 mm. Normal phase operation was used: the machine was filled with stationary aqueous (lower) phase and the mobile phase was the organic (upper) phase. Run conditions were typically 800 and 1200 rpm with flow rates in the range 40–80 mL/min. A UV detector was used at 235 nm.

HPLC Analysis for Glucoraphanin and Glucoiberin

Analysis of fractions for glucoraphanin (GR) content and purity was performed by HPLC using a 3 μm, 100 × 4.6 mm polyhydroxyethyl asparamide hydrophilic interaction column (HILIC) manufactured by PolyLC, Columbia, USA using isocratic mobile phase 30 mM ammonium formate, 85% acetonitrile, pH 5.4, flow rate 2 mL/min, detection at 235 nm.[9]

RESULTS AND DISCUSSION

Extract 1

The concentrated methanol extract was loaded in both mobile phase and stationary phase under varying conditions of flow rate (40, 60, 80 mL/min) and rotational speed (800 and 1200 rpm). This led to a selection of a rotational speed of 1200 rpm and flow rate of 40 mL/min. Figure 2 shows a typical

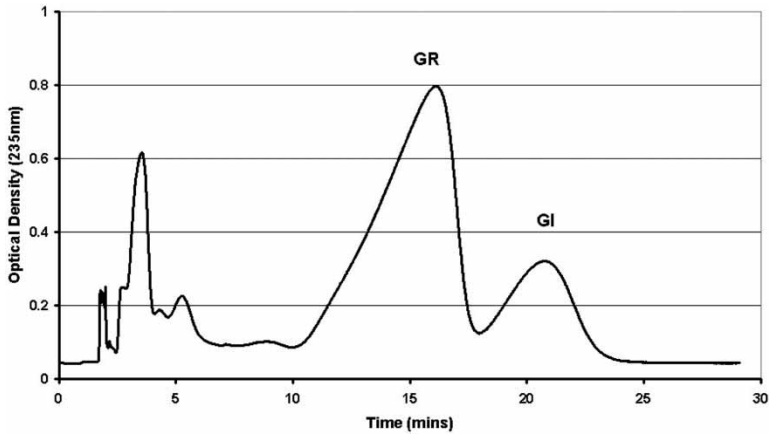

Figure 2. Dynamic extraction chromatogram of Extract 1 prepared from broccoli seed. DE-MIDI centrifuge: normal phase operation with 1-propanol/acetonitrile/ammonium sulphate; rotational speed 1200 rpm; flow rate 40 mL/min. GR—glucoraphanin; GI—glucoiberin.

chromatogram: the GR peak at 16 min is well separated from the glucoiberin (GI) peak at 21 min.

This was very similar to the separations achieved by Fahey et al.,[2] but most important, the present separations were achieved within 30 min compared with the latter's 3 h separations on the Pharma-Tech instrument.

However, analysis of 20 mL fractions taken through the GR and GI peaks (Figure 3) revealed a co-eluting glucosinolate contaminant, which could be resolved from GR by HPLC. This contaminant was identified as methylbutyl glucosinolate (compounds 53, 54 or 55 in Table 1 of Fahey et al.[1]) by mass spectroscopy (J.W. Fahey, data not presented). This minor glucosinolate contaminant was also identified in the seed feedstock and was not an artifact of extraction or chromatography.

Extract 2

Rather than develop a new phase system to separate this contaminant from GR whilst maintaining the separation of GR from GI, an alternative seed source, confirmed to be free of methylbutyl glucosinolate, was identified. In addition, Extract 2 was a methanol extract, rather than a water extract, thus enabling us to avoid the need for spray-drying.

A loading mix of 5 g of methanolic syrup dissolved in 10 mL of the CCC phase system (ratio of stationary phase:mobile phase = 6:4 as described by Fahey et al.[3]) was used. A loading study was performed by increasing the sample volume from 10 mL (*ca* 1% column volume) to 50 mL (*ca* 5% column volume; Figure 4).

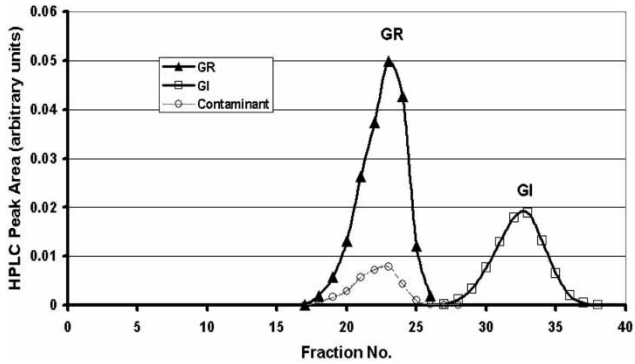

Figure 3. The presence of a contaminant co-eluting with glucoraphanin. Dynamic extraction chromatogram of Extract 1 of broccoli seeds was performed on 10 mL (~10% column volume) of load mix (prepared as 25 g of syrup plus 50 mL stationary phase) on a DE-MIDI centrifuge: normal phase operation with 1-propanol:acetonitrile: ammonium sulphate; rotational speed 1200 rpm; flow rate 40 mL/min. HPLC analysis of fractions was used to identify methylbutyl glucosinolate (MBGS), GR, and GI.

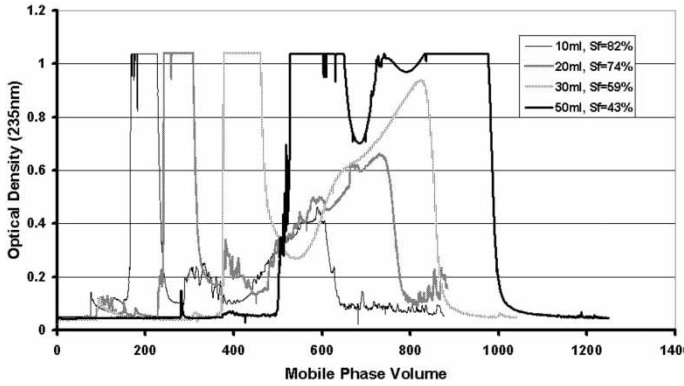

Figure 4. Effect of sample volume on the dynamic extraction separation efficiency of chromatograms. Sample volumes of broccoli seed Extract 2 ranging from 10 mL to 50 mL (1–5% of coil volume) were fractionated on a DE-MIDI centrifuge: normal phase operation with 1-propanol/acetonitrile/ammonium sulphate; rotational speed 1200 rpm; flow rate 40 mL/min.

A large amount of material eluted at the solvent front. The glucoraphanin peak was very broad and ramped, but nevertheless, HPLC analysis showed it to contain only glucoraphanin. There was no detectable GI in the GR peak, and none eluting after this peak. With increasing load, the large peak eluted later. This increase in the solvent front indicated that the retention of stationary phase was being progressively decreased. This was confirmed by measurement of stationary phase retention: Figure 5 shows that the percentage

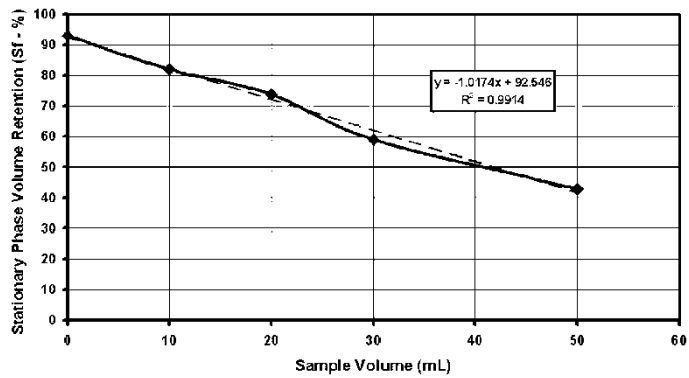

Figure 5. Variation of stationary phase volume retention (Sf) with sample volume. Sample volumes of broccoli seed Extract 2 ranging from 10 mL to 50 mL (1–5% of coil volume) were fractionated on a DE-MIDI centrifuge: normal phase operation with 1-propanol:acetonitrile:ammonium sulphate; rotational speed 1200 rpm; flow rate 40 mL/min.

retention of stationary phase volume reduced approximately linearly with the sample volume applied; by 50 mL (*ca* 5% column volume) the retention (Sf) had fallen below 50%. We have considered that this might be due to a possible surfactant effect of the material in the early eluting peak.

A 30 mL load (*ca* 3% column volume) was selected for manufacturing runs. Figure 6 shows examples of 6 successive runs with an injection volume of 30 mL, plotted from the point of sample injection. There does not appear to be a significant baseline shift and any differences may be due to inconsistencies with the sample loaded, more than in the process itself. After the initial loss of stationary phase due to the surfactant effect of the first highly concentrated contaminant peak, there was very little stationary phase lost in the following runs.

Comparison of Extract 1 with Extract 2: Influence of Seed Batches on CCC Behaviour

The flow optimisations were performed using Extract 1. It was found that flows could be increased up to 80 mL/min. CCC of Extracts 1 & 2, each with a loading volume of 10 mL (1% column volume), were compared (Figure 7). The solvent front eluted earlier for the first sample, maintaining a stationary phase volume retention of 90%. The extremely concentrated peak at the solvent front for Extract 2 had a surfactant effect on the phase system, resulting in significant loss of stationary phase and final stationary phase retention at pump-out of 82%. If continuing with Extract 1, a flow of 60 mL/min would thus have been ideal, but due to the surfactant effect

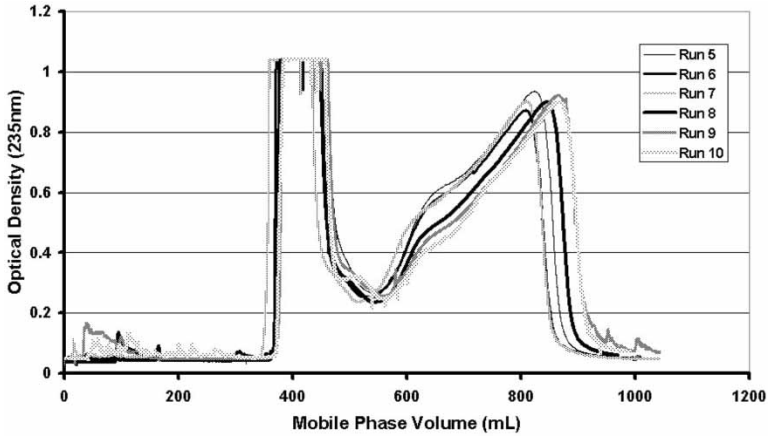

Figure 6. Six successive dynamic extraction chromatograms show the repeatability of the separations. Six-30 mL aliquots of broccoli seed Extract 2 (3% of column volume) were fractionated on a DE-MIDI centrifuge: normal phase operation with 1-propanol: acetonitrile:ammonium sulphate; rotational speed 1200 rpm; flow rate 40 mL/min.

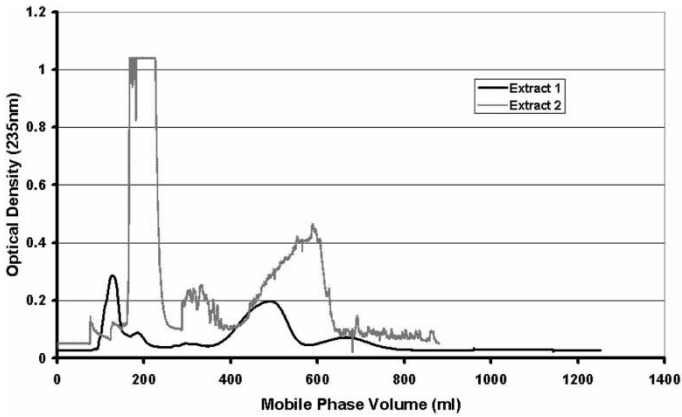

Figure 7. Comparison of dynamic extraction chromatograms between broccoli seed Extracts 1 and 2 for otherwise identical conditions: DE-MIDI centrifuge, speed 1200 rpm, flow 40 mL/min., sample volume 10 mL, concentration 0.5 g extract plus 1 mL phase.

encountered with Extract 2, it was decided to maintain the flow at 40 mL/min to preserve retention.

Manufacturing Conditions and Results

Manufacturing conditions finally selected were to use a 30 mL load (3% column volume) of a ~50% w/w preparation of the Extract 2 in phase system (ratio of stationary phase:mobile phase 6:4). Thirty-four runs were performed over 3 days, changing the stationary phase every 6 runs. The extract (589 g) was processed with an average load of 17 g per run (approximately 1.5 g of glucoraphanin per run).

The GR peaks were collected and confirmed to be of acceptable purity by HPLC. GR was recovered from the united fractions by rotary evaporation to dryness at 50°C, extraction with dry methanol, filtration, and solvent removal by rotary evaporation at 50°C. The overall yield from 589 g of extract loaded was 52.6 g of a pale yellow powder (8.9%).

Analysis of this material by HPLC, UV-visible spectroscopy, myrosinase hydrolysis, and elemental analysis gave a glucoraphanin purity of greater than 98%.

CONCLUSIONS

The technology developed at Johns Hopkins for purifying glucoraphanin was transferred to BIB and within 3 days a 50× scale up was demonstrated.

Manufacturing scale operations were identified and 53 g of glucoraphanin of acceptable, high purity was isolated from 589 g of extract in 34 runs over 3 days.

Further work is continuing to develop much larger scale preparation (ca 1 kg) using the DE-MAXI centrifuge based on loading studies established with the DE-MINI centrifuge, since conditions for the scale up of 1000 fold from the DE-MINI to the DE-MAXI centrifuge have now been established.[6]

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