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Article

Simultaneous Analysis of Glucosinolates and Isothiocyanates by Reversed-Phase Ultra-High-Performance Liquid Chromatography–Electron Spray Ionization–Tandem Mass Spectrometry

Silvia Andini, Carla Araya-Cloutier, Mark Sanders, and Jean-Paul Vincken*

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ABSTRACT: A new method to simultaneously analyze various glucosinolates (GSLs) and isothiocyanates (ITCs) by reversed-phase ultra-high-performance liquid chromatography–electron spray ionization–tandem mass spectrometry has been developed and validated for 14 GSLs and 15 ITCs. It involved derivatization of ITCs with *N*-acetyl-L-cysteine (NAC). The limits of detection were 0.4–1.6 μM for GSLs and 0.9–2.6 μM for NAC–ITCs. The analysis of *Sinapis alba*, *Brassica napus*, and *Brassica juncea* extracts spiked with 14 GSLs and 15 ITCs indicated that the method generally had good intraday (≤10% RSD) and interday precisions (≤16% RSD). Recovery of the method was unaffected by the extracts and within 71–110% for GSLs and 66–122% for NAC–ITCs. The method was able to monitor the enzymatic hydrolysis of standard GSLs to ITCs in mixtures. Furthermore, GSLs and ITCs were simultaneously determined in Brassicaceae plant extracts before and after myrosinase treatment. This method can be applied to further investigate the enzymatic conversion of GSLs to ITCs in complex mixtures.

KEYWORDS: *LC–MS analysis, glucosinolate breakdown product, validation, mustard, dithiocarbamate, thioglucosidase*

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INTRODUCTION

Glucosinolates (GSLs) are widely distributed in all plant tissues within the Brassicaceae family. They play an important role to defend the plants against pathogen and insect attacks.[1] This defense system is assisted by myrosinase. Just like GSLs, myrosinase is found in all tissues of Brassicaceae plants, but it is located in different cells.[2] Myrosinase gets in contact with GSLs when the plant tissues are damaged.[3] In an environment of pH 5–7, this interaction mostly yields the conversion of GSLs into microbiologically active isothiocyanates (ITCs) (Figure 1A).[4−6] Previous studies reported that depending on the structure, ITCs could inhibit the growth of fungi and bacteria pathogenic to plants[7,8] and humans.[9−11] This underlines the potential of ITCs as a new class of plant-derived antimicrobial compounds.

The chemical diversity of GSLs, imparted by various side chains (R-group), determines which ITCs can be formed.[12] In general, the R-group can be an aliphatic, benzenic, or indolic group. Based on the type of R-group, GSLs and ITCs can be classified at least into eight and seven subclasses, respectively (Table 1). ITCs have one subclass less, because those with an indole R-group are unstable.[13] Although ITCs share the R-group of GSLs, ITCs have a completely different core structure than GSLs (Figure 1A). Consequently, ITCs and GSLs have different volatility and ionization ability, making simultaneous analysis difficult. Therefore, GSLs and ITCs have frequently been analyzed separately by mainly liquid chromatography (LC) and gas chromatography (GC).[14]

GSLs are highly polar because of a thioglucosyl group (−SGlc) and a strong acid residue (SO_4^{2-}). The latter causes GSLs to be spontaneously ionized as an anion. Removal of the strong acid residue, that is, desulfation, is often required in various LC–mass spectrometry (MS) methods.[15−19] However, this step could result in incomplete desulfation, self-dimerization, and self-degradation.[20−22] LC–MS methods for direct quantitative analysis of individual intact GSLs without desulfation were developed.[23−26] However, efficient LC–MS analysis of intact GSLs was historically challenging[27] and most methods included only few GSLs and do not cover the analysis of ITCs.

The volatility of ITCs makes GC a convenient technique for ITC analysis. However, several ITCs were thermally unstable. Allyl ITC (AITC) and 4-(methylsulfinyl)butyl ITC (4-MSITC) were transformed into allyl thiocyanate and 3-butenyl ITC (BuITC), respectively, in the GC injection port.[28,29] LC analysis of ITCs was developed previously employing derivatization with thiol compounds, which can overcome the limitation of GC analysis.[30−32] In particular, Pilipczuk et al.[31] successfully developed an LC–MS method to quantify various individual ITCs derivatized with N-acetyl-L-cysteine (NAC), a safe derivatization reagent. This derivatization allowed the ionization of ITCs, while keeping their R-groups intact (Figure 1B). However, this LC–MS method did not cover the analysis of GSLs.

Tsao et al.[33] developed a method to simultaneously analyze allyl GSL (AGSL) and AITC by using high-performance liquid

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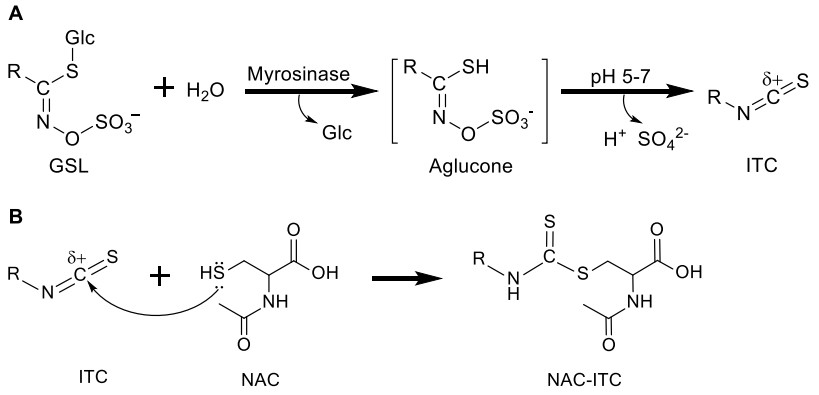

Figure 1. Conversion of GSL to ITC by myrosinase at pH 5−7 (A) and derivatization reaction of ITC with NAC (B). Carbon atom in the ITC functional group is electrophilic.

chromatography (HPLC) with UV detection at 228 nm for AGSL and 242 nm for AITC. This method is, however, not suitable for analyzing GSLs in a mixture because many compounds absorb at 228 nm. Franco et al.[34] developed a simultaneous method for specific pairs of GSLs/ITCs, without precolumn derivatization, by using reversed-phase (RP) HPLC−electron spray ionization (ESI)−tandem mass spectrometry (MS/MS). This method was applicable to analyze the compounds in complex matrices. However, it was only evaluated for 4-(methylsulfinyl)butyl GSL/ITC and 4-(methylthio)butyl GSL/ITC. Another HPLC-based method developed to simultaneously analyze GSLs and ITCs was also evaluated only for few pairs, for example, AGSL/ITC, phenyl GSL/ITC, and benzyl GSL/ITC.[35] Moreover, one study described the simultaneous analysis of only few pairs of GSLs/ITCs by another separation technique, namely, capillary electrophoresis micellar electrokinetic chromatography (CE-MEKC).[36] Altogether, to date there is no method which has been evaluated to analyze many more GSLs and ITCs in complex mixtures in a single analytical run.

In this study, we aimed at developing and validating an LC−MS method which can simultaneously analyze various GSLs and ITCs in complex mixtures. Derivatization of ITCs with NAC was included in the method to enhance the ionization ability of ITCs.[31] The method would enable the monitoring of the in vitro enzymatic hydrolysis of GSLs and the formation of ITCs in standard mixtures and plant extracts upon myrosinase treatment.

MATERIALS AND METHODS

Standard Compounds and Other Chemicals. Authentic standards of 14 GSLs listed in Table 1 were purchased from PhytoLab GmbH & Co (Vestenbergsgreuth, Germany). Authentic standards of nine ITCs (with peak numbers in boldface according to Table 1): 3-(methylsulfinyl)propyl ITC (3-MSITC, **I1**), 4-(methylsulfinyl)butyl ITC (4-MSITC, **I2**), 6-(methylsulfinyl)hexyl ITC (6-MSITC, **I3**), 9-(methylsulfinyl)nonyl ITC (9-MSITC, **I4**), 4-(methylsulfinyl)-3-butenyl ITC (4-MS-3-en-ITC, **I5**), 3-(methylsulfonyl)propyl ITC (3-MSoITC, **I6**), 3-(methylthio)propyl ITC (3-MTITC, **I7**), 4-(methylthio)butyl ITC (4-MTITC, **I8**), and 5-(methylthio)pentyl ITC (5-MTITC, **I9**) were purchased from Abcam (Cambridge, UK). Authentic standards of the other six ITCs: propyl ITC (PITC, **I10**), AITC (**I11**), 3-butenyl ITC (BuITC, **I12**), 4-pentenyl ITC (PeITC, **I14**), benzyl ITC (BITC, **I15**), and phenethyl ITC (PhEITC, **I17**) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany). NAC-4-MSITC of an authentic standard was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). NAC−BITC and NAC−PhEITC of an authentic standard were purchased from Abcam (Cambridge, UK).

NAC, NaOH, KH_2PO_4, myrosinase, *n*-hexane, and *tert*-butanol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Duchefa Biochemie (Haarlem, The Netherlands). Isopropanol (IPA) (ULC/MS grade), acetonitrile (ACN) acidified with 0.1% (v/v) formic acid (FA) (ULC/MS grade), water acidified with 0.1% (v/v) FA (ULC/MS grade), and methanol (MeOH) (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). High-purity water was produced in-house using a Milli-Q A10 Gradient system (18.2 MΩ·cm, 3 ppb TOC) (Merck Millipore, Darmstadt, Germany).

Plant Materials. Seeds of *Sinapis alba* (yellow mustard "Emergo", 393,810), *Brassica napus* ("Helga", 392,600), and *Brassica juncea* var. rugosa rugosa (Chinese mustard/amsoi, 160,400) were purchased from Vreeken's Zaden (Dordrecht, The Netherlands). *B. juncea* var. rugosa rugosa is referred to as *B. juncea* in the following text.

Preparation of Stock Solutions of GSLs, ITCs, and NAC. Stock solutions (5 mM) of GSLs and ITCs were prepared in phosphate buffer pH 7 (0.1 M) and in IPA, respectively. All standard GSLs and ITCs were weighed on a Mettler XP6 microbalance (Mettler-Toledo International Inc., U.S.A.). Stock solutions were kept at −20 °C before use. A stock solution of NAC (100 mM) was freshly prepared in phosphate buffer, pH 7.0 (0.1 M).

Extraction of GSLs from Brassicaceae Seeds. Ground seeds of *S. alba*, *B. napus*, and *B. juncea* were extracted in a SpeedExtractor E-916 (Büchi, Flawil, Switzerland) by using absolute methanol at 65 °C as described in a previous study.[37] Afterward, the extract was evaporated under reduced pressure (Syncore Polyvap, Büchi), resolubilized in *tert*-butanol, and freeze-dried. The dried extracts were stored at −20 °C.

Derivatization of ITCs with NAC. Four different experiments were carried out in which NAC was combined with (i) standard mixtures of 14 GSLs and 15 ITCs, (ii) plant extracts spiked with mixtures of 14 GSLs and 15 ITCs, (iii) a mixture of standard GSLs and myrosinase, and (iv) mixtures of plant extracts and myrosinase.

Derivatization experiments of the mixtures of standard ITCs and GSLs were performed for making calibration curves of each ITC and GSL, to evaluate the linearity range, to determine limit of detection (LOD) and limit of quantification (LOQ), and to quantify GSLs and ITCs in the samples. NAC was added into the mixtures of 14 GSLs and 15 ITCs. The final molar concentration of NAC was five times higher than that of total ITCs. IPA was added to the mixtures to a concentration of 25% (v/v), in which both GSLs and ITCs were soluble (Figure S1). During preparation, all solutions were kept in an ice bath. All reaction mixtures were incubated at 50 °C for 2 h with constant mixing at 900 rpm. Then, the samples were chilled and analyzed by RP ultra-high-performance liquid chromatography (UHPLC)-MS. The completeness of the derivatization after 2 h incubation was established for 4-MSITC, BITC, and PhEITC by

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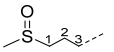
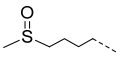
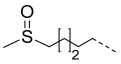
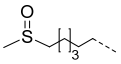
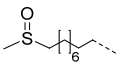
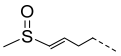
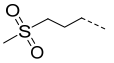
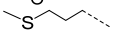
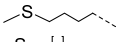
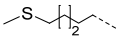
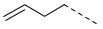
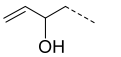
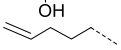
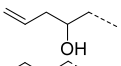
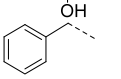
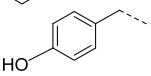
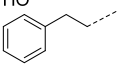
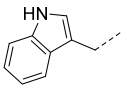
Table 1. GSLs and ITCs in the Study

subclass	structure of R-group	(semi)systematic name of R-group	abbreviation of GSL or ITC	trivial name of GSL or ITC	code[a]	retention time (tR, min)
aliphatic						
(methylsulfinyl)alkyl		3-(methylsulfinyl)propyl	3-MSGSL 3-MSITC	glucoiberin iberin	**G1*** **I1***	1.99 17.53
		4-(methylsulfinyl)butyl	4-MSGSL 4-MSITC	glucoraphanin sulforaphane	**G2*** **I2***	3.11 19.88
		5-(methylsulfinyl)pentyl	5-MSGSL 5-MSITC	glucoalyssin alyssin	**G19** **I19**	4.37 23.44
		6-(methylsulfinyl)hexyl	n.a.[b] 6-MSITC	n.a. hesperin	- **I3***	- 27.51
		9-(methylsulfinyl)nonyl	n.a. 9-MSITC	n.a. 9-(methylsulfinyl)nonyl ITC	n.a. **I4***	- 36.53
(methylsulfinyl)alkenyl		4-(methylsulfinyl)-3-butenyl	4-MS-3-en-GSL 4-MS-3-en-ITC	glucoraphenin sulforaphene	**G5*** **I5***	3.36 20.44
(methylsulfonyl)alkyl		3-(methylsulfonyl)propyl	3-MSoGSL 3-MSoITC	glucocheirolin cheirolin	**G6*** **I6***	2.33 19.60
(methylthio)alkyl		3-(methylthio)propyl	3-MTGSL 3-MTITC	glucoibervirin ibervirin	**G7** **I7***	12.60 32.49
		4-(methylthio)butyl	4-MTGSL 4-MTITC	glucoerucin erucin	**G8*** **I8***	15.08 35.78
		5-(methylthio)pentyl	5-MTGSL 5-MTITC	glucoberteroin berteroin	**G9*** **I9***	18.73 39.15
alkyl		propyl	n.e.[c] PITC	n.e. propyl ITC	- **I10***	- 29.04
alkenyl		2-propenyl (= allyl)	AGSL AITC	sinigrin allyl ITC	**G11*** **I11***	2.90 25.87
		3-butenyl	BuGSL BuITC	gluconapin 3-butenyl ITC	**G12*** **I12***	7.82 31.66
		2-hydroxy-3-butenyl	2-OH-BuGSL n.e.	progoitrin n.e.	**G13*** -	2.67 -
		4-pentenyl	PeGSL PeITC	glucobrassicanapin 4-pentenyl ITC	**G14*** **I14***	14.05 36.04
		2-hydroxy-4-pentenyl	2-OH-PeGSL n.e.	gluconapoleiferin n.e.	**G20** -	9.30 -
benzenic		benzyl	BGSL BITC	glucotropaeolin benzyl ITC	**G15*** **I15***	14.72 36.53
		*p*-hydroxybenzyl	*p*-OH-BGSL *p*-OH-BITC	glucosinalbin *p*-hydroxy-benzyl ITC	**G16*** **I16**	3.83 30.15
		phenethyl	PhEGSL PhEITC	gluconasturtiin phenethyl ITC	**G17*** **I17***	18.54 39.38
indolic		indole-3-ylmethyl	I3MGSL n.e.	glucobrassicin n.e.	**G18***	16.59 -

[a]Codes in the chromatograms for GSLs start with **G** and for ITCs (analyzed as NAC−ITCs) start with **I**. Asterisk (*) is to indicate that the authentic standard was available in the study. [b]n.a. refers to GSLs or ITCs which were not available in this study. [c]n.e. refers to GSLs or ITCs which have never been reported.

comparing the amount of NAC derivatives, which were formed, to that of their respective standards (NAC-4-MSITC, NAC−BITC, and NAC−PhEITC) (Figure S2). Based on this, it was assumed that the derivatization of the 12 other ITCs was also completed in 2 h.

Derivatization experiments of extracts spiked with mixtures of 14 GSLs and 15 ITCs were performed for evaluating the applicability of the method for analyzing GSLs and NAC−ITCs in extracts, in terms of precision and recovery. The approach described earlier was applied in which a mixture of 14 GSLs and 15 ITCs was added to *S. alba*, *B. napus*, and *B. juncea* seed extracts. Two final concentrations at the mid-levels of calibration range for each GSL and ITC were applied, that is, 10 and 30 μM. Therefore, the final concentrations of NAC were 0.75 mM (5 × 10 μM × 15 ITCs) and 2.25 mM (5 × 30 μM × 15 ITCs), respectively. The final concentration of the extracts was 5 mg/mL prepared from the dried extracts solubilized in DMSO (final concentration of 5%) in phosphate buffer, pH 7.0. The mixtures were conditioned in phosphate buffer, pH 7.0, and incubated for 2 h at 50 °C with a constant mixing at 900 rpm. Dilution of extracts in phosphate buffer, pH 7.0, was made for analyzing GSLs and ITCs present at a higher concentration than 60 μM (the upper limit of the calibration range).

Derivatization experiments of a mixture of standard GSLs and myrosinase, that is, simultaneous enzymatic hydrolysis of GSLs and derivatization of ITCs, were performed for testing the applicability of the method to monitor the hydrolysis of GSLs and the formation of ITCs. The mixture of 14 standard GSLs (30 μM each) and myrosinase (0.01 U/mL, 1 unit of enzymatic activity was defined as the amount of enzyme that releases 1 μmol glucose per min with AGSL as the substrate, at pH 6.0 and 25 °C) were incubated in phosphate buffer, pH 7.0 (0.1 M) at 50 °C in the presence of NAC (2.1 mM). GSLs and ITCs were monitored over time until all GSL peaks disappeared, and the intensity of all NAC−ITC peaks did not

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increase anymore, that was up to 4 h. After incubation, IPA was added to a concentration of 25% (v/v), the samples were chilled, and analyzed by RP-UHPLC−ESI−MS.

The GSL hydrolysis rates (μM/h) were defined as the decrease of GSL concentration over time and determined as the slope of the plot of GSL concentration (μM) versus time (h). The NAC−ITC formation rates (μM/h) were defined as the increase of NAC−ITC concentration over time and determined as the slope of the plot of NAC−ITC concentration (μM) versus time (h).

Derivatization experiments of mixtures of Brassicaceae seed extracts and myrosinase were performed with the same incubation as before to test the applicability of the method to analyze GSLs and ITCs in plant extracts upon myrosinase treatment and NAC derivatization. Shortly, the dried extracts were dissolved in DMSO and then 10 times diluted in phosphate buffer, pH 7.0 (0.1 M). Plant extracts 5 mg/mL, myrosinase 0.05 U/mL, and NAC at 5× estimated concentration of ITCs were incubated at 50 °C for 4 h, with a final concentration of DMSO of 5%. Under this condition, all GSL peaks disappeared within 4 h (data not shown). The concentration of ITCs in *B. napus*, *B. juncea*, and *S. alba* seed extracts was estimated from the original GSL concentration analyzed previously.[37] Afterward, IPA was added to a concentration of 25% (v/v). The samples were chilled and analyzed by RP-UHPLC−ESI−MS. When the concentration of GSLs and ITCs exceeded 60 μM (the upper limit of the calibration range), they were diluted in phosphate buffer, pH 7.0.

Simultaneous RP-UHPLC-MS^n Analysis of GSLs and NAC−ITCs. Analysis of GSLs and NAC−ITCs was performed on an Accela UHPLC system (Thermo Scientific, San Jose, CA, U.S.A.) equipped with a pump, autosampler, and photodiode array (PDA) detector. An LTQ Velos ESI ion trap mass spectrometer (Thermo Scientific) was coupled to the LC system.

The sample (1 μL) was injected onto an Acquity UPLC-BEH shield RP18 column (2.1 mm i.d. × 150 mm, 1.7 μm particle size; Waters, Milford, MA, U.S.A.) with an Acquity UPLC BEH shield RP18 VanGuard precolumn (2.1 mm i.d. × 5 mm, 1.7 μm particle size; Waters). Water acidified with 0.1% (v/v) FA, eluent A, and ACN acidified with 0.1% (v/v) FA, eluent B, were used as solvent at a flow rate of 300 μL/min. The temperature of the sample tray was controlled at 4 °C to prevent further reaction. Different column oven temperatures were tested: 25, 35, and 45 °C. The PDA detector was set to monitor absorption at 200−400 nm. The elution gradient used was: 0−6.7 min, isocratic on 0% (v/v) B; 6.7−12.5 min, linear gradient to 8% B; 12.5−24.2 min, linear gradient from 8 to 16% B; 24.2−41.8 min, linear gradient from 16 to 40% B; 41.8−43.5 min, linear gradient from 40 to 100% B; 43.5−50.5 min, isocratic on 100% B; 50.5−52 min, linear gradient from 100 to 0% B; 52−59 min, isocratic on 0% B.

MS analysis was performed on an LTQ Velos equipped with a heated ESI-MS probe coupled to RP-UHPLC. The spectra were acquired in an m/z (mass to charge ratio) range of 92−1000 Da in both positive (PI) and negative ionization (NI) modes. The PI mode was used only for identification of NAC−ITCs to complement the data from NI mode. Data-dependent MS^n analysis was performed on the most intense (product) ion with normalized collision energy of 35%. Nitrogen was used as sheath and auxiliary gas. The ion transfer tube temperature was 300 °C, and the source voltage was 4.0 kV (PI) or 4.5 kV (NI).

The identification and quantification of peaks were performed in Xcalibur (v.2.2, Thermo Scientific). The identification was based on UV and MS spectra. GSLs were detected in NI and their fragmentation pattern can be referred to in Andini et al.[37] NAC−ITCs have UV_{max} at 268 nm and were detected in NI and PI modes. The diagnostic fragment ion for NAC−ITCs was $[NAC\text{-}H]^-$ (m/z 162, the most abundant) in NI mode and $[NAC + H]^+$ (m/z 164, >15% abundance) in PI mode. The quantification of GSLs and NAC−ITCs was based on the response in NI mode. The quantification of 3-(methylthio)propyl GSL (3-MTGSL), 5-(methylsulfinyl)pentyl GSL (5-MSGSL), 2-hydroxy-4-pentenyl GSL (2-OH-PeGSL), 5-(methylsulfinyl)pentyl ITC (5-MSITC), and *p*-hydroxybenzyl ITC (*p*-OH-BITC) (i.e., the compounds without standards) was performed by using the calibration equation of the standard GSL or ITC from the same subclass with the closest structural resemblance and molecular weight (Table S1).

Linearity of the Calibration Curves. Calibration series of 14 GSLs and 15 ITCs (i.e., NAC−ITCs) were prepared at concentrations of 2 μM (for GSLs) or 3 μM (for ITCs) to 60 μM. The calibration curves, consisting of eight data points, were obtained by plotting concentration versus NI-MS chromatographic peak area of the analyte at m/z of its molecular ion. The linearity of the calibration curves was indicated by the coefficient of determination (R^2).

Limits of Detection and Quantification. The LOD was determined from the calibration curves.[38] The LOD is the smallest concentration of analyte in the test sample that can be reliably distinguished from zero.[39] The root mean square error (RMSE) approach was applied to calculate the LOD. According to Bernal & Guo,[38] this approach uses both the variability of the blank and of the measurement values. The following formulas were used to calculate the LOD

$$\mathrm{LOD} = \frac{3.3\ \mathrm{RMSE}}{a} \tag{1}$$

$$\mathrm{RMSE} = \left(\frac{\sum_{i=1}^{n}(y - y(x))^2}{n-2}\right)^{0.5} \tag{2}$$

where RMSE is the residual standard deviation of the calibration curve, a is the slope of the calibration curve, y is the measured peak area, $y(x)$ is the theoretical peak area calculated from the calibration equation, and n is the number of regression points. The LOD should meet the following two requirements: (i) LOD < C_{min}, and (ii) 10 × LOD > C_{min}. The LOQ was calculated according to Pilipczuk et al. and FDA.[31,40]

$$\mathrm{LOQ} = 3 \times \mathrm{LOD} \tag{3}$$

Precision. For the standard mixtures of 14 GSLs and 15 ITCs, the intraday precision was determined by replicate analysis (n = 3) at five concentrations ranging from 5 to 50 μM in one day. The interday precision was determined by replicate analysis in three separate days (n = 3). The standard mixtures were subjected to the NAC-derivatization. The intra- and interday precisions for the standard mixtures were expressed as a percentage of overall relative standard deviation (% RSD) of the NI-MS chromatographic peak areas of the analytes. The overall precisions (% RSD) were calculated using eq 4, where RSD_i (%) is RSD (i.e., coefficient of variation, CV) for the i-th concentration point, and n is the number of calibration points.[31]

For the spiked extracts of *S. alba*, *B. napus*, and *B. juncea*, the intra- and interday precisions were determined simultaneously: two replicates in day 1 and another two replicates in day 2. The extracts were spiked with a mixture of 14 GSLs and 15 ITCs (each analyte at 30 μM). The spiked extracts were subjected to the NAC-derivatization. The intra- and interday precisions for the spiked extracts were expressed as percentage of overall relative standard deviation (% RSD) of the concentrations of the spiked analytes and evaluated simultaneously by one-way ANOVA.[40,41]

$$\mathrm{RSD} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}\mathrm{RSD}_i^2} \tag{4}$$

Recovery. To further evaluate the applicability of the method for analyzing extracts, recovery was determined by analyzing the three different Brassicaceae seed extracts (i.e. *S. alba*, *B. napus*, and *B. juncea*), spiked with 14 GSL and 15 ITC standards (each at two levels: 10 and 30 μM), derivatized with NAC. Recovery (%) was calculated as

$$R(\%) = \frac{x_{\mathrm{spiked}} - x_{\mathrm{unspiked}}}{x_{\mathrm{ref}}} \times 100 \tag{5}$$

where x_{spiked} was the measured concentration of the analyte in the spiked experiment, $x_{unspiked}$ was the measured concentration of the

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analyte in the unspiked experiment, and x_{ref} was the true concentration which was spiked to the extract. Four repetitions were performed.

According to FDA,[40] a good recovery for the working analyte concentrations in this study should be between 80 and 110%. To test whether the recovery was within the range, the recovery whose average outside the range was statistically evaluated by analysis of variance (ANOVA) one-sample *t*-test using IBM SPSS Statistic v.23 software (SPSS Inc., Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Development of RP-UHPLC-PDA−ESI−MS^n Method for GSL and ITC Analysis. Simultaneous quantitative analysis of different GSLs and ITCs was performed using RP-UHPLC-PDA−ESI−MS^n. The method included derivatization of ITCs with NAC, which was based on the method developed by Pilipczuk et al.,[31] enabling detection of ITCs in MS. The method of Pilipczuk et al.[31] was modified to also enable analysis of very polar GSLs.

To ensure a good separation of the very polar GSLs, two important parameters were optimized. First was the column oven temperature as it influences the elution; the lower the temperature, the more delay in elution, which benefits the separation of polar compounds. Three different column oven temperatures, i.e. 25, 35, and 45 °C, were tested. At column oven temperature of 25 °C, sharp peaks were obtained and the seven most polar GSLs (**G1**, **G6**, **G13**, **G11**, **G2**, **G5**, and **G16**), which were eluted within 5 min, were separated (Figure 2A). Column oven temperature of 25 °C was also applied in previous research analyzing intact GSLs by LC−ESI−MS.[24] In contrast, higher column oven temperatures, which were 35 and 45 °C, resulted in poor separation for these seven most polar GSLs (data not shown). Column oven temperatures lower than 25 °C might improve the separation, but as this would extend the analysis time and cause high backpressure, this was not further elaborated.

Second, the polarity of the solvent is important for analyzing both GSLs and NAC−ITCs. All GSLs are soluble in water, but not all NAC−ITCs are soluble in water. IPA has been found to be better than methanol and ethanol to dissolve ITCs.[31] Furthermore, to dissolve both GSLs and ITCs in one mixture, 25% (v/v) aqueous IPA was found to be a better solvent than IPA 50%. This was indicated by the elution profiles of representative GSLs and ITCs in both solvents shown in Figure S1 (**B**, **D** vs **C**, **E**).

Figure 2A shows a good baseline separation of 15 NAC−ITC peaks (retention time, tR, of 17−40 min) in IPA 25%. Because quantification was based on the MS signal, coelution (e.g., **I4** and **I15**) and partial peak overlap (e.g., **G15** and **G8**) did not affect the quantification, as the compounds have different *m*/*z*.

Linearity of the Calibration Curves. The results of the regression analysis of 14 GSLs and 15 ITCs are listed in Table 2. Statistical analysis of the calibration data of GSL (2−60 μM) and ITC (3−60 μM) showed a high linearity ($R^2 \geq 0.994$).

Limits of Detection and Quantification. All LOD and LOQ values are presented in Table 2. The LOD values for GSLs and ITCs were within a range of 0.4−2.6 μM. With regard to GSL analysis, our LC−MS method has higher sensitivity than the method with CE-MEKC-UV (LOD up to 30 μM),[36] and this is in line with a previous study using the LC−MS method.[34] With regard to NAC−ITCs, our analytical protocol generated lower LOD values than those obtained by Pilipczuk et al.[31] (1.7−4.9 μM). The LOQ values of our method for GSLs and ITCs were within a range of 1.2−7.8 μM. Overall, our new analytical method can be applied for quantification of GSLs and NAC−ITCs at low concentrations.

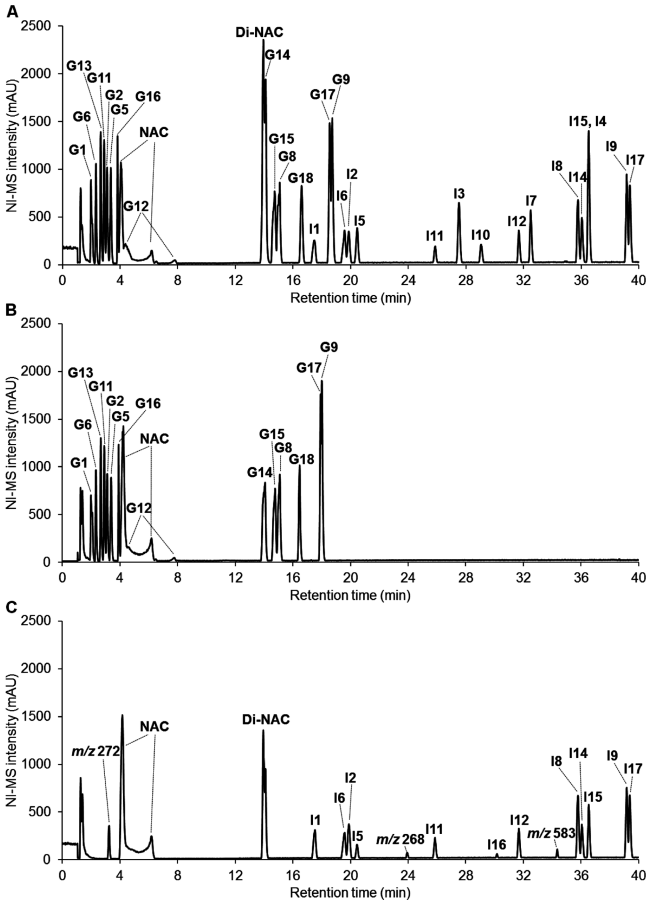

Figure 2. UHPLC-MS negative ion chromatograms of a mixture of 14 different GSLs (each 30 μM) and 15 different ITCs (each 30 μM) derivatized with NAC (2.25 mM) in 25% (v/v) aqueous IPA at 50 °C for 2 h (A); a mixture of 14 GSLs (each at 30 μM), myrosinase, and NAC upon incubation at 50 °C at 0 h (B) and 4 h (C). The chromatograms refers to base peak chromatograms. The analyses were performed at a column oven temperature of 25 °C. **G12** and NAC each appeared in two peaks, but the identification and quantification of the GSL were still feasible. The elution was depending on the solvent; in the absence of organic solvent, they were eluted as one peak (Figure S3).

Precision. The intraday and interday precisions of the developed method are displayed in Table 2. For the analyte concentration applied in our study, good intraday and interday precisions should be ≤10% RSD and 16% RSD, respectively.[40]

For GSL analysis in the standard mixtures, the intra- and interday precisions of the method for all GSLs (≤8.9% RSD and ≤14.8% RSD, respectively) complied with the FDA requirement.

For GSL analysis in the spiked extracts, the intraday and interday precisions of most GSLs (0.6−10.0% RSD and 2.0−16.0% RSD, respectively) were within the permitted FDA range. PeGSL (**G14**) and *p*-OH-BGSL (**G16**) had lower intra- and interday precisions than the other GSLs in the three spiked extracts.

For NAC−ITC analysis in the standard mixtures, the intra- and interday precisions of the method (7.2−10.0% RSD and 10.8−16.0% RSD, respectively) complied with the FDA

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Table 2. Validation Data of 14 Different GSLs and 15 Different ITCs Analyzed by RP-UHPLC-MS^n with Pre-Column NAC-Derivatization

					intra-day precision (% RSD)[b]				inter-day precision (% RSD)				recovery (%) (means ± SD)[c]		
code	analyte	R^2	LOD (μM)	LOQ (μM)[a]	SM	*Sa*	*Bn*	*Bj*	SM	*Sa*	*Bn*	*Bj*	*Sa*	*Bn*	*Bj*
							GSL								
G1	3-MSGSL	0.9989	1.3	3.9	5.9	3.9	8.8	10.3	8.0	4.2	9.1	10.6	93.3 ± 3.2	99.1 ± 8.7	84.9 ± 8.4
G2	4-MSGSL	0.9985	1.6	4.7	6.4	2.4	5.4	8.4	12.5	5.0	7.1	9.2	100.2 ± 5.7	108.6 ± 8.2	101.2 ± 10.0
G5	4-MS-3-en-GSL	0.9992	1.2	3.5	7.4	3.1	4.3	5.8	12.5	4.0	5.3	7.3	99.7 ± 4.3	110.1 ± 6.1	100.9 ± 7.9
G6	3-MSoGSL	0.9988	1.3	4.0	7.7	2.2	7.8	4.0	9.1	3.6	8.3	4.4	93.8 ± 3.7	106.2 ± 7.5	99.9 ± 4.6
G8	4-MTGSL	0.9989	1.3	3.8	7.4	3.2	4.6	7.7	14.8	3.5	5.6	10.0	93.3 ± 3.4	104.7 ± 6.2	94.9 ± 10.1
G9	5-MTGSL	0.9999	0.4	1.2	8.9	2.4	5.3	2.5	12.2	2.6	6.3	4.6	87.5 ± 2.3	102.8 ± 6.8	98.0 ± 5.0
G11	AGSL	0.9989	1.3	3.9	8.3	4.0	3.5	3.9	12.3	5.5	4.2	4.2	94.4 ± 5.6	101.8 ± 4.4	99.5 ± 8.6
G12	BuGSL	0.9983	1.6	4.9	4.9	7.6	8.9	9.0	13.2	8.4	9.3	9.4	107.2 ± 9.2	99.1 ± 4.3	96.9 ± 3.8
G13	2-OH-BuGSL	0.9998	0.7	2.0	8.0	3.2	4.5	9.6	10.4	3.6	5.8	10.8	93.4 ± 3.4	98.8 ± 3.6	97.5 ± 8.9
G14	PeGSL	0.9994	1.0	2.9	8.0	8.8	24.2	13.1	11.8	9.8	27.0	14.1	76.9 ± 5.6	71.0 ± 18.3	80.6 ± 9.4
G15	BGSL	0.9991	1.2	3.6	7.2	1.8	5.7	2.2	11.0	2.0	5.9	3.1	97.3 ± 2.0	107.3 ± 5.9	101.2 ± 3.3
G16	p−OH−BGSL	0.9999	0.6	1.9	7.7	9.8	10.5	19.8	13.7	10.9	11.6	22.1	94.8 ± 7.9	100.6 ± 8.9	89.1 ± 14.5
G17	PhEGSL	0.9992	1.1	3.4	7.4	0.6	8.5	3.4	11.5	2.1	8.8	4.5	96.4 ± 2.3	99.9 ± 8.0	99.5 ± 4.7
G18	I3MGSL	0.9999	0.6	1.8	7.3	2.7	7.4	3.9	10.2	9.7	8.2	5.3	108.9 ± 12.1	101.1 ± 6.3	95.6 ± 5.4
							ITC								
I1	3-MSITC	0.9978	1.4	4.2	8.2	4.0	7.8	5.7	12.1	5.3	14.3	6.3	99.6 ± 5.6	105.6 ± 16.7	111.6 ± 5.2
I2	4-MSITC	0.9982	1.7	5.2	10.0	3.8	9.3	5.2	14.6	11.5	13.7	5.6	99.8 ± 13.1	101.7 ± 15.1	110.9 ± 5.0
I3	6-MSITC	0.9952	1.3	3.8	9.2	3.0	13.8	6.1	17.9	3.3	15.3	6.8	96.1 ± 3.3	102.3 ± 16.1	108.6 ± 5.5
I4	9-MSITC	0.9996	0.9	2.8	9.0	3.6	18.4	5.0	12.9	3.8	18.5	5.1	99.1 ± 3.4	96.1 ± 17.6	105.3 ± 5.4
I5	4-MS-3-en-ITC	0.9981	1.4	4.2	8.2	2.8	8.7	6.2	15.6	5.5	12.9	6.9	101.0 ± 6.3	103.9 ± 14.5	110.1 ± 5.7
I6	3-MSoITC	0.9961	2.0	5.8	8.6	6.9	11.6	7.7	11.8	7.1	12.0	8.3	112.7 ± 7.4	121.9 ± 14.8	117.9 ± 8.0
I7	3-MTITC	0.9953	1.9	5.6	8.2	3.9	9.7	6.6	15.0	7.7	13.8	7.4	92.9 ± 8.0	95.6 ± 14.3	103.1 ± 5.7
I8	4-MTITC	0.9981	1.5	4.5	9.7	4.1	6.9	4.8	12.2	6.1	13.3	5.0	92.0 ± 6.1	92.0 ± 13.6	102.0 ± 4.6
I9	5-MTITC	0.9995	0.9	2.7	7.9	10.0	14.0	4.4	11.4	10.5	16.1	4.9	88.0 ± 8.2	86.0 ± 14.4	94.1 ± 4.7
I10	PITC	0.9947	1.9	5.5	11.9	13.6	16.4	9.3	16.5	14.7	20.7	10.3	66.0 ± 7.8*	74.9 ± 18.4	75.0 ± 5.8
I11	AITC	0.9970	1.9	5.8	9.2	6.8	13.6	6.0	12.3	7.6	15.9	6.5	95.7 ± 5.4	95.2 ± 15.7	97.0 ± 5.1
I12	BuITC	0.9991	1.3	3.8	9.5	10.7	11.4	6.1	11.8	10.8	16.4	6.8	80.3 ± 8.5	81.9 ± 13.0	85.1 ± 4.3
I14	PeITC	0.9936	2.6	7.8	10.5	8.3	11.4	4.5	12.3	9.2	16.2	5.0	72.5 ± 5.0	79.0 ± 8.1	78.1 ± 2.9
I15	BITC	0.9983	1.6	4.8	7.2	3.3	7.1	4.2	12.1	4.7	7.4	4.7	101.5 ± 5.2	102.6 ± 6.7	101.2 ± 3.6
I17	PhEITC	0.9978	2.1	6.2	8.9	8.7	12.3	4.6	10.8	9.0	15.8	5.2	110.0 ± 7.5	110.9 ± 15.0	105.6 ± 3.6

[a] R^2, LOD, and LOQ were obtained from a series of standard mixtures containing the GSLs and ITCs in 8 different concentrations within a range of 2−60 and 3−60 μM. [b] The intra- and inter-day precisions for standard mixtures (SM) were expressed as % RSD of the chromatographic peak areas of the analytes, whereas those for spiked extracts (*Sinapis alba* (*Sa*), *Brassica napus* (*Bn*), and *B. juncea* (*Bj*) seeds) were expressed as % RSD of the concentration of the analytes. [c] ANOVA one-sample *t*-test was performed for comparing means of % recovery of the lowest and the highest to the standard guideline (the min. 80% or the max. 110%) (FDA, 2015). *The recovery of NAC−PITC (**I10**) in *S. alba* seed extract was significantly lower than the minimum guideline (80%, $p < 0.05$). The rest of GSLs and ITCs in the three extracts had recovery within the range of the standard guideline.

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requirement for most ITCs. Only two ITCs had a slightly lower intra- and interday precision (up to 11.9% RSD and 17.9% RSD, respectively). The intraday precision of nine NAC−ITCs in a previous study was higher (1.8−5.0% RSD).[31] In our study, the calibration series of NAC−ITCs was made from the NAC-derivatization of the authentic standards of ITCs, and not from the purified or authentic standard NAC−ITCs as in Pilipczuk et al.[31] Therefore, the intraday precision obtained in our study reflected the variation not only of the actual LC−MS analysis, but also of the NAC-derivatization process.

For NAC−ITC analysis in the spiked extracts, the intraday precisions of the method tended to vary among extracts rather than among analytes. NAC−ITC analysis in the spiked *S. alba* and *B. juncea* extracts had intra- (2.8−10.0% RSD) and interday precisions (3.3−14.7% RSD) within the FDA requirement, except for the intraday precision of **I10** and **I12** in *S. alba* extract (up to 13.6% RSD). However, the analysis of 9 of 15 NAC−ITCs in the spiked *B. napus* extract had lower intraday precision (11.4−18.4% RSD) than the FDA requirement. This might be due to the presence of other low-molecular-weight thiol compounds (such as glutathione (GSH), the dominant thiol compound in most plant cells[42,43]) in the extract, which might compete with NAC in reacting with ITCs. Nevertheless, this competition was not expected in our study, because GSL extraction was done in absolute methanol where GSH is insoluble.[44] When the extraction is done in aqueous solvent, coextracting GSH, the level of this thiol compound in the extract should be considered. Despite this relatively low intraday precision of NAC−ITCs in the spiked *B. napus* extract, the interday precision of the majority (10 NAC−ITCs) complied with the FDA.

Recovery. The recovery of 14 GSLs and 15 NAC−ITCs spiked in *S. alba*, *B. napus*, and *B. juncea* extracts was evaluated. Two concentration levels were applied, which were 10 and 30 μM. The chromatograms of *B. napus* extracts, as a representative, are shown in Figure S4. All GSLs and ITCs added to the extract were distinguishable, and their intensity increased upon increasing concentration, except for **G12** and **G13** as they were constitutively present in high abundance (>80 μM, outside the range of concentrations used for calibration).

The recovery is the closeness of agreement between the true value of the analyte concentration and the experimental result.[40,41] According to FDA,[40] a good recovery for our working analyte concentrations should be between 80 and 110%.

The recoveries for GSLs ranged from 71 ± 18 to 110 ± 6% (Table 2), complying with the FDA requirement ($p > 0.05$). PeGSL (**G14**) had a tendency to have a lower recovery than the other 13 GSLs in all the three spiked extracts.

The recoveries of NAC−ITCs ranged from 66 ± 8 to 122 ± 15% (Table 2). NAC−PITC (**I10**) had a tendency to have a lower recovery, whereas NAC-3-MSoITC (**I6**) had a tendency to have a higher recovery than the other 14 NAC−ITCs in all three spiked extracts. Only NAC−PITC (**I10**) in *S. alba* extract had a recovery lower than the minimum FDA requirement (80%, $p < 0.05$). Pilipczuk et al.[31] found satisfactory recoveries (83−104%) for nine NAC−ITCs, including NAC−methyl ITC and NAC−ethyl ITC (shorter-chained analogues of PITC). Considering ITC's physicochemical properties, for example, boiling point and reactivity, we found no plausible explanation for the low recovery of PITC.

Overall, our method is well suited to quantify various GSLs and ITCs (as NAC−ITCs) in complex mixtures, such as plant extracts, with a high precision and recovery for most analytes. The method also offers an alternative way of making calibration series of NAC−ITCs, which is from the fresh NAC-derivatization of standard ITCs.

Detection of Various Enzymatic Hydrolysis Products of GSLs. At neutral conditions, most GSLs are degraded to form ITCs upon myrosinase treatment.[4−6] Our new analytical method was applied to monitor simultaneously the decrease of the concentration of 14 different standard GSLs and the increase of the concentration of their corresponding ITCs, in the form of NAC derivatives, during enzymatic hydrolysis. In our study, ITCs were the default rearrangement products of the aglucones (Figure 1) in the GSL extracts treated with the commercial myrosinase, supporting previous studies.[45,46] Figure 2B shows the presence of 14 GSLs before hydrolysis with myrosinase. Figure 2C indicates that after incubation with myrosinase and NAC at 50 °C for 4 h, all 14 GSLs were hydrolyzed, peaks corresponding to 12 NAC−ITCs and 3 unknown peaks appeared.

Upon myrosinase treatment 2-hydroxylated alkenyl GSLs and indolic GSLs form unstable ITCs which further form other types of products.[13,47] (*R*)-2-OH-BuGSL or progoitrin (**G13**) is known to form unstable 2-OH-3-butenyl ITC, which spontaneously cyclizes forming 5-ethenyl-1,3-oxazolidine-2-thione (i.e., goitrin)[47] (Figure S5A). However, the reaction product of a mixture of **G13**, myrosinase, and NAC is unknown in literature. Therefore, it remains unclear whether goitrin or the unstable 2-OH-3-butenyl ITC had reacted with NAC (Figure S5B,C), which might depend on the rates of cyclization of the ITC and of reaction between the ITC and NAC. In addition, the reaction product between goitrin and NAC has never been described in literature. A peak at a tR of 34.3 min and *m/z* of 583 (Figure 2C) might correspond to a dimer of reaction product between NAC and the hydrolysis product of **G13** with a molecular formula of $C_{20}H_{32}N_4O_8S_4$ (584 Da) (Figure S5D). The fragmentation gave an ion at *m/z* of 291 (possibly the monomer) at the most abundant, and this was fragmented to an ion at *m/z* of 162 (possibly the NAC). Meanwhile, indol-3ylmethyl (I3M) GSL (**G18**) forms various hydrolysis products, for example, indole-3-acetonitrile and I3M ITC, which further reacts with water to form indole-3-carbinol.[13,48,49] However, these compounds and their possible reaction products with NAC were not detected in our LC−MS analysis.

Furthermore, Figure 2C indicates two other peaks at 3.25 min (*m/z* 272) and 23.94 min (*m/z* 268), which were related to **G5** and **G16**, which were GSLs partially converted to their corresponding ITCs (**I5** and **I16**) (Table 3). The presence of the first peak was never indicated previously, and the annotation requires structural elucidation by NMR spectroscopy. A previous study found that **G5** was hydrolyzed to form **I5**, which was unstable and rapidly converted to a water-soluble degradation product, namely 6-[(methylsulfinyl)-methyl]-1,3-thiazinan-2-thione, at 25 °C, pH 7.[50] However, this compound was not detected in our LC−MS analysis. The second peak was tentatively annotated as $C_{12}H_{15}NO_4S$ (269 Da), which was possibly an ester from *p*-OH-benzyl alcohol and NAC (Figure S6), and observed after 3-h incubation. **G16** formed not only **I16** (13% conversion, Table 3), but also *p*-

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Table 3. Rates (μM/h) of Enzymatic Hydrolysis of GSLs and Formation of NAC−ITCs from ITCs Released upon Hydrolysis and Simultaneous NAC-Derivatization, and Conversion of GSLs to ITCs

		hydrolysis of GSL		formation of NAC−ITC		
GSL or ITC	R-group[a]	rate	R^2	rate	R^2	conversion (%)
subclass (methylsulfinyl)alkyl (MS)						
G1, I1	3-MS	13.6	0.981	11.7	0.968	100
G2, I2	4-MS	11.2	0.987	10.7	0.922	100
subclass (methylsulfinyl)alkenyl (MS-en)						
G5, I5	4-MS-3-en	11.9	0.986	7.4	0.945	63
subclass (methylsulfonyl)alkyl (MSo)						
G6, I6	3-MSo	17.3	0.966	10.4	0.965	100
subclass (methylthio)alkyl (MT)						
G8, I8	4-MT	18.4	0.982	10.1	0.952	100
G9, I9	5-MT	19.7	0.991	9.7	0.968	100
subclass alkenyl						
G11, I11	A	20.6	0.990	11.1	0.959	100
G12, I12	Bu	25.4	0.994	9.0	0.866	100
G13, n.a.	2-OH-Bu	28.4	0.999	n.a.[b]	n.a.	n.a.
G14, I14	Pe	20.3	0.966	9.3	0.870	100
subclass benzenic						
G15, I15	B	26.9	0.989	9.1	0.809	100
G16, I16	*p*-OH-B	29.7	0.959	n.d.[c]	n.d.	13
G17, I17	PhE	20.8	0.985	11.0	0.919	100
subclass indolic						
G18, n.a.	I3M	25.7	0.983	n.a.	n.a.	n.a.

[a]R-groups are presented in the abbreviations, referring to Table 1. [b]n.a. stands for not available, due to the degradation products were not ITCs. [c]n.d. indicates that the concentration of the compound cannot be determined due to lack of standard. Therefore, the conversion (13%) was calculated by taking the concentration BITC equivalent for this *p*-OH-BITC.

OH-benzyl alcohol, which is in line with the finding of Buskov et al., confirming the structure by NMR spectroscopy.[51]

Overall, our results underline that the amount of ITCs formed upon hydrolysis of GSLs does not necessarily equal that of hydrolyzed GSLs, and this is influenced by the side chain. Therefore, the amount of ITCs formed upon hydrolysis of GSLs should be determined appropriately.

Quantitative Monitoring of the Enzymatic Conversion of Standard GSLs to NAC−ITCs. Figure 3A (no organic solvent) and 3B (25% IPA) demonstrate the progress of hydrolysis of an aliphatic GSL (**G1**) and an aromatic GSL (**G15**), as representatives, and the progress of NAC-derivatization of the corresponding ITCs (**I1** and **I15**). The graphs for the other GSLs and NAC−ITCs are displayed in Figure S7. In general, the hydrolysis of all GSLs at 50 °C, pH 7.0, occurred to completion at 1.5−2.5 h, whereas the complete conversion to their respective NAC−ITCs lasted longer (2.5−3.5 h). Table 3 shows the rates of GSL hydrolysis and NAC−ITC formation for all tested compounds. The hydrolysis rates of the 14 GSLs were within 11.2−29.7 μM/h. A previous study using the same commercial *S. alba* myrosinase, but different incubation temperature (37 °C), found much higher hydrolysis rates (369−800 μM/h).[35] The lower hydrolysis rates found in our study in comparison with this previous study[35] were probably because we used a lower concentration of myrosinase (0.05 U/mL vs 1.10−14.52 U/mL). The formation rates of NAC−ITCs were within 7.4−11.7 μM/h. The slower rate of NAC−ITCs formation compared to GSL hydrolysis might be explained by the effect of solvent as the solubility of ITCs are favored in organic solvent. Figure 3B indicates that when the hydrolysis of GSLs and the formation of NAC−ITCs co-occurred in IPA 25%, the rates were comparable. However, the hydrolysis of 3-MSGSL in IPA 25% was not fully accomplished in 4 h (Figure 3B). Therefore, the rest of experiments on the simultaneous enzymatic hydrolysis of GSLs and NAC−ITC formation occurred in the absence of IPA.

Based on the hydrolysis rates (Table 3), the alkenyl, benzenic, and indolic GSLs were the most preferred substrates of myrosinase (hydrolysis rate of 20.3−29.7 μM/h), followed by (methylthio)alkyl and (methylsulfonyl)alkyl GSLs (17.3−19.7 μM/h). The least preferred substrates were (methylsulfinyl)alkyl and (methylsulfinyl)alkenyl GSLs (11.2−13.6 μM/h). In our study, the myrosinase used was originally isolated from *S. alba* seed. *p*-OH-BGSL (**G16**) is the most abundant GSL in this seed and had the highest hydrolysis rate (29.7 μM/h). Our finding supports previous studies,[36,52] indicating that myrosinase acts more efficiently on the most abundant GSL present in the plant, to which both myrosinase and GSL belong.

Enzymatic Conversion of Constitutive GSLs to ITCs in Plant Extracts. To further test the applicability of our method, simultaneous enzymatic hydrolysis of GSLs and NAC-derivatization of ITCs was also performed in the three Brassicaceae seed extracts. The condition of this hydrolysis was similar to that of the hydrolysis of standard GSLs in our study (50 °C, buffer pH 7.0), except for the presence of DMSO 5% to assist the solubilization of the extracts. Under this condition, all GSLs were hydrolyzed completely within 4 h (similar to the experiments without DMSO; data not shown).

Figure 3C−E indicates the concentration (in logarithmic scale) of GSLs in extracts of each species before hydrolysis and of NAC−ITCs after 4 h of hydrolysis. The predominant GSLs in *S. alba*, *B. napus*, and *B. juncea* seed extracts at 5 mg/mL were *p*-OH-BGSL (**G16**) 1857 μM, 2-OH-BuGSL (**G13**) 128 μM, and BuGSL (**G12**) 2012 μM, respectively, which equaled 61, 4, and 66 μmol/g DW seed, respectively. The result was in agreement with a previous study.[37]

For GSLs which form only ITCs upon myrosinase treatment in neutral pH solution, for example, 3-MSGSL (**G1**), 4-MTGSL (**G8**), AGSL (**G11**), and BuGSL (**G12**), the concentration of ITCs formed was comparable to that of the corresponding GSLs (Figure 3C−E), consistent with our results from the hydrolysis of standard GSLs (Figures 3A and S7). *p*-OH-BGSL (**G16**) partially formed ITC (Figure 3C), two hydroxylated alkenyl GSLs (i.e., 2-OH-BuGSL **G13**, 2-OH-PeGSL **G20**) and I3M GSL (**G18**) formed no ITC (Figure 3C−E), in accordance with the findings obtained in the hydrolysis of standard GSLs (section Detection of Various Enzymatic Hydrolysis Products of GSLs).

Our method enabled analysis of plant extracts with different GSL and ITC compositions. If the plant extract would contain excessive amounts of ITC, then the amount of NAC for derivatization might become limiting. In such a case, several dilutions of the plant extract should be prepared, while keeping the concentration of NAC constant, for example, 2.25 mM (as applied in the spike experiments), to confirm that the amount of NAC is sufficient to react with all ITCs.

Several methods to analyze GSLs and ITCs simultaneously were developed previously,[33−36] some of which were with high

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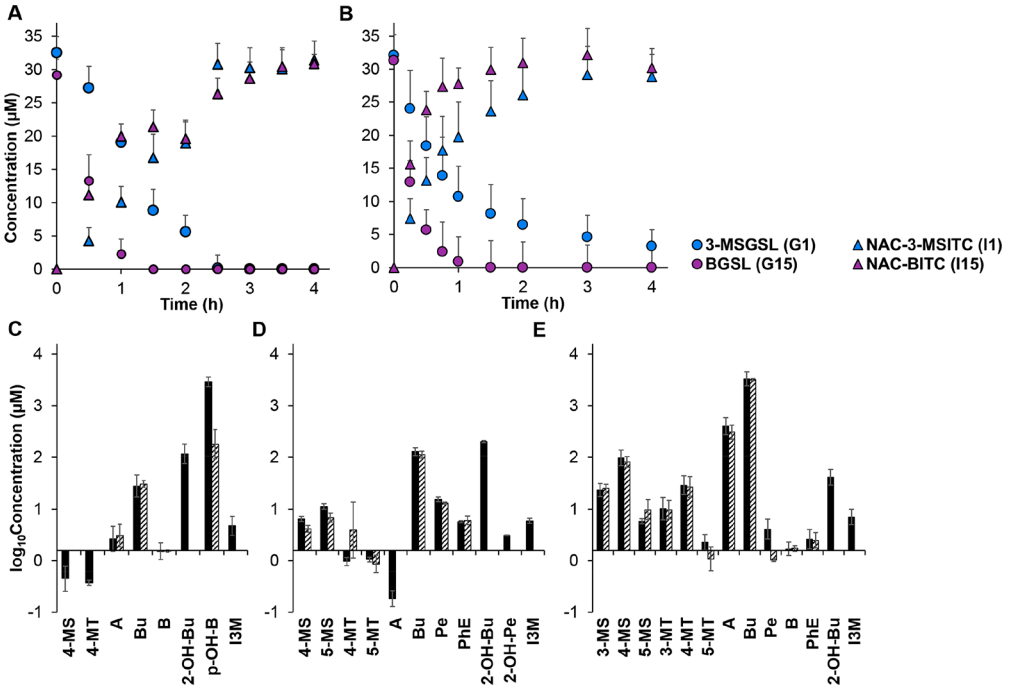

Figure 3. Simultaneous enzymatic hydrolysis of GSLs and NAC-derivatization of ITCs that were released: the decrease in concentration of GSLs (circles) and the increase in concentration of NAC−ITCs (triangles) during enzymatic hydrolysis of standard GSLs in the absence of organic solvent (A), in IPA 25% (B); concentration (in logarithmic scale) of constitutive GSLs (filled bars) before hydrolysis and their corresponding ITCs (hatched bars) in *S. alba* (C), *B. napus* (D), and *B. juncea* (E) seed extracts (5 mg/mL) after 4 h hydrolysis at 50 °C. Hydroxylated aliphatic GSLs (2-OH-BuGSL and 2-OH-PeGSL) and indole GSL (I3MGSL) did not form ITCs upon myrosinase treatment. *p*-OH-BITC was not the only hydrolysis product of *p*-OH-BGSL (**G16**). The error bars are the standard deviations for the means, taken from three independent experiments.

sensitivity and precision.[34,36] In our study, we have developed an RP-UHPLC−ESI−MS^n method able to analyze: (i) a more extensive set of GSLs and ITCs simultaneously comprising eight different subclasses, most of them with representatives of different chain lengths; (ii) complex mixtures, for example, plant extracts, by MS detection (more specific than UV detection); (iii) GSLs and ITCs directly after hydrolysis and NAC-derivatization, that is, simultaneous hydrolysis and analysis. Overall, this method is valuable for understanding the in vitro enzymatic conversion of GSLs to ITCs under various conditions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.9b07920.

Base peak negative ion UHPLC−MS chromatograms of representative GSLs and ITCs in different solvents; base peak negative ion UHPLC−MS chromatograms of NAC-ITCs; base peak negative ion UHPLC−MS chromatograms of NAC in the absence of an organic solvent; base peak negative ion UHPLC−MS chromatograms of extracts of *B. napus* seeds, nonspiked and spiked with standards of 14 GSLs and 15 ITCs at different concentrations; hydrolysis schemes of (*R*)-2-OH-3-butenyl GSL and *p*-OH-benzyl GSL; decrease in concentration of GSLs and increase in concentration of ITCs; and GSLs and NAC-ITCs whose calibration curves were employed to quantify analytes (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Jean-Paul Vincken − *Laboratory of Food Chemistry, Wageningen University, 6700 AA Wageningen, The Netherlands;* orcid.org/0000-0001-8540-4327; Phone: +31 317 482234; Email: jean-paul.vincken@wur.nl

Authors

Silvia Andini − *Laboratory of Food Chemistry, Wageningen University, 6700 AA Wageningen, The Netherlands; Department of Chemistry, Satya Wacana Christian University, Salatiga 50711, Indonesia*

Carla Araya-Cloutier − *Laboratory of Food Chemistry, Wageningen University, 6700 AA Wageningen, The Netherlands*

Mark Sanders − *Laboratory of Food Chemistry, Wageningen University, 6700 AA Wageningen, The Netherlands*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.9b07920

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