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Optimization and validation of analytical RP-HPLC methods for the quantification of glucosinolates and isothiocyanates in *Nasturtium officinale* R. Br and *Brassica oleracea*

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ABSTRACT

Watercress contains a considerable amount of vitamins, minerals and secondary metabolites and is used in food and for its medicinal properties. These latter are mainly attributed to glucosinolates which are precursors of bioactive compounds such as isothiocyanates. Broccoli is an edible vegetable of the family Brassicaceae that is rich in vitamins and secondary metabolites. Because of the biological activity of sulforaphane, broccoli became a popular food supplement. However, broccoli also contains the glucosinolate progoitrin and its derivative goitrin, an antithyroid compound and goitrogen, which can enlarge the thyroid gland. To avoid these effects, the daily intake of progoitrin and goitrin are limited to 20 mg and 5 mg. Since the quality of the food supplements of watercress and broccoli depends on the content of glucosinolates and their derivatives, quantitative HPLC methods were developed and validated conform the ICH guidelines to analyse the glucosinolates, phenylethyl isothiocyanate and goitrin. The methods were linear in the ranges 0.084–0.418 μmol/mL(gluconasturtiin), 0.013–1.04 μmol/mL (phenylethyl isothiocyanate), 0.044–1.312 μmol/mL (glucotropaeolin) and 0.0077–3.0965 μmol/mL (goitrin). The precision of the methods with respect to time and concentration was acceptable. A recovery of 99.2% and 100.6% was obtained for progoitrin and goitrin.

1. Introduction

Herbal preparations have been used for centuries as herbal medicines or as food supplements. Recently, plant based preparations are gaining popularity and are consumed increasingly worldwide. *Nasturtium officinale* R. Br and *Brassica oleracea* are used as food supplements because of their positive health effects like antioxidant, anticancer, antibacterial, anti-inflammatory and cardio protective properties (Klimek-Szczykutowicz, Szopa, & Ekiert, 2018; Favela-González, Hernández-Almanza, & De la Fuente-Salcido, 2020). Both species belong to the Brassicaceae family. Just as other members of the Brassicaceae, watercress and broccoli contain high concentrations of glucosinolates. Glucosinolates are water soluble secondary metabolites, of which already more than 130 varieties are identified. Naturally occurring glucosinolates have a sulfur-linked β-D-glucopyranose part and an amino acid-derived side chain. The side chain is depending on the type of glucosinolate and is very variable as it can be aromatic, heterocyclic or aliphatic. Glucosinolates are stable when present in the vacuole of the plant and are rather nonreactive. When the tissue of the plant is damaged by e.g. bruising, chewing or harvesting, the enzyme myrosinase, a β-thioglycosidase will come into contact with the glucosinolate and hydrolyse the glucoside. After a Lossen rearrangement the corresponding isothiocyanate is formed (Fahey, Zalcmann, & Talalay, 2001; Hofmann et al., 2009). Isothiocyanates are volatile compounds with a typical sharp flavor and scent. These compounds protect the plant against insects and herbivores and have antimicrobial activities. Isothiocyanates also have an effect on the liver metabolism, as they inhibit phase I enzymes and induce phase II enzymes. In this way, toxic or carcinogenic products (e.g. nitrosamines) will be formed slower and the elimination of these toxic metabolites will occur faster (Francisco et al., 2009; Liu et al., 2014).

The medicinal properties of *Nasturtium officinale* R. Br are mainly attributed to glucosinolates which are precursors of bioactive compounds. The most abundant glucosinolate in watercress is gluconasturtiin or 2-phenylethylglucosinolate (Fig. 1a). Phenylethyl isothiocyanate (PEITC, Fig. 1b), as a degradation product of gluconasturtiin, is the most abundant isothiocyanate in watercress (Klimek-Szczykutowicz et al., 2018). Since the quality of a food supplement

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of watercress depends on the content of glucosinolates and isothiocyanates, an analytical method for these compounds is needed for quality control. ISO 9167-1 (1992) describes an HPLC method for the analysis of glucosinolates in rapeseed. This method was adapted by Heyerick (2010, pp. 1–27) for the analysis of glucosinolates in watercress. The work of Heyerick (2010, pp. 1–27) also describes a method for the analysis of isothiocyanates in watercress. Both methods were further optimized and validated.

The biological activity of broccoli is mostly determined by the isothiocyanate sulforaphane (Fig. 1c). It is derived from the glucosinolate glucoraphanin (Fig. 1d) and has a wide range of activities. Sulforaphane down-regulates the expression of heat shock proteins, inducing apoptosis in breast cancer cells (Jaman & Sayeed, 2018). It also inhibits Phase I enzymes, thereby lowering the levels of the carcinogens interacting with DNA (Sestili & Fimognari, 2015). In addition, sulforaphane induces apoptosis and cell cycle arrest in different cancer cell lines derived from blood, brain, bladder, colon, ovary, prostate, pancreas and skin (Manchali, Chidambara Murthy, & Patil, 2012; Hecht, 1999; Elkashty & Tran, 2021). As mentioned above, glucosinolates and especially isothiocyanates are related with health promoting effects. Nevertheless, some of their derivatives have negative health effects, e.g. progoitrin (Fig. 1e), a glucosinolate present in broccoli. This glucosinolate is converted to progoitrin-isothiocyanate by myrosinase. As this isothiocyanate is unstable, a spontaneous cyclisation to goitrin (Fig. 1f) will occur (Felker, Bunch, & Leung, 2016).

Progoitrin and goitrin both have goitrogenic or thyroid gland enlarging effects. By competition in the transport mechanism, the uptake of iodine in the thyroid gland will be reduced. Goitrin also interferes with the synthesis of the thyroid hormone. These two effects are resulting in thyroid hypertrophy (Felker et al., 2016). Due to the antioxidative effects of sulforaphane, broccoli became a popular food supplement. However, the negative effects of progoitrin and goitrin also need to be taken into consideration. To avoid these effects, the Belgian Royal Decree of January 24, 2017 limits the daily intake of progoitrin and goitrin to 20 mg and 5 mg, respectively.

In order to assure the quality of food supplements and to comply with related legislation, the need for appropriate analytical methods is high. In this study, the analytical methods for the quantification of glucosinolates in watercress, as described by Heyerick (2010, pp. 1–27) and which was based on the ISO 9167-1 method, was further optimized and validated. The analysis of isothiocyanates in watercress was described in the work of Heyerick (2010, pp. 1–27), but was optimized and validated in this work. The analysis of progoitrin in broccoli was optimized, based on the method of the glucosinolates in watercress. For the analysis of goitrin in broccoli, the HPLC method described by Wang et al. (2013) was validated. Finally, some products available on the Belgian market were tested for their progoitrin and goitrin content.

2. Material and methods

2.1. Chemicals and reagents

Ultrapure water with a resistivity of 18.2 MΩ cm at 25 °C was generated with a Millipore™-purification system (Merck Millipore, Darmstadt, Germany). HPLC-grade methanol, ethanol, *n*-hexane, acetic acid, formic acid and ammonia 25% were purchased from Fisher Scientific (Leicestershire, United Kingdom). Acetonitrile far UV and isopropanol, both HPLC-grade, and sodium acetate were delivered by Acros Organics (Geel, Belgium). Imidazole and sodium acetate were purchased from Sigma (St. Louis, USA). DEAE Sephadex A-25 was purchased from GE Healthcare (Uppsala, Sweden). Glucosinolates were desulfated with sulfatase type H-1, which was supplied by GE Healthcare (batch number 10055929, Uppsala, Sweden).

Before use, 75.0 mg sulfatase from *Helix pomatia* (Sigma-Aldrich, St. Louis, USA) was dissolved in 6.0 mL ethanol 40% and sonicated for 10 min. After centrifugation, the supernatant was removed and the residue was dissolved in 5.0 mL water. This diluted sulfatase solution was used for the analysis.

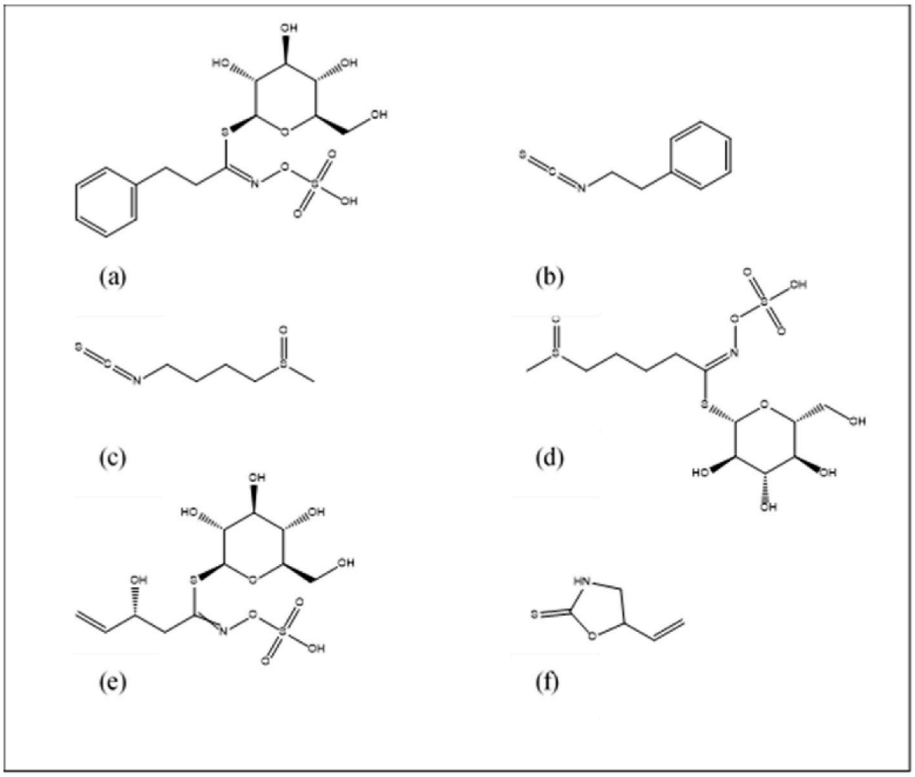

Fig. 1. Structures of gluconasturtiin (a), PEITC (b), sulforaphane (c), glucoraphanin (d), progoitrin (e) and goitrin (f).

2.2. Plant material and standards

Lyophilized watercress was provided by Cressana (Zwalm, Belgium), a Belgian company that develops food supplements containing watercress. The standards sinigrin monohydrate, or 2-propenylglucosinolate, and PEITC were purchased from Sigma (St. Louis, USA). Powder from broccoli (batch number WN32G-BRO03719A) was obtained from Frutarom (Londerzeel, Belgium). Several commercially available food supplements were analysed for their goitrin and progoitrin content, in triplo on 500 mg product: product A is a product with lyophilized broccoli juice and rice flour., product B contains a broccoli extract and additives, product C is a product that contains *Tropoleum majus*, *Origanum vulgare*, *Origanum majorana*, *Pinus sylvestris*, *Thymus vulgaris* and *Lavendula angustifolia,* product D is an alcoholic tincture of *Isatis tinctoria.* Analytical standards of progoitrin and glucotropaeolin were purchased from Carl-Roth (Karlsruhe, Germany). An analytical standard of goitrin was provided by Santa-Cruz Biotechnology (Heidelberg, Germany). The stock solutions of goitrin, glucotropaeolin and sinigrin monohydrate were prepared in water; PEITC was dissolved in *n*-hexane.

2.3. Quantification method for the determination of glucosinolates in watercress

For the optimization of the extraction, an existing method for the determination of gluconasturtiin was adapted [ISO 9167-1 (1992); Heyerick, 2010, pp. 1–27). Several parameters were evaluated in order to completely extract all glucosinolates from the lyophilized watercress. The volume of the extraction solvent (2.0 mL, 3.0 mL and 5.0 mL), extraction time (10 and 20 min), number of extractions (2 and 3) and desulfatation process (diluted, overnight and 10 times concentrated, 2 h) were tested (Fig. 2).

As a final method the following procedure was performed. Approximately 150 mg of lyophilized watercress was accurately weighed. The sample was heated at 75 °C in a water bath for 1 min. Then 200 μL internal standard (sinigrin monohydrate, 20 mM) was added. Subsequently, 5.0 mL boiling methanol 70% (v/v) was added to the sample. This mixture was stirred for 10 min at 75 °C, placed in an ultrasonic bath at 55–60 °C for 10 min and centrifuged (10 min, 1180 g). Afterwards, the supernatant was collected. The residue was extracted a second and a third time with 5.0 mL boiling methanol 70% (v/v) and all supernatants

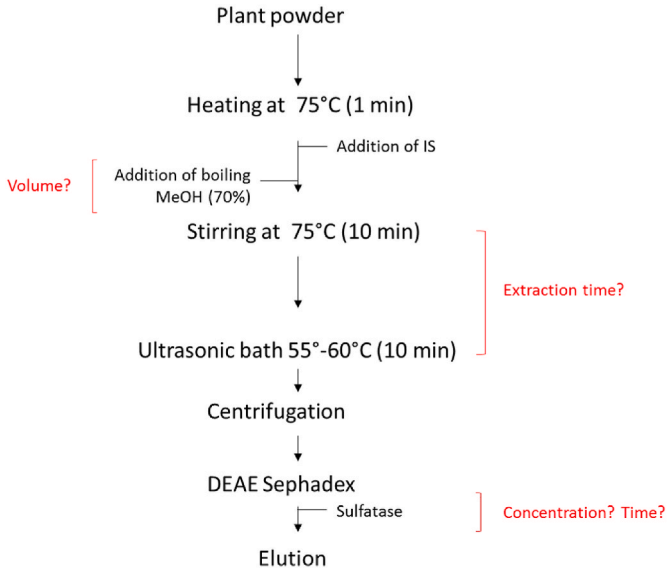

Fig. 2. Different steps in the extraction of glucosinolates from watercress. The steps that were optimized are marked in red. (IS = internal standard). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were combined. This extract was purified using the following procedure on a DEAE Sephadex A-25 ion exchange resin: 1.0 mL resin suspension was brought on a glass filter; the column was rinsed with 2 mL imidazole formate 6 M and 2 times 1 mL water; 1.0 mL test solution was brought on the column and the column was rinsed twice with 1 mL sodium acetate buffer. Before analysis, gluconasturtiin was desulfated with diluted sulfatase (see 2.1.; 2.0 mL) and incubated at 37 °C overnight. Lastly, desulphogluconasturtiin was eluted with water (2 x 1.0 mL) and the solution was analysed with HPLC-DAD (Agilent 1200 Liquid Chromatography System, Agilent Technologies N.V., Diegem, Belgium).

For the HPLC analysis, 20 μL of the extraction solution was injected on a Lichrospher RP 18e column (4.6 mm × 250 mm, 5 μm) (Merck, Darmstadt, Germany). The temperature of the column was set at 40 °C and a flow rate of 1.0 mL/min was chosen. The mobile phase solvents consisted of water +0.025% formic acid (A) and acetonitrile far UV + 0.025% formic acid (B). The gradient was set as follows: at the start 98% A, this condition was kept for 3 min, afterwards 75% A was reached in 35 min, this condition was kept for 2 min whereupon the starting conditions were reached again in 3 min (98% A) and hold for 7 min . For detection, a diode array detector (G1315 D, Agilent Technologies, Diegem, Belgium) was set at 229 nm.

2.4. Quantification method for the determination of isothiocyanates in watercress

The extraction method was optimized starting from an existing method described by A. Heyerick (2010, pp. 1–27). Different parameters were tested in order to completely extract the isothiocyanates from the freeze-dried watercress. The volume of the extraction solvent *n*-hexane (5.0 mL, 7.0 mL and 10.0 mL), time of extraction (30 s and 2 min) and sample clean-up method (filtration with a Chromafil RC, 0.2 μm filter versus centrifugation) were tested.

As a final method the following procedure was executed: approximately 200 mg of lyophilized watercress was accurately weighed. Firstly, 5.0 mL *n*-hexane was added. Secondly, the mixture was vortex mixed during 30 s. After centrifugation the supernatant was collected. The extraction was executed for a second time (5.0 mL n-hexane, 30 s) and both supernatants were combined. To avoid loss of PEITC during the analysis, the isothiocyanate was converted to its thiourea derivative by adding 2 mL 2M ammonia in isopropanol. The mixture was incubated overnight at 25 °C. Afterwards, the sample was evaporated with nitrogen gas and the residue was dissolved in 2.5 mL acetonitrile:water (3:2, v/v). After incubation overnight, the sample was analysed with HPLC-DAD.

For the HPLC analysis, 20 μL of the extraction solution was injected on a Lichrospher RP 18e column (4.6 mm × 250 mm, 5 μm) (Merck, Darmstadt, Germany). The temperature of the column was set at 40 °C and a flow rate of 1.0 mL/min was used. The mobile phase solvents consisted of water +0.025% formic acid (A) and acetonitrile far UV + 0.025% formic acid (B). An isocratic gradient was used: 40% A during 10 min. For detection, the diode array detector was set at 242 nm.

2.5. Quantification method for the determination of progoitrin in broccoli

The method was based on the analytical method for the analysis of glucosinolates in watercress as described in 2.3. Different extraction parameters were evaluated in order to fully extract the compound out of the broccoli powder. Extraction on 500.0 mg of the sample was compared with 1.0 g and 250.0 mg. The extraction was carried out on three different temperatures; 70 °C, 75 °C and 80 °C.

As a final method the following procedure was performed. 500.0 mg broccoli seed powder was heated at 75 °C in a water bath for 1 min 5.0 mL boiling methanol (70% v/v) and 200 μL internal standard (glucotropaeolin, 20 mmol/L) were added. This mixture was stirred for 10 min at 75 °C and then sonicated for 10 min at 60 °C. It was centrifuged (10 min at 1180 g) and the supernatant was transferred to a calibration flask

of 20.0 mL. The residue was extracted a second and a third time with 5.0 mL boiling methanol 70%. The calibration flask was completed to 20.0 mL with water. Purification and desulfatation of the glucosinolates was done on a DEAE Sephadex A-25 ion exchange column. 2.0 mL of the test solution and 100 μL diluted sulfatase from *Helix pomatia* were brought on the ion exchange column and incubated overnight at 37 °C. The column was eluted with 2 times 1.0 mL water.

The HPLC-DAD analysis was carried out on an Agilent Technologies 1200 series apparatus (Agilent Technologies N.V., Diegem, Belgium). For the HPLC analysis, 20 μL of the extraction solution was injected on a Superpher® RP 18e Kinetex column (250 × 4.0 mm, 5 μm) (Phenomenex, Utrecht, The Netherlands). The temperature of the column was set at 40 °C. The mobile phase solvents consisted of water +0.025% formic acid (A) and acetonitrile far UV + 0.025% formic acid (B). The gradient was set as follows: at the start a flow rate of 0.8 mL/min with 98% A was used, at 7 min the flow rate and % A were still 0.8 and 98%, afterwards the flow rate was increased to 1.0 mL/min and at 38 min the percentage A is 75%, going to 0% A in 2 min. This condition was kept until 45 min, the flow rate decreased to 0.8 again and the starting conditions are reached at 47 min. These conditions are kept until 55 min. For detection, a diode array detector (Agilent Technologies, Diegem, Belgium) was set at 229 nm.

2.6. Quantification method for the determination of goitrin in broccoli

The method was based on an optimized and validated method of Wang et al. (2013). 250.0 mg broccoli seed powder was accurately weighed and 10.0 mL water was added. The mixture was sonicated for 30 min and subsequently centrifuged for 10 min (1180 g). The supernatant was kept overnight. After filtration through a syringe filter (0.45 μm), this solution was used for HPLC-DAD analysis. 10 μL of the solution was injected on a Kinetex® RP C18 column (250 × 4.0 mm, 5 μm) at room temperature and a flow rate of 0,8 mL/min was chosen. The mobile phase solvents consisted of 0.025% FA (v/v) in water (A) and 0.025% FA (v/v) in ACN far UV (B). The gradient was set as follows: the starting condition was 95% A, the % A was decreased to 88% over 25 min, at 30 min 25% A was reached, this condition is kept for 2 min, at 35 min 95% A is reached again which is kept for 5 min. The detector was set at 240 nm.

2.7. Method validation

All methods were validated according to the International Council for Harmonization (ICH) guidelines on validation of analytical methods (ICH Harmonised tripartite guideline 1994). Firstly, the calibration model of the internal standard was examined. Therefore, six to ten concentration levels of the standards were prepared. All solutions were analysed in duplicate. The repeatability of the injection was tested by analysing one sample six times. Secondly, the repeatability and the intermediate precision were investigated. Six independently prepared samples (100%) were analysed according to the above described methods. For the intermediate precision, this was repeated on three different days. The third day, another analyst executed the method. The precision on different concentration levels was determined by analysing six samples weighing 50% of the prescribed mass and six samples weighing 150% of the mass. All the solutions used were freshly prepared each day. For goitrin and progoitrin the recovery was investigated with standard addition at 3 concentration levels in triplo: to 50% sample 25%, 50% and 75% of standard was added.

3. Results and discussion

3.1. Quantification method for the determination of glucosinolates in watercress: optimization and validation

Since *Nasturtium officinale* R. Br. is known for its beneficial effects in the treatment of inflammation and infections, it is widely used for its medicinal properties. These beneficial effects are mainly attributed to glucosinolates and its main degradation products, isothiocyanates. Since the quality of a food supplement of watercress depends on the content of gluconasturtiin and phenylethylisothiocyanate, it is necessary to have a validated method in order to quantify these compounds in a reliable way.

For the analysis of gluconasturtiin, sinigrin monohydrate was added as internal standard. This glucosinolate is structurally related to gluconasturtiin, but it is not present in watercress itself. The main compound present in the chromatogram of the lyophilized watercress was identified as desulfogluconasturtiin, the desulfated derivative of gluconasturtiin, by comparison with literature data. The other peak present in the chromatogram was identified as desulfosinigrin, a derivative of the internal standard (Fig. 3).

The optimization experiments showed that the yield of the extraction improved when the extraction volume was increased. A volume of 5.0 mL methanol 70% (v/v) was used in the final method. The average amount of gluconasturtiin was equal after two and three extractions. However, the variation of the results was lower when an additional extraction was performed. The extraction time was prolonged from 10 to 20 min in order to achieve a more complete extraction with a smaller standard deviation. The desulfatation step with the diluted sulfatase and overnight incubation gave the best results.

The calibration model of the internal standard (0.084–0.418 μmol/mL) was investigated. The regression line was constructed, the equation generated (y = 7,1719x + 33,739) and the correlation coefficient (0.997) was calculated (Fig. 4). The residuals were graphically evaluated. A visual assessment of the regression line and residuals plot showed that the method was linear and homoscedastic. The determination coefficient (R^2) was 0.995. The slope and intercept of the regression line were investigated with a Student's t-test. This test showed that the slope was significant and that the 95% confidence interval of the intercept included the point (0,0). Since the line passed through the origin, a single-point calibration is justified (Table 1).

The precision of the method was investigated by calculating the mean, standard deviation and the relative standard deviation (RSD%) for each day as well as for each concentration level. Additionally, the overall mean, standard deviation and RSD% were calculated for the three days and for the three concentration levels. All results are shown in Table 5.

The repeatability and intermediate precision were evaluated by an ANOVA single factor test. Since this test uses variances, the variances for the different days as well as for the different concentration levels should not differ significantly. The variances were compared using a Cochran test. The calculated Cochran (C_{calc})value was smaller than the critical value (C_{crit}) for both the different days and the different concentration levels, implying that the variances are not significantly different. ANOVA showed that the calculated F-value (F_{calc}) was smaller than the critical F-value (F_{crit}) for the different days and for the different concentration levels. Additionally, the values of RSD_{within} and $RSD_{between}$ are in the same range. However, the RSD% is rather high, 9.90% for the intermediate precision and 9.05% for the repeatability on different concentration levels. Despite the optimization of the method, the variation was still relatively high. However, this variation is inevitably associated with the complexity of the method. Based on the results, 10% is considered as maximal value for the RSD. This value was comparable with results from previous investigation (ISO 9167-2019, Heyerick, 2010, pp. 1–27). Based on the results of the 30 analysed samples, the average amount of gluconasturtiin was 20.13 ± 1.80 μmol per gram of lyophilized watercress.

3.2. Quantification method for the determination of isothiocyanates in watercress: optimization and validation

The main compound present in the chromatogram of the freeze-dried

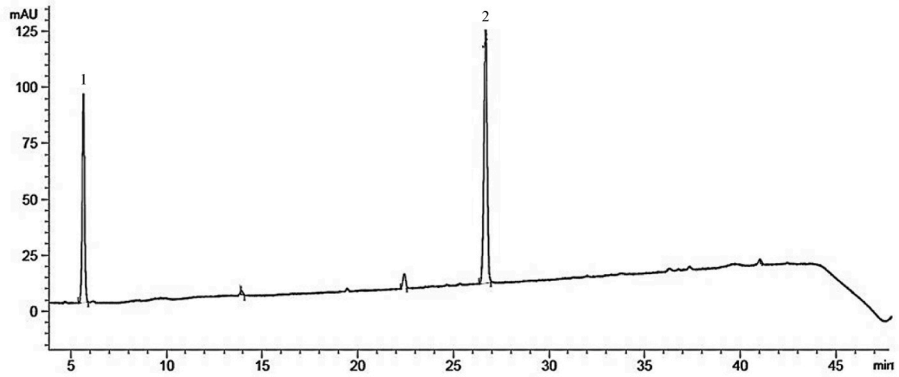

Fig. 3. Chromatographic profile (detection wavelength 229 nm) of the lyophilized watercress. Peak 1 was identified as desulfosinigrin, a derivative from the internal standard. Peak 2 was identified as desulfogluconasturtiin.

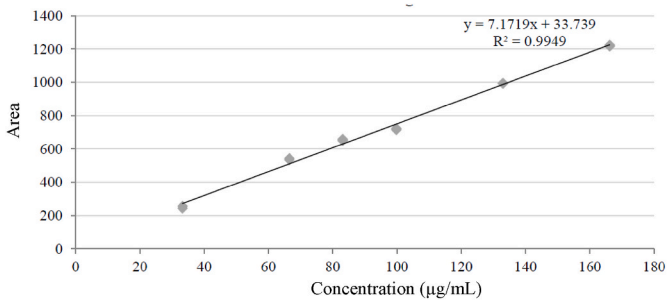

Fig. 4. Calibration curve of sinigrin.

Table 1
Data of the linear regression of sinigrin monohydrate.

Sinigrin monohydrate	
Correlation coefficient	0.9974
Slope ±standard error	7.17 ± 0.16
Intercept ±standard error	33.74 ± 17.25
Confidence interval (95%)	−4.70–72.17

watercress was identified as phenylethylthiourea a derivative of phenylethylisothiocyanate, by comparison with literature data (Fig. 5).

The extraction experiments showed that the best result was obtained when 5.0 mL *n*-hexane was added as extraction solvent. When the volume was increased to 7.0 or 10.0 mL the derivatization reaction was not quantitative. This is probably due to the small volume (2.0 mL) of the derivatization reagent. An extraction time of 30 s gave a similar result compared to an extraction time of 2 min. Therefore, the method was executed with an extraction time of 30 s. Cleaning-up the sample using centrifugation led to a higher amount of PEITC compared to filtration. The lower amount obtained with the latter method might be due to adsorption to the filter.

The calibration model of the PEITC standard (0.013–1.04 µmol/mL) was estimated by analysing the regression line, the equation and the correlation coefficient (Fig. 6). A visual evaluation of the regression line and residuals plot showed that the method was linear and homoscedastic. The determination coefficient was 0.999. The slope and intercept of the regression line were investigated with a Student's t-test. This test

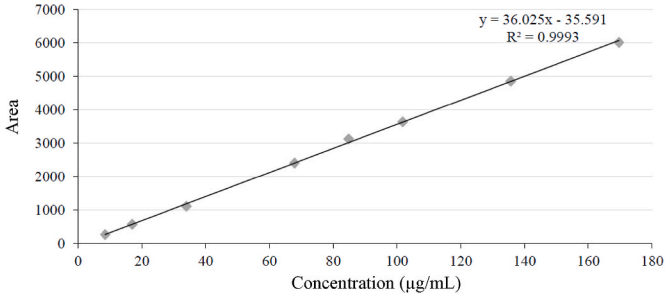

Fig. 6. Calibration curve of PEITC.

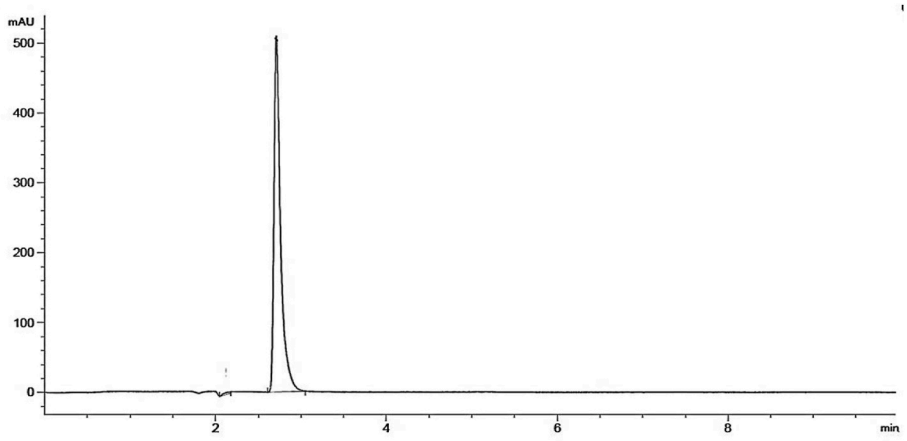

Fig. 5. Chromatographic profile (detection wavelength 242 nm) of the lyophilized watercress. The peak was identified as phenylethylthiourea, a derivative of phenylethylisothiocyanate.

showed that the slope was significant and the 95% confidence interval of the intercept included the point (0,0). Since the line passes through the origin, a one point calibration can be used (Table 2).

For the evaluation of the precision; the mean, the standard deviation and the RSD% were calculated for every day as well as for each concentration level. Additionally, the overall mean, standard deviation and RSD% were calculated for the three days and for the three concentration levels. All results are shown in Table 5.

Before evaluating the repeatability and intermediate precision by an ANOVA single factor test, the variances were compared by a Cochran test. Since the calculated Cochran value was smaller than the critical value for both the different days and the different concentration levels, the variances are not significantly different. ANOVA indicated that the calculated F-value was smaller than the critical F-value as well as for the different days as for the different concentration levels. The RSD% is rather high, 12.67% for the intermediate precision and 13.10% for the repeatability on different concentration levels. During the optimization and validation of the method to analyse isothiocyanates, there was a high degree of variation as well. This variation is caused by the volatility of PEITC, which makes it challenging to analyse. To circumvent additional loss during the analysis, PEITC was converted to the phenylethyl thiourea derivative. The first experiment (three different concentration levels tested in duplicate) rendered an RSD% of 7.77%. However, the complete validation resulted in an RSD% of 12.67% for the intermediate precision and 13.10% for the repeatability on different concentration levels. Due to the volatility of PEITC, the values were considered as acceptable. Based on the result of the 30 analysed samples, the average amount of PEITC was 0.904 ± 0.118 μmol per gram of lyophilized watercress.

3.3. Quantification method for the determination of progoitrin in broccoli: optimization and validation

Fig. 7 shows the chromatographic profile of the progoitrin analysis. The peaks of progoitrin and glucotropaeolin in the chromatogram could be identified based on the retention time of their standards. The extraction experiments showed that the highest yield of progoitrin was obtained by carrying out the extraction on 500.0 mg broccoli powder with 5.0 mL boiling methanol. The variation in extraction temperature had no influence on the yield.

The calibration model of the internal standard glucotropaeolin (0.044–1.312 μmol/mL) was investigated. The regression line was created, the equation generated and correlation coefficient calculated (Fig. 8). The slope and intercept were studied using a Student's t-test. A graphical representation of the residuals was evaluated. Visual inspection of the regression line revealed a linear relation between the concentration and the obtained peak area. The residual plot showed that the residuals were randomly scattered and homoscedastic. The determination coefficient was higher than 0.999. The slope of the regression line was significantly different from zero. The intercept of the line included the point (0,0), therefore a single-point calibration is justified (Table 3).

The precision of the method was investigated by calculating the mean, the standard deviation and the RSD% for each day and each concentration level. The calculations were done with the internal standard glucotropaeolin, as described in ISO 9167-1 (1992) with the correction factor 1.09 for glucotropaeolin – progoitrin. The overall mean, standard deviation and RSD% were calculated for the three days and the three concentration levels. The results, shown in Table 5, were compared within and between the different groups. In order to know if the variances are the same for all the groups, a Cochran test was carried out. For both, the different days as well as for the different concentration levels, the Cochran value was smaller than the critical value, implying that the variances are not significantly different. Therefore, a one-way ANOVA could be done. The ANOVA test showed a significant difference between the 3 days and the 3 concentration levels. Since the RSD% between days (4.26%) and RSD% between levels (4.77%) is smaller than 5% and there is no dependency on days or concentration observed, the method is still accepted. The average amount of progoitrin was 0.521 ± 0.046 μmol/g broccoli seed powder. The recovery of progoitrin was 99.2% with a confidence interval 93.8–104.6%.

Table 2
Data of the linear regression of PEITC.

PEITC	
Correlation coefficient	0.9997
Slope ±standard error	36.64 ± 0.20
Intercept ±standard error	−19.74 ± 16.00
Confidence interval (95%)	−53.28–13.80

3.4. Quantification method for the determination of goitrin in broccoli: validation

By using a standard of goitrin, the corresponding peak in the chromatogram could be identified. The chromatographic profile is shown in Fig. 9. The calibration model of goitrin was investigated. The regression line (0.0077–3.0965 μmol/mL) was created, the equation generated and correlation coefficient calculated (Fig. 10). The slope and intercept were studied using a Student's t-test. A graphical representation of the residuals was evaluated. A visual inspection of the regression line revealed a linear relation between the concentration and the obtained peak area. The residual plot showed that the residuals were randomly scattered and homoscedastic. The determination coefficient was higher than 0.999. The slope of the regression line was significantly different from zero. The intercept of the line included the point (0,0): the use of a single-point calibration is justified (Table 4).

The precision of the method was investigated by calculating the mean, the standard deviation and the RSD% for each day and each concentration level. The overall mean, standard deviation and RSD% were calculated for the three days and the three concentration levels. The results, shown in Table 5, were compared within and between the different groups. In order to know if the variances are the same for all the groups, a Cochran test was carried out for both the different days and the different concentration levels. The Cochran value was smaller than the critical value, implying that the variances are not significantly different. Subsequently, a one-way ANOVA-test was carried out.

For the precision of the method on different days, the calculated and theoretical F-values are shown in Table 5. The calculated F-value is higher than the critical F-value. According to these statistical results, the concentrations of goitrin differ significantly between the groups and cannot be considered as the same. However, the method is considered to be suitable, since the $RSD_{Between}$ (2,51%) is less than 5%, which is accepted as the RSD_{max}. This implies that the eighteen samples do not differ significantly in terms to the average content. Also for the precision on different concentration levels, the calculated F-value is higher than the critical F-value. The $RSD_{Between}$ (3.06%) is again smaller than the RSD_{max} (5%). It can be concluded that the average contents of goitrin in thirty samples are not significantly different. The average amount of goitrin was 0.34 ± 0.02 μmol/g broccoli seed powder. The recovery of goitrin was 100.6% with a confidence interval 99.2–102.0%.

3.5. Analysis of commercial products

The content of progoitrin and goitrin in commercially available products was analysed with the validated methods. In product A an average of 0.744 mg/g progoitrin and 0.0039 mg/g goitrin could be determined. The daily dose of product A is 3 times a tablet of 500 mg, hence the limits of daily intake, according to the Belgian Royal Decree (24.01.2017): respectively 20 mg and 5 mg per day, are not exceeded. In product B progoitrin was not detected (limit of detection (LOD) progoitrin 0.08 mg/capsule). For goitrin there was an interfering compound in the chromatogram; if goitrin was present, it would be less than 0.055

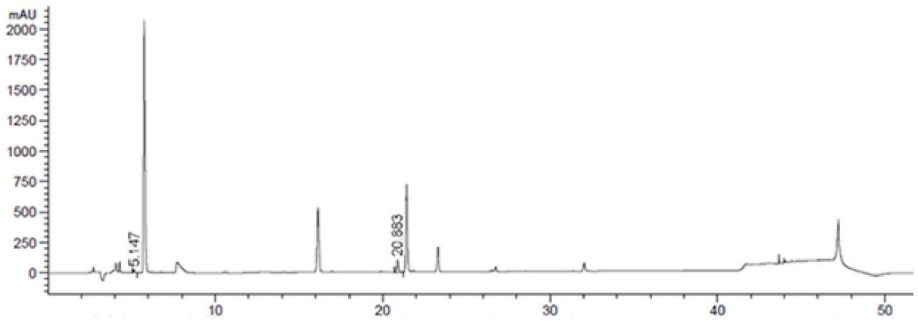

Fig. 7. Chromatographic profile (detection wavelength 229 nm) of broccoli, with the peaks of progoitrin and glucotropaeolin at retention times 5.15 and 20.88 min respectively.

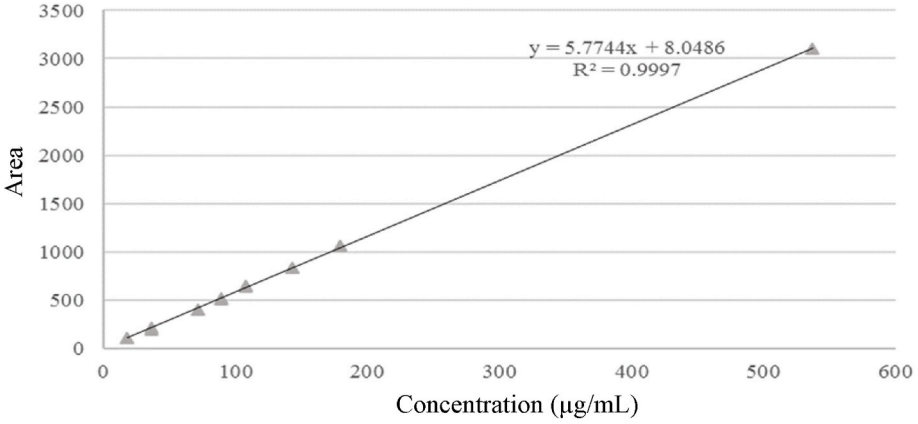

Fig. 8. Calibration curve of glucotropaeolin.

Table 3
Data of the linear regression of glucotropaeolin.

Glucotropaeolin	
Correlation coefficient	0.9999
Slope ±standard error	5.77 ± 0.03
Intercept ±standard error	8.05 ± 5.48
Confidence interval (95%)	−3.71–19.80

mg/caps. Analysis of product C showed no detectable amounts of progoitrin and goitrin. The limits of detection were 0.12 mg/tablet and 7 µg/tablet for progoitrin and goitrin, respectively. Product D contains an average of 26.5 µg/mL progoitrin and 53.8 µg/mL goitrin. The daily dose is 45 drops, which is about 750 µL. The limit for both progoitrin and goitrin is not exceeded.

4. Conclusions

In order to assess the quality and safety of food supplements containing glucosinolates and isothiocyanates, such as *Nasturtium officinale* R. Br. (watercress) and broccoli, both belonging to the Brassicaceae family, quantitative analytical methods were optimized and validated for the analysis of glucosinolates, phenylethyl isothiocyanate and goitrin, conform the ICH guidelines. The methods of watercress were found to be linear in a range of 0.084–0.418 µmol/mL (gluconasturtiin) and 0.013–1.04 µmol/mL (PEITC). The repeatability on different days and concentrations was accepted despite the higher variations. These variations are due to the complexity of the method (glucosinolates) or the volatility of the compound (isothyocyanates). The methods of broccoli were linear in the range 0.044–1.312 µmol/mL (glucotropaeolin) and 0.0077–0.0965 µmol/mL (goitrin). The repeatability on different days and concentrations was accepted. Some selected samples available on the Belgian market were analysed and the levels of progoitrin and

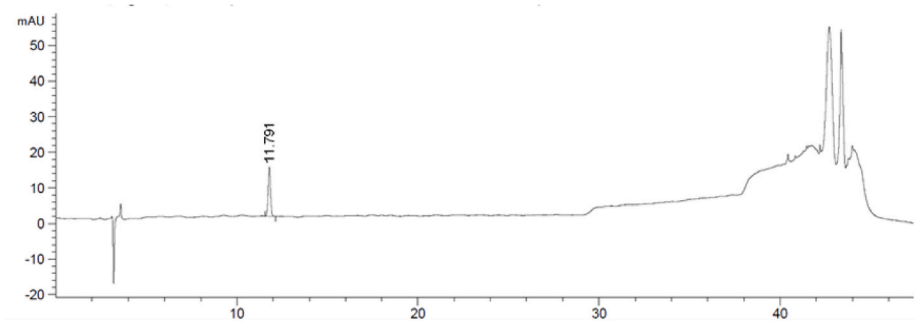

Fig. 9. Chromatogram (detection wavelength 240 nm) of the goitrin standard; retention time is 11.79 min.

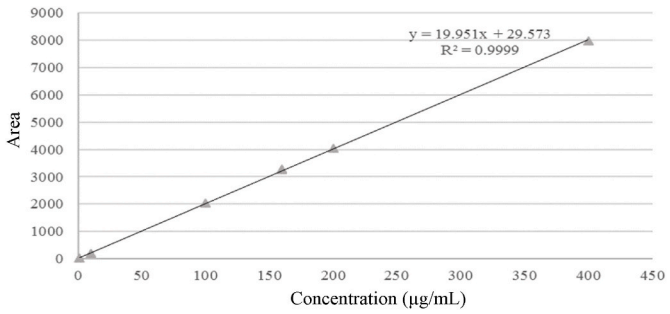

Fig. 10. Calibration curve of goitrin.

Table 4
Data of the linear regression of goitrin.

Goitrin	
Correlation coefficient	0.9999
Slope ±standard error	19.95 ± 0.08
Intercept ±standard error	29.57 ± 15.31
Confidence interval (95%)	−4.54–63.69

Table 5
Precision results of gluconasturtiin, PEITC, progoitrin and goitrin on different days (n = 18, C_{crit} = 0.71, F_{crit} = 3.68) and different concentration levels (n = 30, C_{crit} = 0.51, F_{crit} = 2.76).

Gluconasturtiin	Mean (µmol/g)	RSD %	RSD_{within}	$RSD_{between}$	C_{calc}	F_{calc}
50%	19.53	6.47	8.50%	9.05%	0.45	1.81
150%	19.44	7.20				
100% - Day 1	20.51	5.58	9.34%	9.90%	0.60	1.73
100% - Day 2	19.55	9.06				
100% - Day 3	21.61	11.91				
PEITC						
50%	0.955	15.19	12.98%	13.10%	0.31	1.12
150%	0.904	13.26				
100% - Day 1	0.846	12.92	11.90%	12.67%	0.39	1.81
100% - Day 2	0.953	11.99				
100% - Day 3	0.862	10.62				
Progoitrin						
50%	0.551	3.22	3.29%	4.77%	0.257	7.6
150%	0.512	2.59				
100% - Day 1	0.520	3.74	3.51%	4.26%	0.386	3.85
100% - Day 2	0.499	3.90				
100% - Day 3	0.527	2.85				
Goitrin						
50%	0.357	2.58	1.72%	3.06%	0.48	13.9
150%	0.342	1.39				
100% - Day 1	0.350	0.63	1.27%	2.51%	0.65	18.3
100% - Day 2	0.337	1.80				
100% - Day 3	0.337	1.14				

goitrin were below the legally established thresholds.

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CRediT authorship contribution statement

Mart Theunis: Conceptualization, Supervision, Writing – original draft. **Tania Naessens:** Investigation. **Laura Peeters:** Investigation, Writing – original draft. **Maxime Brits:** Investigation, Writing – original draft. **Kenn Foubert:** Conceptualization, Supervision, Writing – review & editing. **Luc Pieters:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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