Screening, identification and quantification of glucosinolates in black radish (*Raphanus sativus* L. *niger*) based dietary supplements using liquid chromatography coupled with a photodiode array and liquid chromatography - mass spectrometry

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ABSTRACT

The glucosinolate profile of black radish (*Raphanus sativus* L. *niger*) based dietary supplements has been investigated by HPLC-PDA, LC-ESI-MS/MS and LC-APCI-MS/MS systems. Optimization of the MS/MS parameters and LC conditions was performed using sinigrin reference standard and rapeseed certified reference material (BC190) respectively. An LC-ESI-MS/MS system was used to detect (screen) and identify the naturally occurring intact glucosinolates (GLs). The intact GLs identified were then desulfated and quantified on an HPLC-PDA system as desulfo-glucosinolates (DS-GLs). Prior to quantification, the DS-GLs were identified using an APCI-MS/MS. The HPLC-PDA method performance criteria were evaluated using glucotropaeolin potassium salt. The validated method was applied for the analysis of six dietary supplements. In total, six glucosinolates were identified and quantified in the dietary supplements; glucoraphasatin (0.2–0.48 mg/g), glucosisaustricin (0.37–0.91 mg/g), glucoraphenin (0.84–1.27 mg/g), glucoputrajivin (0.14–0.28 mg/g), glucosisymbrin (0.70–0.99 mg/g) and gluconasturtiin (0.06–0.12 mg/g). Glucoraphenin was the most abundant glucosinolate in all samples.

1. Introduction

Over the last two decades the use of dietary supplements has increased dramatically throughout the world. In 2000, world sales reached billions of dollars with the European market estimated at 30% of the global sales. Of all dietary supplements, 42% are derived from medicinal plants. As a matter of fact the traditional use of medicinal plants, which dates back centuries ago, serves for the prevention and treatment of diseases of the most vulnerable people (the young, old, pregnant and immuno-compromised). Within the European Union, Directive 2002/46/EC adopted in 2002 is aimed to harmonise the legislation on food supplements and to ensure that these products are safe and appropriately labelled so that consumers can make informed choices. According to this Directive, a dietary supplement is a product that is intended to supplement the diet and which is a concentrated source of nutrients or other substances with a nutritional or physiological effect, alone or in combination and it is intended to be taken by mouth as a pill, capsule, pastille, tablet or liquid [1].

Besides the beneficial effects of dietary supplements, fatal health consequences have also been reported as a result of intake of these products. In 1998, reports from 11 poison control centres in the USA revealed a 33.3% incidence of moderate to adverse effects, including three deaths from a total of 348 symptomatic ingestions of dietary supplements by adults [2]. It is also worth noting that the composition of dietary supplements has been reported to vary widely from one manufacturer to another. Harkey et al. [3] analyzed 25 commercially available ginseng products and revealed that the concentration of ginseng ranged from 10.8% to 327% of the labelled concentration. This variability in the composition of dietary supplements could eventually lead to significant dose variation between manufacturers, and as a consequence, could result in adverse health consequences in humans.

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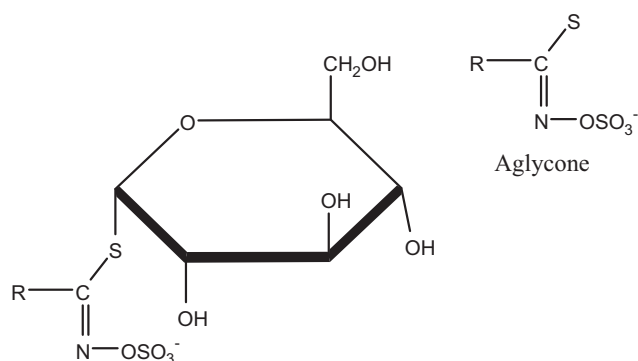E-mail address: emmanuel.njumbeediage@ugent.be (E. Njumbe Ediage).

Fig. 1. General structure of intact GLs (R = functional group).

Plant-derived dietary supplements include among others *echinacea*, garlic, St John's Wort, *Ginkgo biloba* and ginseng. Black radish (*Raphanus sativus* L. var. *niger*), a small-rooted, short seasoned vegetable can also be added to this list. Black radish roots differ in shape, flesh and skin colour and are thought to originate from Asia [4]. Black radish (BR) is not only a root vegetable but also an important source of medicinal compounds. It has been used since antiquity to treat different hepatic, urinary, biliary, gastrointestinal, cardiovascular and respiratory disorders. There is definitely no doubt that radishes are rich in a variety of bioactive components. However, the most studied group of these bioactive molecules are the glucosinolates [5].

Glucosinolates (GLs) are sulfur-rich secondary plant metabolites (β-D-thioglucosides), common in many agriculturally important crops (cabbage, cauliflower, horseradish, turnip, mustard, rapeseed, and BR), belonging to the genus *Brassica* of the family *Brassicaceae* (syn *Cruciferae*). These compounds have also been reported in 15 other families of dicotyledonous angiosperms [6]. GLs are characterized by a sulfated thiohydroximate group, which is conjugated to glucose, and a further R-group (Fig. 1). The glucose and the central carbon atom of GLs are often further modified. Based on the nature of the R group, GLs are broadly classified into alkyl, aromatic, benzoate, indole, multiple glycosylated and sulfur-containing side chains, which result in a wide range of biological activity and polarity of these compounds [7]. At the moment, the number of GLs reported in scientific literature now approaches 200 [8].

Disruption of cellular structures of *Brassica* vegetables by cutting, chewing, cooking, and freezing results in hydrolysis of GLs [9]. Hydrolysis (degradation) of GLs, a reaction catalyzed by the enzyme myrosinase (β-thioglucosidase), yields a variety of compounds, the nature of which depends on several factors such as pH, temperature, metal ions, protein, co-factors and the R group of the glucosinolate [10,11]. Thiohydroximates, thiocyanates, nitriles and thiones have been identified as the main degradation products of GLs. Interestingly, the thiohydroximates are well known for their nematocidal, fungicidal, and bactericidal properties, and have recently attracted intense research interest because of their cancer preventive attributes [12]. Intact (naturally occurring) GLs can also be degraded by aryl sulfatase (an enzyme present in extracts of horse radish plants) leading to the release of a sulfate moiety and desulfoglucosinolates (DS-GLs).

Liquid chromatography (LC) analysis of intact GLs is not straight forward due to the relatively high polarity of these compounds. A common approach is to convert the intact GLs into desulfo derivatives which can be more easily analyzed by reversed phase LC. The original work describing the conversion of GLs via sulfatase to DS-GLs was published in 1982 and was harmonized into an official method ISO 9167-1 [13]. ISO 9167-1 describes a high performance liquid chromatography coupled to photodiode array detector (HPLC-PDA) method for the determination of GLs in rape-

seed (RS) samples. This remains in widespread use with suggested improvements [14,15]. However, this method does not work for GLs with substituents on the thioglucose part or with acidic groups in the R chain [16,17].

Within the last two decades, over 100 different cruciferous plants have been screened for their glucosinolate content. Despite the numerous research carried out in this field, reference standards exist just for a few of these GLs, which renders identification and quantification of unknown GLs (for which no reference standards exist) difficult. For this reason, qualitative and quantitative data are available only for a few selected plant species examples of which include RS, broccoli, mustard and rocket salad. These species are often selected because of their potential health benefits and most importantly due to the fact that the glucosinolate profile in these species is similar to those reported and described for RS, for which certified reference material is commercially available. Thus identification of unknown peaks was often carried out by matching the UV absorption spectra and retention time of the DS-GLs in unknown samples to those in RS [15]. Once identified, quantification is performed by taking into consideration the relative response factors (rRF) for each of the corresponding DS-GLs. rRFs for the different DS-GLs identified in RS were originally specified in the ISO 9167-1 document. This list has been revised and further extended by Wathelet et al. [18]. However, for the other cruciferous vegetables, the glucosinolate profile can be very different from that of RS which renders identification of unknown peaks very challenging. There is therefore a need to use a more specific detection technique such as tandem mass spectrometry (MS/MS) to specifically identify the different DS-GLs prior to quantification [19–21].

In general, LC-MS/MS has recently emerged as the technique of choice for the determination of GLs due to its superior specificity [22,23], compared to other analytical methods (gas chromatography, colorimetry, X-ray fluorescence, ultraviolet spectroscopy and capillary electrophoresis) used in the past to screen and quantify GLs. Almost all LC-MS/MS methods reported in the literature were developed for determination of GLs in vegetative tissues. Only a few of these articles have reported the identification of GLs in radish roots [24–26]. To the best of our knowledge no report on identification of GLs in BR based dietary supplements has been published yet. The primary aim of this study was therefore to develop an analytical strategy based on liquid chromatography-electrospray ionization (LC-ESI-MS/MS), atmospheric pressure chemical ionization (APCI-MS/MS), and HPLC-PDA for screening, identification and quantification of GLs in BR based dietary supplements. This approach enables us to overcome the identification challenges previously mentioned. An LC-ESI-MS/MS system was used to detect (screen) and identify the naturally occurring intact GLs. The intact GLs identified were then desulfated and quantified by an HPLC-PDA system as DS-GLs. Prior to quantification, the DS-GLs were identified using an APCI-MS/MS system. Furthermore, reports that some herbal products contain widely varying amounts of the same bioactive component, prompted us to analyze commercially available BR based dietary supplements, with the aim of understanding the variation of glucosinolate content in different brands of dietary supplements.

2. Materials and methods

2.1. Reagents and chemicals

Methanol, ethanol and n-hexane, all HPLC grade were purchased from VWR International (Zaventem, Belgium). LC-MS grade MeOH was supplied by Biosolve (Valkenswaard, the Netherlands). Dichloromethane and ethylacetate were from Acros Organics (Geel, Belgium). Acetic acid, formic acid and concentrated ammonia, were

supplied by Merck (Darmstadt, Germany). Bond Elut SAX (500 mg) solid phase extraction (SPE) cartridges were obtained from Varian (Sint-Katelijne Waver, Belgium). Water was purified on a Milli-Q Plus apparatus (Millipore, Brussels, Belgium). Ultrafree ®-MC centrifugal filter devices (0.22 μm) of Millipore (Bredford, MA, USA) were used for the filtration of the sample extract. Tetramethylammonium chloride, sodium acetate, imidazole, aryl sulfatase from *Helix pomatia* (100 KUnits/g) and reference standard sinigrin (SIN) monohydrate from horse radish were all purchased from Sigma–Aldrich (Bornem, Belgium). Glucotropaeolin (GTL) potassium salt with 98.7% purity was obtained from LGC Standards (Molsheim Cedex, France).

2.1.1. Preparation of standards

SIN was used for tuning of the MS parameters (see Sections 2.4.1 and 2.4.2) as well as an internal standard (IS). Eight milligram (8 mg) of SIN monohydrate was dissolved in 1 mL of Milli-Q water to prepare a stock standard solution (8 mg/mL), which was stored at 4 °C until use. Further dilutions were then made and used as tuning solutions. As reference material, certified RS (European Reference Material ERM®-BC190, high purity grade with expanded uncertainty of 14.52%), from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) was used. GTL potassium salt was used as validation standard. 1 mg of GTL potassium salt was dissolved in 1 mL of methanol. The solution was stored at −20 °C for three months.

2.1.2. Purification of enzyme (aryl sulfatase)

Purification of the enzyme aryl sulfatase was performed based on the principle of alcohol precipitation. This was done as follows: 100 mg of the lyophilised enzyme was accurately weighed and approximately 6 mL of ethanol/water (40:60, v/v) was added. The solution was centrifuged at 2540 g for 6 min. The supernatant was transferred to a 20 mL test tube to which 6 mL of fresh ethanol solution was added to precipitate the enzyme. The resulting suspension mixture was further centrifuged at $2540 \times g$ for 6 min. The supernatant was discarded and the sediment (purified enzyme), dissolved in 5 mL of water (20 mg/mL). The purified enzyme was further divided into eppendorf tubes and stored at −20 °C as stock solution from which further dilutions could be made before use.

2.2. Sample preparation

2.2.1. Extraction

Six BR based dietary supplements were purchased through the Internet as well as from the Belgian market. Prior to analysis, a representative amount of capsules or tablets from each dietary supplement was taken and carefully homogenised, from which a representative sample was obtained for analysis.

The same extraction procedure was applied to both BR based dietary supplements and RS reference material (BC190). For each dietary supplement, the amount of product corresponding to 200 mg of plant material was weighed accurately into two separate test tubes. Table 1 gives for each sample analyzed the amount of powder that needs to be weighed in order to get 200 mg of plant material. Both tubes were placed in a water bath, at 75 °C and heated for 1 min. Three milliliters of boiling methanol/water mixture (10:90, v/v) was added to each of the tubes immediately followed by addition of 200 μL of 5 mmol/L of SIN (IS) into one of the tubes. Both tubes were again placed in a water bath at 75 °C for 15 min, while shaking at regular intervals. Centrifugation was carried out at $3170 \times g$ for 10 min. The supernatant from each tube was then transferred into a new tube and the extraction procedure was repeated without the addition of IS. Both extracts were recombined, placed in a water bath at 60 °C and dried under a gentle stream of

Table 1
Amount of powder corresponding to 200 mg of plant material for different black radish dietary supplements.

Sample	Amount of powder per capsule (mg)	Labelled amount of plant material per capsule (mg)	Actual amount of powder weighed (mg) for extraction
Sample 1	321 ± 4	240	266
Sample 2	373 ± 3	373	200
Sample 3	248 ± 10	248	200
Sample 4	384 ± 7	200	384
Sample 5	270 ± 11	270	200
Sample 6	250 ± 7	250	200

nitrogen. The residue was then reconstituted in 5 mL of methanol. At this point, the extract was ready for further clean-up.

2.2.2. Sample clean-up

The sample clean-up protocol used in this study was adapted from that described in the ISO 9167-1 method. Matrix components were first precipitated from the sample extract by adding 10 mL of dichloromethane/hexane (50:50, v/v). The solution was centrifuged at $3170 \times g$ for 10 min. The supernatant was brought into a new test tube, evaporated and redissolved in 5 mL of methanol. SAX solid phase extraction (SPE) cartridges were used for the sample clean-up instead of Sephadex DEAE ion-exchange resin described in ISO 9167-1. The SAX adsorbent was activated by adding 6 mL of imidazole formate (6 mol/L), followed by 2 mL of demineralized water at a flow rate of one drop per second. To the activated SAX cartridge, 1 mL of the sample extract was loaded and allowed to drain. The cartridge was further washed with 5 mL of dichloromethane/hexane (50:50, v/v), and dried by aspirating the cartridges. Intact GLs were eluted with 5 mL of methanol followed by 1 mL demineralized water. The eluate was evaporated to dryness in a water bath at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of injection solvent consisting of methanol/water (5:95, v/v) and 1 mM ammonium formate adjusted to pH 5. Ultrafree® MC centrifugal devices (0.22 μm) were used to filter (15 min at $14,000 \times g$) the resulting solution prior to injection into the LC-MS/MS system.

2.2.3. Desulfation of intact GLs
2.2.3.1. Experimental design.
The desulfation process was optimized using a central composite design with 27 runs. The different parameter settings of the experimental design are given in Table 2. Modde 8.0 software (Umetrics, Umea, Sweden) was used to analyze the statistical data.

The statistical relationship between a response Y and the experimental variables X_i, X_j is of the following form:

$$Y = \beta_0 + \beta_i X_i + \beta_j X_j + \beta_{ij} X_i X_j + \beta_{ii} X_i^2 + \beta_{jj} X_j^2 + \dots \varepsilon \tag{1}$$

where the βs are the regression coefficients and ε is the overall experimental error. The linear coefficients β_i and β_j describe the quantitative effect of the respective variables. The cross coefficient β_{ij} measures the interaction effect between the variables and the square terms $\beta_{ii} X_i^2$ and $\beta_{jj} X_j^2$ describe the non linear effects on the response. The response Y is the peak area of desulfosinigrin.

Table 2
Parameter settings applied in the central composite design corresponding to low (−), central (0) and high values (+).

Parameter	Low (−)	Middle (0)	High (+)
pH of buffer	4	4.9	5.8
Incubation time (h)	12	24	36
Volume of enzyme solution (μL)	100	200	300
Concentration of enzyme (mg/mL)	2	11	20

2.2.3.2. Optimized desulfation protocol. For the desulfation of GLs, the sample extract was first treated as described in Section 2.2.2, but the GLs were not eluted from the SAX SPE cartridge. After aspirating the SPE cartridge, 5 mL (20 mM) sodium acetate buffer (pH 5.0) was added on the cartridge and allowed to drain at a flow rate of one drop every 5 s. This was followed by addition of 200 μL (20 mg/mL) of the enzyme solution. The SPE cartridge was then incubated at 37 °C for 24 h. To prevent drying of the cartridges during incubation, each cartridge was wrapped in aluminum foil. The DS-GLs were eluted with 4 mL of methanol followed by 2 mL of Milli-Q water. The eluate was evaporated to dryness in a water bath at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of injection solvent consisting of methanol/water/formic acid (4.7:95:0.3, v/v/v).

2.3. Chromatographic conditions

2.3.1. Screening of intact GLs

Waters Acquity UPLC apparatus (Waters, Zellik, Belgium) was used for the chromatographic analysis of intact GLs. A reversed phase Zorbax SB-18 (C18, stable bond), 5 μm, 250 mm × 4.6 mm I.D. (Agilent, Diegem, Belgium) column, maintained at room temperature was used for separation of the analytes. As mobile phases, water (eluent A) and methanol (eluent B) were used; both containing 10 mM ammonium formate at pH 5.0. The sample injection volume was fixed at 20 μL. A gradient elution was applied as follows: 95% A for 1 min, decreasing to 93.1% A between 1 to 10 min; and kept at 93.1% A for 3 min. From 13 to 23 min A was decreased to 79.8%, and finally back to initial conditions (95%A) after 33 min. A flow rate of 1 mL/min was applied for HPLC-PDA analysis, while a post-column split flow was used to introduce 300 μL/min of the mobile phase for LC-ESI-MS/MS analysis.

2.3.2. Analysis of DS-GLs

The LC apparatus for the analysis of DS-GLs consisted of a Waters 2695 binary pump, an autosampler, and a thermostatted column compartment (Waters, Zellik, Belgium). The stationary phase was the same as that described in Section 2.3.1. Twenty μL of sample was injected onto the column via a sample loop. Eluent A consisted of water, while eluent B consisted of methanol, with both containing 0.3% formic acid. A gradient elution profile was applied as follows: 91% A for 1 min; 91% A to 83% A from 1–10 min; 10–30 min 83% A to 55% A; 30–50 min 55% A to 20% A, and finally 50–60 min: 91% A. A flow rate of 1 mL/min was applied.

2.4. Detection

2.4.1. Intact GLs

For MS investigation of intact GLs, a Micromass Quatro Micro triple quadrupole mass spectrometer (Waters, Zellik, Belgium) was used. The MS parameters were optimized using a standard solution of SIN (10 ng/μL) infused at a flow rate of 0.03 mL/min. The optimum conditions were obtained in the ESI negative ion mode and were as follows: cone voltage 35 V; capillary voltage 2.8 kV; source and desolvation temperature 150 °C and 350 °C respectively; extractor voltage was at 2 kV; collision gas 15 eV; cone nitrogen and desolvation gas flows were at 200 and 500 L h^{-1} respectively. Masslynx software was used for data processing.

2.4.2. DS-GLs

A Micromass Quatro triple quadrupole mass spectrometer (Waters, Zellik, Belgium) was used for investigation of DS-GLs. Using a syringe pump desulfosinigrin was infused into the running mobile phase via a T-connector at a flow rate of 0.3 mL/min. The optimized MS conditions were as follows: cone voltage 30 V; capillary voltage 2.8 kV; source and desolvation temperature 150 °C

and 650 °C respectively; coronary pin voltage and collision voltage were at 2.8 eV and 20 eV respectively. Masslynx software was used for data processing.

For HPLC-PDA analysis, a Waters 2996 PDA detector was used for data acquisition. An HPLC-PDA scanning (200–400 nm) was performed with desulfosinigrin and desulfoglucotropaeolin to select the best wavelength for detection of DS-GLs. Empower 2.0 software was used for data processing.

2.5. HPLC-PDA method validation

The validation parameters for the quantification method (HPLC-PDA) were evaluated using GTL potassium salt. Sample preparation and desulfation were according to the protocol described in Section 2.2. To determine the linearity and trueness of the analytical method, fortified samples were prepared at five concentration levels by adding 0.07, 0.41, 0.74, 1.47, 2.2 nmol/g GTL to a chosen dietary supplement. Each concentration level was spiked in triplicate. The five (fortified) concentration levels were used to construct a calibration curve. A sixth sample spiked at a predetermined concentration was then used to evaluate the trueness of the analytical method. The response (ratio of peak area of desulfoglucotropaeolin to the peak area of desulfosinigrin) was plotted against the spiked concentration levels and the resulting calibration curve was used to determine the trueness and linearity of the analytical method.

Extraction efficiency (recovery) was assessed by using two sets of samples of a chosen dietary supplement, in triplicate; one spiked with known concentration of GTL (0.07 nmol/g) prior to extraction (spike begin) and the other spiked just before the sample clean-up (spike end). The ratios of the peak areas of the spike begin and spike end were used to calculate the extraction recovery. In the same way, the efficiency of the SAX cartridge to retain intact GLs was also evaluated. One set of samples was spiked with known concentrations of GLT (0.07 nmol/g) prior to loading on the pre-conditioned SAX SPE cartridge (spike begin) while the other set was spiked at the end of the sample clean-up protocol (spike end). The ratios of the peak areas were used to calculate the percentage GLT that was retained on the SAX cartridge.

To determine the precision (intra-day data) of the HPLC-PDA method, relative standard deviations (RSDs) of the desulfoglucotropaeolin peak area were evaluated by fortifying six different samples of a chosen dietary supplement, with GTL potassium salt, in triplicate at the same concentration level (0.41 nmol/g), under the same experimental conditions on the same day. Inter-day precision was calculated from results obtained by the analysis of samples with the same concentration (0.41 nmol/g, $n = 3$) on three different days.

The method limit of detection (LOD) and limit of quantification (LOQ) were obtained from signal-to-noise (S/N) ratios of 3:1 and 10:1 respectively.

2.6. Measurement uncertainty

An estimate of the combined measurement uncertainty (Uc) associated with the results was obtained for desulfoglucotropaeolin and desulfosinigrin by taking into consideration the within-laboratory reproducibility standard deviations (S_{RW}), the uncertainty associated with the purity of the standards ($U(\text{Cref})$), the uncertainty associated with the measurement of the rRF (U_{rRF}) as well as the uncertainty associated with the mean recovery (S_{bias}) as follows:

$$(Uc)^2 = (S_{RW})^2 + U^2(\text{Cref}) + U_{rRF}^2 U(S_{bias})^2$$

Because the uncertainty associated with the purity of the standard $U^2(\text{Cref})$ was not available from the suppliers, an arbitrary value
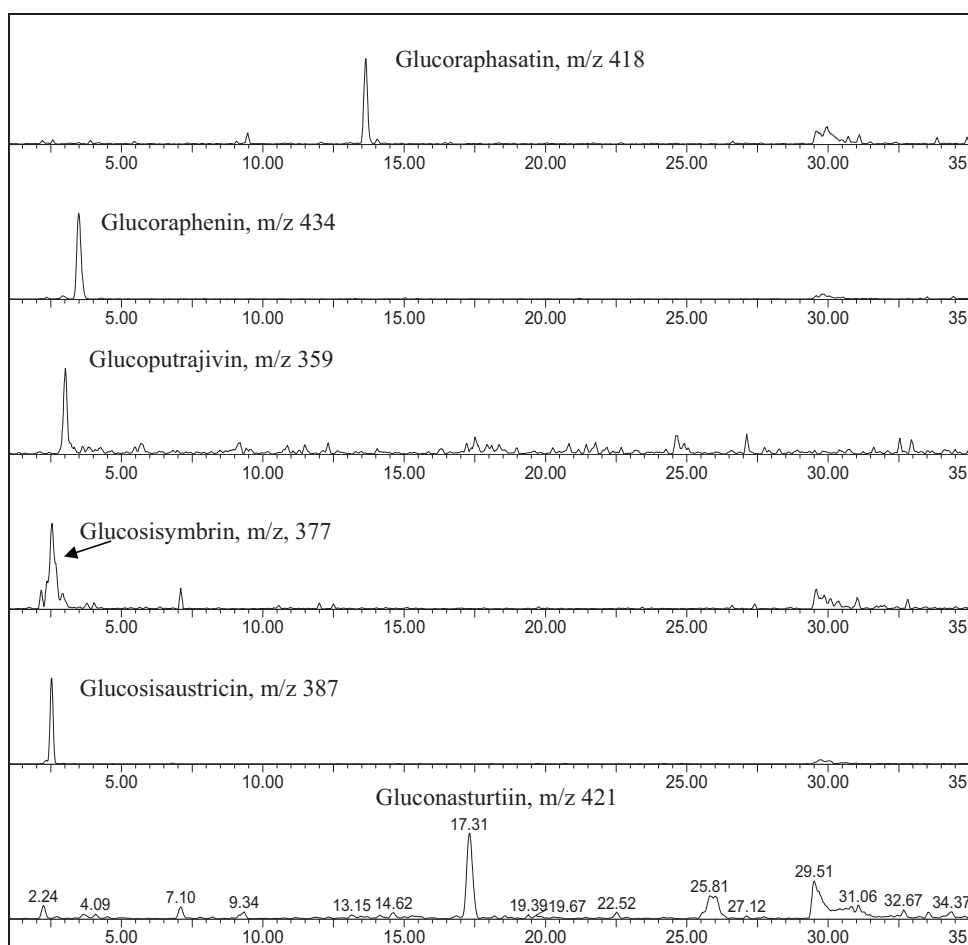
Fig. 2. Extracted ion chromatogram of intact GLs in BR based dietary supplements obtained in the ESI negative mode.

of 10% was taken into consideration. Similarly, because the uncertainty associated with the measurement of the rRF was also not available, an arbitrary value of 10% was also taken into consideration. An estimate of the expanded uncertainty (U) corresponding to a confidence interval of approximately 95% was obtained by multiplying the combined uncertainty (Uc) by a factor of 2, i.e. $U = Uc \times 2$.

3. Results and discussion

3.1. Optimization of chromatographic separation

Due to the high polarity of the GLs sulfonate moiety, it is difficult to achieve satisfactory retention of this analytes on a conventional reversed phase C18 chromatographic column. This problem is even more complicated when complex plant extracts are analyzed. As a consequence several approaches are currently being investigated aiming at achieving sufficient chromatographic retention for this group of analytes on a C18 reversed phase chromatographic column. One of the approaches involves the use of ion-pairing agents. Ion-pairing agents interact with the charged analytes, forming neutral pairs, which in turn interact strongly with the stationary phase, thus, rendering the separation, a property of the variable R group. In this study, tetramethylammonium chloride at low percentages (1%, 2%, 5% and 10%, w/v) was evaluated. Furthermore, to control the elution rate of the analytes, a mobile phase containing a suitable organic solvent is often required. Acetonitrile was preferred to methanol because of its dielectric constant and its capacity to compete with the ion pairs. The suitability of the ion-pairing agent was tested by analyzing a solution of pure solvent and extract of

BR fortified with SIN. Slight retention of SIN (1.8 min compared to 1.2 min with no ion-pairing agent) on the reversed phase chromatographic column was achieved at 5% (w/v) of ion-pairing agent (data not shown). Higher percentages (10%, w/v) of ion-pairing agent did not improve the retention of SIN but rather led to a decrease in the retention time. Such phenomenon was also observed previously [27].

Alternatively, the use of columns claimed to be suitable for polar compounds such as Waters Atlantis dC18 (150 mm × 2.1 mm
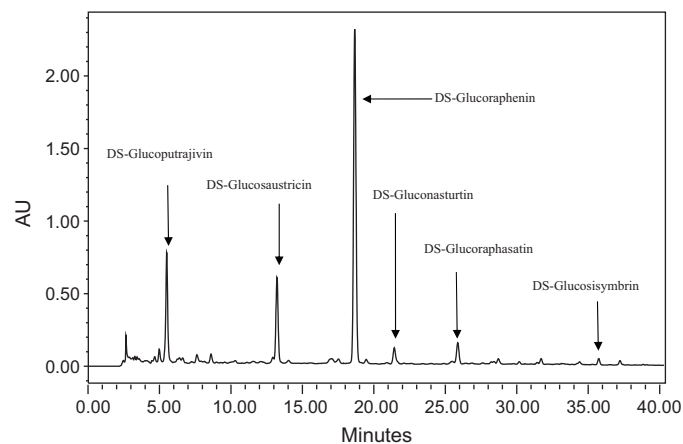

Fig. 3. Typical HPLC-PDA chromatogram of a desulfated BR based dietary supplement sample.

I.D., 3.5 μm), previously shown to be suitable for retention of acrylamide [28] and Waters HILIC XBridge™ (150 mm × 2.1 mm, 3.5 μm) were also investigated. The use of these columns did not improve the retention and chromatographic separation of the analytes of interest (GLs).

In further development, the suitability of a Zorbax SB-18 (C18, stable bond) reversed phase, 5 μm, 250 mm × 4.6 mm I.D. (Agilent, Diegem, Belgium) column was tested. This column did not only show sufficient retention of both the intact and desulfated analytes but also proved to be robust with excellent reproducibility. This stationary phase was chosen for this study. The mobile phase composition as well as the gradient program was optimized using RS reference material which is reported to contain more than 7 major

(known) GLs. The LC conditions as described in Sections 2.3.1 and 2.3.2 were finally selected. For BR based dietary supplements, satisfactory chromatographic separation was achieved within 17 min for the intact GLs (Fig. 2) and 40 min for DS-GLs (Fig. 3). Meanwhile for RS reference material good analyte separation was achieved within 33 min and 45 min for the intact GLs (Fig. 4a) and DS-GLs (Fig. 4b) respectively.

3.2. Screening of GLs

The determination of GLs in both RS reference material and BR based dietary supplements was performed by first screening for the intact forms (GLs) using an ESI-MS/MS system (see the following
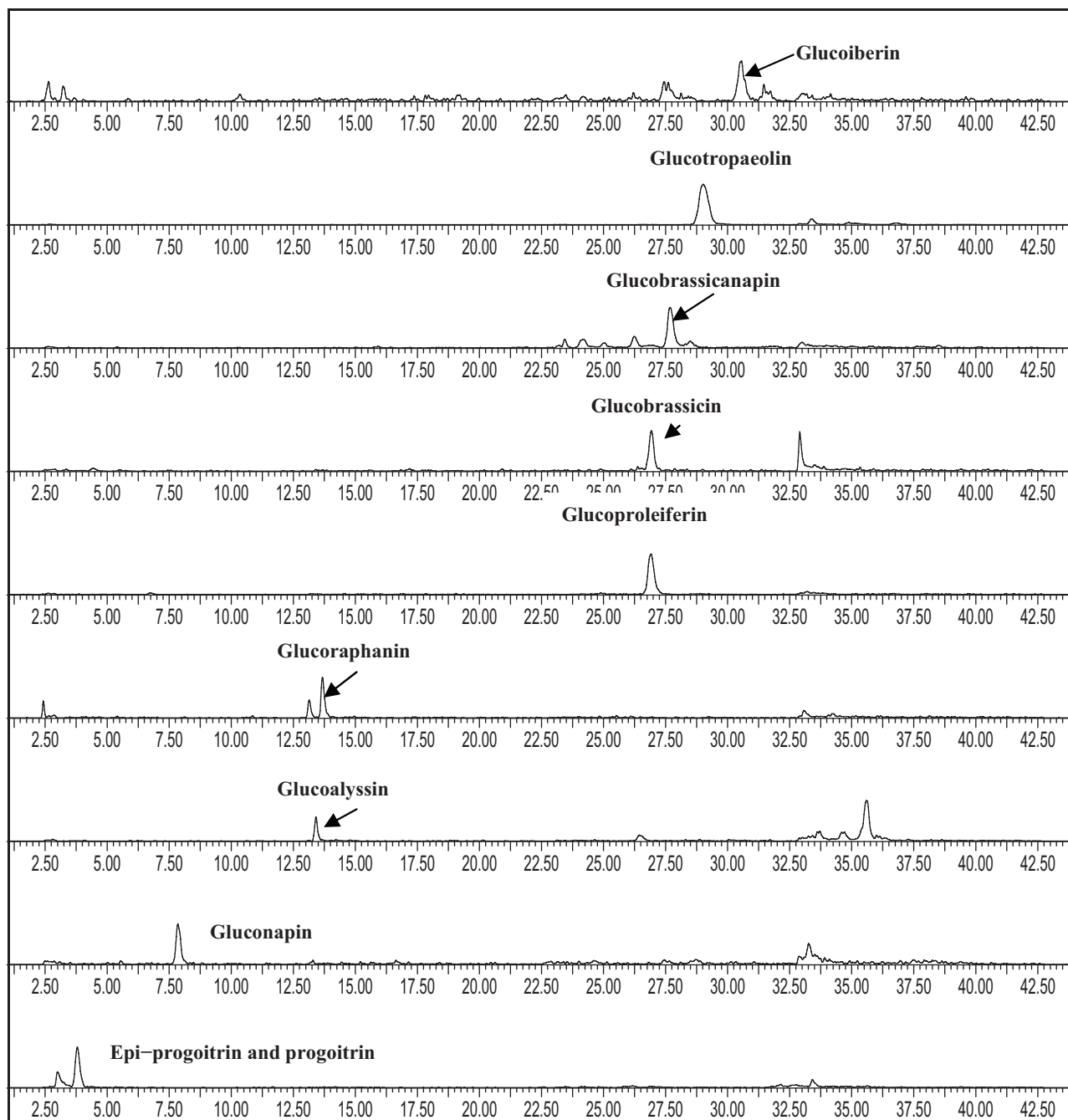

Fig. 4. (a) Extracted ion chromatogram of intact GLs in unspiked RS reference material obtained in the ESI-MS/MS mode. Peaks (glucoerucin, neo-glucobrassicin and sinigrin) for which very low signals were obtained are not displayed. (b) Extracted ion chromatogram of DS-GLs in RS extract obtained in the APCI-MS/MS mode. Peaks for which very low intensities were obtained are not represented.
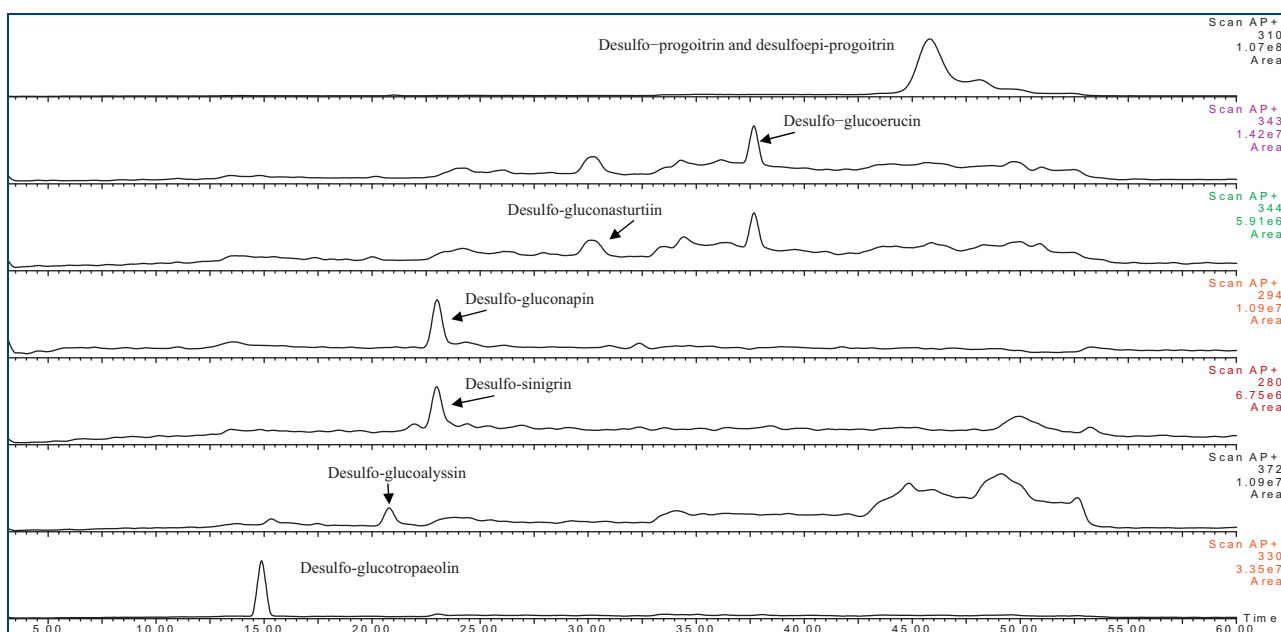
Fig. 4. (*Continued*)

paragraphs). Optimization of the MS conditions was performed by infusion of standard solutions of SIN and GTL as described in Section 2.4.1. ESI in both the positive and negative ionization modes was investigated. The best signal for both SIN and GTL was obtained in the negative ESI mode. The use of 10 mM ammonium formate as organic modifier in the mobile phase gave excellent ionization and chromatographic separation of the intact GLs. With these optimal conditions, a single ion monitoring (SIM) scan for SIN and GTL spiked in extracts of RS reference material and BR based dietary supplements was performed. SIN and GTL showed precursor ions with mass-to-charge (*m/z*) values of 358 and 408 respectively, corresponding to their respective deprotonated molecular ions, [M−H]⁻. Meanwhile, a full MS scan (with scan range of 200–600 *m/z*) was also performed with unspiked extracts of the RS reference material and BR based dietary supplements. The *m/z* values of all the naturally occurring GLs identified in vegetative tissues, as specified in Fahey et al. [6] were used as a database for the screening of possible GLs in the BR based dietary supplements. Similarly, for RS reference material, the GLs (major GLs) specified on the certificate of analysis were considered as target GLs. However, screening for other GLs not mentioned on the certificate of analysis was also performed. The chromatogram for each of the targeted glucosinolate was mass extracted and displayed. All the GLs identified revealed the presence of the molecular ion [M−H]⁻.

Twelve and six GLs were identified in extracts of RS reference material and BR based dietary supplements respectively. Further confirmation of the identified GLs was done by performing a product ion scan. Each of the GL was mass selected through the first analyzer and fragmented through a combination of cone voltages and collision energies (daughter scan) to yield product ions. As a fact, GLs share the same fragmentation pathway by undergoing a loss of 80 Da and/or 97 Da. All the suspected GLs revealed product ion(s) of *m/z* 80 Da and/or 97 Da (loss of SO₃⁻ and HSO₄⁻ respectively), confirming the presence of a sulfate moiety in their structure. Indeed, based on a study carried out with over 100 cruciferous plant species, Mellon et al. [29] concluded that plant sulfates other than GLs do not occur naturally in cruciferous plant species. Although fungi metabolite containing sulfate(s) (e.g. zearalenone-sulfate) has been reported in plant materials [30,31], this molecule could not be considered as the possible source for the sulfate moieties detected during the product ion scan. Indeed in a related study, Diana Di Mavungu et al. [32] screened these same dietary supplements for 25 different fungi metabolites (mycotoxins) and zearalenone (ZON) was not detected. It is therefore unlikely that the corresponding metabolite, ZON-sulfates reported to occur at much lower concentration (molar ratios from 1:12 to 1:2 of ZON [30]) be present in these samples. Thus, with regard to the method reported herein, there is definitely no doubt that the product ion related to the sulfate moiety observed (formed) during the fragmentation process originated from an intact glucosinolate, these product ions were used as identification criteria.

Furthermore, the complete MS/MS fragmentation spectrum of each glucosinolate previously identified was compared with those already reported in the literature. Only literature, for which the same fragmentation source (triple quadruple MS) was used, was selected for this comparative analysis. The spectra of all the GLs identified in extracts of RS reference material matched with those reported in literature for most of the abundant product ions. In accordance with other researchers [33,34], our study also revealed that the MS/MS fragmentation of GLs gives other four diagnostic ions with *m/z* values of 162, 195, 259 and 275. However, the relative abundances of these four diagnostic product ions were not consistent for all the GLs. All fragmentations were performed with the collision energy fixed at 20 eV for all the GLs. These four diagnostic ions were considered and used as identification points for further confirmation of the unknowns. All the 6 GLs (suspected) previously identified in BR based dietary supplements were confirmed as they produced at least three of the diagnostic product ions each. Fig. 2 shows the extracted ion chromatogram of intact GLs, identified and confirmed in extracts of BR based dietary supplement.

Table 3 gives the occurrence, structural formulae of the side chain (R-group), mass of precursor ion, retention time and the diagnostic product ions of intact GLs identified in extracts of RS reference material and BR based dietary supplement. All the major GLs in RS reference material as specified on the certificate of analysis were detected and confirmed except for 4-hydroxyglucobrassicin that was not detected. Along with the two most common GLs (glucoraphenin (*m/z* 434) and glucoraphasatin (*m/z* 418)) frequently reported in radish roots, four other less targeted GLs were identified in BR based dietary supplements samples. They included

Table 3
Intact GLs identified in RS and BR based dietary supplements using the negative ESI-MS/MS mode.

Compound	R group	Occurrence	Retention time (min)	m/z of precursor ion of intact GLs	Diagnostic product ions of intact GLs
Glucosisymbrin	$HO–CH_2–CH(CH_2)–$	BR	2.41	377	80, 97, 162, 195, 201, 223, 259, 275, 277, 278, 302, 336
Glucoputrajivin	$(CH_3)_2–CH–$	BR	2.58	359	80, 97, 162, 195, 200, 221, 259, 275, 306, 341, 377
Sinigrin	$CH_2=CH–CH_2–$	RS, reference standard	2.59	358	80, 97, 118, 136, 162, 195,227, 259, 275, 278, 289
Glucosisaustricin	$HO–CH_2–CH(CH_2CH_3)–$	BR	2.65	387	80, 97, 162, 179, 195, 210, 244, 282, 295, 311, 341, 361
Glucoraphenin	$CH_3–SO–CH=CH–CH_2–CH_2–$	BR	3.87	434	80, 97, 162, 195, 259, 275, 291, 331, 354, 419, 429
Epi-progoitrin	$CH_2=CH–CH(OH)–CH_2–$	RS	3.74	388	80, 97, 161, 179, 180, 195, 219, 259, 265, 275,278, 291, 291
Progoitrin	$CH_2=CH–CH(OH)–CH_2–$	RS	3.77	388	80, 97, 161, 195, 210, 259, 275, 332
Gluconapin	$CH_2=CH–CH_2–CH_2–$	RS	7.82	372	80, 97, 162, 179, 188, 195, 259, 275, 292, 318, 348
Glucoerucin	$CH_3–S–CH_2–CH_2–(CH_2)_2–$	RS	10.27	419	80, 97, 161, 195, 225, 259, 268, 275, 300, 359
Glucoalyssin	$CH_3–SO–(CH_2)_5–$	RS	13.41	450	80, 97, 165, 172, 195, 208, 259, 275, 284, 287, 307, 327, 354,376
Glucoraphanin	$CH_3–SO–(CH_2)_4–$	RS	13.69	435	80, 97, 161, 193, 195, 211, 259, 283, 322, 372, 393
Glucoraphasatin	$CH_3–S–CH=CH–(CH_2)_2–$	BR	14.83	418	80, 97, 161, 195, 230, 275, 264, 270, 366, 370
Gluconasturtiin	$C_6H_5–CH_2CH_2–$	BR	17.31	421	80, 97, 162, 195, 211, 259, 275, 352,
Glucopoleiferin	$CH_2=CH–CH2–CH(OH)–CH_2–$	RS	26.91	402	80, 97, 160, 188, 195, 217, 224, 247, 259, 267, 298, 313, 328, 343, 372, 387
Glucobrassicin	Indol-3-ylmethyl	RS	26.93	447	80, 97, 162, 195, 214, 259, 300, 385
Glucobrassicanapin	$CH_2=CH–(CH_2)_3–$	RS	27.64	386	80, 97, 162, 195, 259, 275, 310
Neo-glucobrassicin	1-methoxyindol-3-ylmethyl–	RS	28.47	476	80, 97, 162, 175, 195, 237, 257, 259, 269, 275, 294, 322, 358, 447,466
Glucotropaeolin	$C_6H_5–CH_2–$	RS, reference standard	29.02	408	80, 97, 162, 195, 215, 230, 259, 275, 278, 328
Glucoiberin	$CH_3–SO–(CH_2)_3–$	RS	30.58	422	80, 97, 162, 206, 229, 241, 257, 275, 294, 310, 371, 375

glucosisaustricin (m/z 387), glucoputrajivin (m/z 359), glucosisymbrin (m/z 377) and gluconasturtiin (m/z 421). It is worth noting that the elution order of the peaks is consistent with the structures assigned to the different GLs. We hereby report for the first time, the occurrence of six GLs in BR based dietary supplements.

3.3. LC-APCI-MS/MS and LC-PDA determination of the identified GLs

3.3.1. Optimization of the enzyme desulfation protocol

The desulfation process that was initially developed for RS (ISO 9167-1, 1992) was optimized for its application to BR based dietary supplements. The four parameters (pH of the sodium acetate buffer, concentration of enzyme, volume of enzyme solution, and incubation time) that have the most influence on the desulfation process were evaluated and optimized by an experimental design using SIN reference standard.

An experimental design with 27 runs (experiments) was executed in one randomized batch. As response, the peak area of desulfosinigrin was carefully investigated. The individual and interaction effects as well as the quadratic effects of different parameters on the response are summarized in Fig. 5. The plot consists of bars, which correspond to the regression coefficients. The magnitude of the variable effect is proportional to the regression coefficients (see Eq. (1)). The bars denoted by variable (*i*)*x* variable (*i*) reflect the regression coefficient for the non linear effect of that particular variable, whereas the bars denoted by variable (*i*)*x* variable (*j*) represent the interaction of the two variables concerned. The 95% limits are expressed in terms of an error line over the coefficient. A regression coefficient smaller than the error line shows that the variation caused by changing the variable is smaller than the experimental error. Therefore the effect of the variable is considered insignificant. A positive regression coefficient stands for a positive effect on the desulfation process, while a negative regression coefficient indi-

cates a negative effect. The coefficients of the terms in the model were estimated by the partial least square (PLS) method.

From the regression coefficient plot presented in Fig. 5, it is observed that the pH has the most important effect on the desulfation process. This effect is negative; meaning that in the range investigated, an increase of pH will reduce the peak area of desulfosinigrin. The other parameters show positive but less significant effect. It should be noted that this plot gives the effects of the individual parameters when the other parameters are maintained at their central value. Positive interactions are observed between pH and incubation time as well as between pH and enzyme concentration, while a negative interaction is observed between incubation time and enzyme concentration as well as between volume of enzyme solution and the concentration of enzyme. An insignifi-
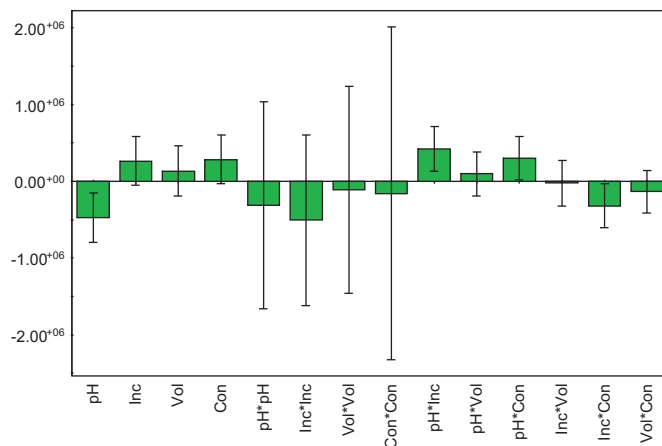

Fig. 5. Regression coefficients for the area of desulfosinigrin. pH = buffer pH; Inc = incubation time; Vol = volume of enzyme solution; Con = concentration of enzyme.
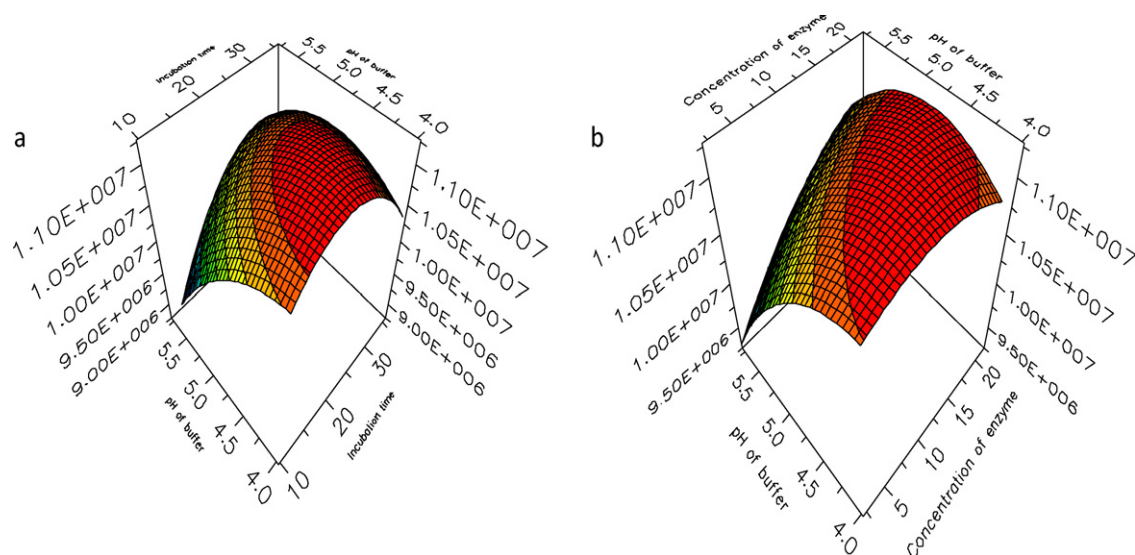
Fig. 6. Response surface plots for the area of desulfosinigrin as a function of (a) buffer pH and incubation time (b) buffer pH and concentration of enzyme.

cant interaction is observed between incubation time and volume of enzyme solution as well as between the pH and the volume of enzyme solution. The interaction effects indicates that the effect observed for a given parameter is not the same through the experimental domain, that is, when going from the lower level to higher level of the other parameter. All the quadratic effects were found not to be significant.

On the other hand, the statistical analysis of the model, gave the coefficient of determination (R^2) value above 0.95. The R^2 measures the fraction of the total variability in the response that is accounted for by the model. The Q^2 value obtained was above 0.85, and this corresponds to the proportion of the variability of the response that can be predicted by the independent variables (factors). These values approach 1, which indicates the suitability of the model in predicting the optimum conditions.

In order to better estimate the influence of the different parameters on the desulfation process, response surface plots (Fig. 6) were constructed. Fig. 6 shows the variation of the peak area of desulfosinigrin as a function of (a) buffer pH and incubation time, (b) buffer pH and enzyme concentration (all other parameters being kept constant at their central values). The selection of the optimum conditions took into consideration the regression plot presented in Fig. 5. Because the interaction effect between buffer pH and volume of enzyme solution was insignificant, the response surface was not constructed.

The response surface plot of the peak area of desulfosinigrin as a function of pH and incubation time (Fig. 6a) shows an increase of the peak area with a decrease in pH until pH of around 5.0. This is followed by a decrease in the peak area or a plateau with further decrease of the pH. The effect of incubation time reaches a plateau around 24 h. Fig. 6b shows an increase of the peak area of desulfosinigrin with an increase in enzyme concentration. Based on the outcome of these analyses, the following conditions were chosen for further analysis of samples: pH 5.0, enzyme volume 200 μL, incubation time 24 h, enzyme concentration 20 mg/mL.

3.3.2. Peak identification

Indeed, as already mentioned, a common approach for GL analysis is to convert the intact GLs into their DS-derivatives prior to quantification with LC-UV. However, because peak identification using HPLC-PDA is very complicated due to the lack of reference standards, an APCI-MS/MS was used for this purpose, taking into consideration data already generated with the ESI-MS/MS system

with intact GLs. As mentioned above, the suitability of MS detection for identification of DS-GLs was assessed. Almost no signal was obtained with ESI in both the positive and negative modes. This can be attributed to the fact that the desulfation process resulted in neutral derivatives that do not readily ionize in the ESI source. On the other hand, APCI that is more sensitive for neutral molecules gave sufficient signal in the positive mode when 1 mM formic acid was used as organic modifier in the mobile phase. No signal was obtained using APCI in the negative mode.

A full MS scan was performed (within the scan range of 200–500 m/z) to select all the possible DS-GLs prior to subsequent identification and confirmation. The total APCI-MS ion count performed with the desulfated extracts of RS reference material and BR based dietary supplements resulted in well separated peaks with very good baseline separation. The m/z values of the intact GLs, confirmed with the ESI-MS/MS, were used to deduce the m/z of the corresponding DS-GLs. The chromatogram of each DS-GL was mass extracted and displayed. The m/z of the detected DS-GLs showed a 78 mass unit shift compared to the corresponding intact GLs. This mass difference is consistent with the loss of a sulfite (SO_3^{2-}) moiety during the desulfation process. Fig. 4b shows the extracted ion chromatogram of DS-GLs detected in desulfated extract of RS reference material

3.4. Linearity, precision, trueness, recovery, LOD and LOQ data

The linearity of the HPLC-PDA method was established by means of linear regression (least square method). The coefficient of determination (R^2) of the calibration curve obtained with desulfoglucotropaeolin was 0.9903 (Fig. 7). This indicates good linearity of the overall analytical method. Intra-day and inter-day precision values were 11.8% and 12.5% respectively, which demonstrates the good precision and reliability of the method over the for-

Table 4
Uncertainty estimate using desulfosinigrin and desulfoglucotropaeolin.

	Desulfosinigrin	Desulfoglucotropaeolin
S_{RW}	11	12.5
S_{bias}	12	8
U^2_{rRF}	10	10
$U(\text{Cref})$	10	10
Combined uncertainty (Uc), %	21.56	20.5
Expanded uncertainty (U), %	43.1	41

Table 5
individual and total glucosinolate content in black radish based dietary supplements.

Compounds	Dietary supplements						
	rRF	Sample 1 (mg/g)	Sample 2 (mg/g)	Sample 3 (mg/g)	Sample 4 (mg/g)	Sample 5 (mg/g)	Sample 6 (mg/g)
Desulfoglucoraphenin	0.9	1.01	1.12	1.27	1.16	1.23	0.84
Desulfoglucoputrajivin	1	0.24	0.2	0.28	0.32	0.17	0.14
Desulfogluconasturtiin	0.95	0.06	0.07	0.12	0.08	0.08	0.10
Desulfoglucosisaustricin	1	0.50	0.91	0.45	0.77	0.37	0.65
Desulfoglucoraphasatin	0.40	0.32	0.20	0.48	0.24	0.36	0.20
Desulfoglucosisymbrin	1.32	0.73	0.76	0.99	0.74	0.80	0.70
Total		2.85	3.26	3.59	3.30	3.00	2.62
Recommended intake[a]		2.052	2.44	4.45	2.64	1.62	1.31

rRF: response factors (ISO 9167-1-1992).

[a] The calculation took into account the recommended number of capsules, the amount of plant material per capsule and the total GL per capsule.

tification range. Trueness and extraction recovery values were 95% and 89% respectively. The recovery of the sample clean-up protocol was 85%, which illustrates the suitability of the SAX cartridges for the retention of the analyte prior to desulfation. The expanded measurement uncertainty associated with desulfosinigrin and desulfoglucotropaeolin were 43.1% and 41% respectively (Table 4). Meanwhile, the method LOD and LOQ were 0.07 nmol/g and 0.41 nmol/g respectively. Thus the validated method proved to be very sensitive.

3.5. Calculations

Due to the lack of reference standards, the retention time of the DS-GLs identified with the APCI-MS/MS was used to match the corresponding peaks obtained with the HPLC-PDA system. The chromatographic method, developed on the LC-APCI-MS/MS system, was transferred to the HPLC-PDA system for quantification of the analytes. UV wavelength set at 300 nm gave the optimum best for viewing the DS-GLs with minimum background. Quantification of GLs in BR based dietary supplements was based on the guidelines established in ISO 9167-1. ISO 9167-1 specifies the method for the determination of GLs content in RS, by quantifying the desulfo derivatives, using HPLC. The rRF values described in Wathelet et al. [18] were applied during quantification. A rRF of 1 was applied to all the other DS-GLs for which no rRFs has been assigned. The content of the individual glucosinolates was calculated as follows:

$$\frac{mg}{g} = RF \times \left(\frac{Area\ g}{Area\ s}\right) \times \left(\frac{n}{m}\right) \times \left(\frac{Mw}{1000}\right) \qquad (2)$$

Area g is the peak area of the desulfoglucosinolate, Area s is the peak area of desulfosinigrin, m is the mass, in g of the test portion, n is the quantity, in micromoles of the internal standard added to the test portion, RF is the response factor of the corresponding DS-GL. Mw, molecular weight of the analyte.
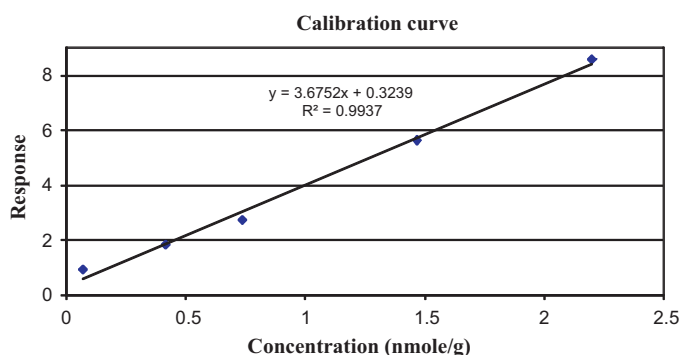

Fig. 7. Calibration curve obtained with HPLC-PDA for desulfoglucotropaeolin. Concentration range: 0.023–2.2 nmol/g. Number of repetition per concentration: 3.

3.6. Analysis of commercial BR based dietary supplements

The validated method was successfully applied to analyze six BR based dietary supplements available on the Belgian market and on the Internet. The quantitative data showed a great diversity in the individual glucosinolates content in the six black radish based dietary supplements. Table 5 gives the individual and total concentrations of GLs in each of the six dietary supplements. Glucosisaustricin, glucosisymbrin and glucoraphenin were the most abundant GLs identified in all dietary supplements.

Glucoraphasatin and glucoraphenin has been reported as the main GLs in radish roots [35]. These GLs were also confirmed in all six BR based dietary supplements. Since glucoraphasatin and glucoraphenin differ only in the degree of oxidation of the sulfur atom in the side chain, direct biological reduction of glucoraphenin to glucoraphasatin during sprouting [36] could explain the possible co-existence of both glucosinolates in radishes. Furthermore the co-existence of glucosisymbrin (1-methyl-2 hydroxyethyl glucosinolate) and glucosisaustricin (1-ethyl-2 hydroxyethyl glucosinolate), both with similar chemical structure, can be explained by the fact that chain elongation of 1-methyl-2 hydroxyethyl glucosinolate to 1-ethyl-2 hydroxyethyl glucosinolate occurs during biosynthesis of the former. Table 5 shows the recommended daily intake of each BR based dietary supplement as recommended by the different manufacturers. According to the data presented herein, sample 3 and sample 6 tend to have the highest and least respectively of the recommended daily intake among the six BR based dietary supplements investigated. This variation in the recommended daily intake clearly indicates the differences that exist between different manufacturer's in terms of product consistency and also the possibility of overdosage or underdosage as a result of the intake of these different products.

4. Conclusions and recommendations

A method based on an innovative combined approach (HPLC-PDA and LC-MS/MS) has been developed for screening, identification and quantification of GLs in BR based dietary supplements. This approach can be applied to investigate the GLs profile in other cruciferous plant species for which little or no data are available. Furthermore, this method provides a new and simple alternative to sample clean-up and enzymatic desulfation protocols when compared to the method described in ISO-9167. SAX cartridges proved to be very efficient for sample clean-up and enzymatic desulfation. The additional advantage of SAX cartridges over sephadex DEAE-A25 is its practicability of use as it does not need any special column preparation.

The analytical method allows for a more comprehensive profiling of GLs in BR based dietary supplements. We report in this study that there are at least two sets of structurally related GLs (glucoraphasatin/glucoraphenin and glucosisymbrin/glucosisaustricin)

present in BR based dietary supplements. The presence of other GLs (glucoputrajivin, glucosisymbrin and glucosisaustricin) that have not yet been naturally associated with BR roots could also indicate product adulteration or falsification during manufacture.

Due to the variation in both the individual and total glucosinolate content in the dietary supplements, there is thus a need for the development of Standard Reference Materials (SRM) for dietary supplements as this will help in achieving product consistency and potency through the characterization of raw materials. Furthermore, quantitative information on the composition of these products will be helpful for future methods development and hence in the quality control of finished products.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.012.

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