

Analytical Methods

Simultaneous determination of individual isothiocyanates in plant samples by HPLC-DAD-MS following SPE and derivatization with N-acetyl-L-cysteine

Tadeusz Pilipczuk^a, Barbara Kusznierevicz^a, Tomasz Chmiel^a, Witold Przychodzeń^b,
Agnieszka Bartoszek^{a,*}

^a Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland ^b Department of Organic Chemistry, Faculty of Chemistry, Gdańsk University of Technology, Narutowicza 11/12, 80-233 Gdańsk, Poland

abstract

The procedure for the isothiocyanates (ITCs) determination that involves derivatization with N-acetyl-L-cysteine (NAC) and separation by HPLC was developed. Prior to derivatization, plant ITCs were isolated and purified using solid-phase extraction (SPE). The optimum conditions of derivatization are: 500 μ L of isopropanolic eluate obtained by SPE combined with 500 μ L of derivatizing reagent (0.2 M NAC and 0.2 M NaHCO₃ in water) and reaction time of 1 h at 50 °C. The formed dithiocarbamates are directly analyzed by HPLC coupled with diode array detector and mass spectrometer if required. The method was validated for nine common natural ITCs. Calibration curves were linear ($R^2 \geq 0.991$) within a wide range of concentrations and limits of detection were below 4.9 nmol/mL. The recoveries were in the range of 83.3–103.7%, with relative standard deviations <5.4%. The developed method has been successfully applied to determine ITCs in broccoli, white cabbage, garden cress, radish, horseradish and papaya.

Keywords: Isothiocyanates, N-acetyl-L-cysteine, Dithiocarbamates, HPLC-DAD-MS SPE, Brassicaceae

1. Introduction

Isothiocyanates (ITCs) are regarded as the most biologically active breakdown products of glucosinolates – secondary metabolites of plants, mainly from *Brassicaceae* family. Upon plant tissue damage induced by pest attack or other disruption events, e.g. cutting or chewing, glucosinolates (GLs) undergo hydrolysis catalyzed by the enzyme myrosinase (β -thioglucoside glucohydrolase, EC 3.2.3.1) to the unstable intermediate which rearranges further to ITCs and related products, such as nitriles, epithionitriles, indoles, thiocyanates or oxazolidine-2-thiones (Mithen, Armah, & Traka, 2011). The formation of specific products depends on the variety of factors, including metal ions, pH, protein cofactors and side chain structure (Wittstock & Burow, 2007). Among GL hydrolysis products, ITCs ensure the most effective barrier against plant pathogenic microorganisms (Aires et al., 2009). This group of compounds triggers also several biological activities that discourage herbivore attacks and thus represents environment friendly biopesticides that can be used in biofumigation process

(Kusznierevicz et al., 2012; Pilipczuk, Piekarska, Kusznierevicz, Bartoszek, & Namieśnik, 2013). However, the feature of ITCs that warranted them the position of the most investigated phytochemical family is their ability to reduce the incidence and progression of human cancers and to prevent inflammation (Mithen et al., 2011). The chemopreventive effect of ITCs involves multiple mechanisms that include inhibition of phase I cytochrome P450 enzymes, stimulation of phase II detoxification enzymes, ceasing inflammatory reactions, inhibition of angiogenesis, induction of cell cycle arrest and apoptosis of tumor cells (Navarro, Li, & Lampe, 2011). Importantly, numerous epidemiological studies have demonstrated the high potency of ITCs in prevention of lung, prostate, breast, bladder, colorectal, pancreatic and other less frequent human cancers (Higdon, Delage, Williams, & Dashwood, 2009).

Equally important are synthetic ITCs, which are widely applied as valuable starting materials for a wide range of chemical reactions. For instance, they are used for synthesis of glycolipids with thiourea- or urea-linkers (Mathiselvam, Loganathan, & Varghese, 2013), 1,3,5-triazine derivatives needed in organic chemistry and medicinal research (Li, Tu, Jiang, Wang, & Tu, 2013) or other compounds with important functional groups such as thiosemicarbazides (Pandurangan, Kitchen, McCabe, & Gunnlaugsson, 2013),

isocyanides (Bhat, Allan, & Rawal, 2011), and guanidines (Smith et al., 2007).

Despite the fact, that ITCs are broadly employed compounds due to their promising chemopreventive properties, application in chemical industry or agriculture, e.g. in crop protection, there is still an open problem of fast and reliable methods for qualitative determination of the content of this class of compounds in samples of various origin. Volatility of ITCs suggests that gas chromatography (GC) should be the most appropriate technique for their analysis. Accordingly, there have been proposed methods utilizing the gas chromatograph coupled with thermoionic-specific detector (TSD) (Trott, Lepage, & Hebert, 2012), flame ionization detector (FID) (Hansch et al., 2012) or mass spectrometer (MS) (Aissani et al., 2013; Matich et al., 2012). Unfortunately, GC has in this case one unavoidable disadvantage, i.e. requirement of high temperature to volatilize analytes. ITCs are not stable at very high temperatures and during GC analysis up to 80% of these compounds may be degraded or transformed into new ITCs, while passing through the GC column (Chen & Ho, 1998; Chiang, Pusateri, & Leitz, 1998). Therefore, results obtained by such methods depend on the composition of ITCs in the samples and the content of certain components may be frequently either over- or underestimated. Liquid chromatography could have solved this problem, because separation of the analytes does not require high temperatures of the column, so several attempts to establish HPLC method of ITCs determination have been undertaken. The most common HPLC-UV-vis method enabling quantification of the total content of ITCs utilizes the reaction of cyclocondensation with 1,2-benzenedithiol to coloured derivative (Zhang, Wade, Prestera, & Talalay, 1996). Unfortunately, this method leads to a single derivatization product, thus provides only information about ITC content, but not their composition in a sample analyzed. Moreover, contaminants such as dithiocarbamates, carbon disulphide and related thiocarbonyl compounds also undergo cyclocondensation reaction with 1,2-benzenedithiol (Zhang et al., 1996) and may lead to overestimation of ITC concentration. As dithiocarbamates are worldwide applied bacteriocides or fungicides (Wesseling, McConnell, Partanen, & Hogstedt, 1997), this method should not be used when ITC content in plants originated from protected fields are to be analyzed. Carbon disulphide is a common reactant in the synthesis of ITCs (Psurski et al., 2012), so yet another potential application of this method, namely monitoring of reactants during organic synthesis, is excluded. Other approaches involving liquid chromatography and precolumn derivatization that enable determination of individual ITCs in different matrix include the use of: ammonia (Agerbirk, De Nicola, Olsen, Müller, & Iori, 2015), mercaptoethanol (Wilson et al., 2011) and *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (Budnowski et al., 2013) as derivatization reagents. However, the proposed methods have been applied to certain types of sample matrix and focused on the determination of only few selected ITCs. Additionally, these analytical procedures have not been optimized as regards sample preparation step for purification, concentration and efficient isolation of target ITCs, which results in rather low sensitivity of these methods.

The purpose of our study was to develop and validate analytical approach that involves purification of the analytes with SPE technique, derivatization of ITCs in the reaction with *N*-acetyl-L-cysteine (NAC) and subsequent HPLC-DAD-MS quantitation and identification of dithiocarbamates formed. The concept of this method has been based on human metabolism of ITCs occurring via mercapturic acid pathway, where initially formed glutathione conjugates are subsequently transformed into dithiocarbamates called also ITC-NAC conjugates (Shapiro, Fahey, Wade, Stephenson, & Talalay, 2001; Zhang, Kolm, Mannervik, & Talalay, 1995), which are the final detoxification products released from the organism. In this study, the applicability of the proposed

NAC-derivatization approach was tested with an array of commercially available ITCs: sulforaphane, methyl, ethyl, allyl, phenyl, phenylethyl, benzyl and 4-(methylthio)butyl isothiocyanate – representative compounds of substantial importance in industry, agriculture and medicine, as well as with real brassica plant samples. We demonstrate that the proposed method enables efficient simultaneous qualitative and quantitative determination of ITCs in complex samples.

2. Materials and methods

2.1. Materials and reagents

Methyl isothiocyanate (MITC), allyl isothiocyanate (AITC), phenyl isothiocyanate (PITC), benzyl isothiocyanate (BITC), phenylethyl isothiocyanate (PEITC), 3-(methylthio)propyl isothiocyanate (3-MTPITC), *D,L*-sulforaphane (SFN), *N*-acetyl-L-cysteine (NAC), 1,2-benzenedithiol (BDT), sodium bicarbonate, HPLC grade methanol and isopropanol were purchased from Sigma-Aldrich (Germany), ethanol and formic acid from Merck (Germany). LC-MS grade acetonitrile was obtained from VWR (USA). High purity water was produced in-house using a Milli-Q System (18.2 M Ω /cm). All other reagents were of analytical grade. Bakerbond SPE Octadecyl C₁₈ (500 mg, 3 mL) cartridges were obtained from J.T. Baker (Greisheim, Germany). 4-(Methylthio)butyl isothiocyanate (4-MTBITC), and the conjugates of NAC with ITCs: AITC-NAC, PITC-NAC, BITC-NAC and PEITC-NAC were synthesized at the Department of Organic Chemistry, Gdańsk University of Technology. Their structures were characterized by chemical and spectroscopic methods (UV, NMR, MS). As judged by HPLC-DAD ($\lambda = 254$ nm), purities of AITC-NAC, PITC-NAC, BITC-NAC and PEITC-NAC stored at -20 °C for at least 6 months were 99.21, 94.36, 99.39, 99.38%, respectively.

2.2. Chemical synthesis and characterization of standards

The details of chemical synthesis, structural data and chromatographic characterization of standard dithiocarbamates are presented in the [Supplementary Material](#) section accompanying this paper. The following synthetic conjugates of NAC with ITCs were used throughout the study as standards for selection of optimal HPLC conditions: AITC-NAC, BITC-NAC, PEITC-NAC and PITC-NAC.

2.3. Plant material

Horseradish (*Armoracia rusticana*, syn. *Cochlearia armoracia*), white cabbage (*Brassica oleracea* var. *capitata* f. *alba*), garden cress (*Lepidium sativum*), radish (*Raphanus sativus*) and papaya fruit (*Carica papaya* L.) were obtained from a local store and Beneforte broccoli florets (*Brassica oleracea* L. var. *italica*) were obtained from Tesco supermarket (Ireland). Vegetables were freeze-dried, ground and stored at -20 °C until investigation.

2.4. Extraction of ITCs by SPE

An aliquot (10 mL) of a water solution of ITC (0.1 mM) or plant water extracts were added to the Bakerbond C18 column preconditioned with 3 mL of MeOH and 3 mL of water. After sample loading, the stationary phase was dried under the stream of air (5 min). Then, the analytes were eluted with 1.0 mL of isopropanol. Among three tested solvents (methanol, ethanol, isopropanol), the isopropanol was chosen as the most efficient for the washout of retained ITCs.

2.5. Determination of the total content of isothiocyanates

The total content of ITCs was determined by the method described previously by Zhang et al. (1996) and utilizing the reaction of 1,2-benzenedithiol (BDT) with ITCs. The sample containing ITCs (standard solutions or plant extracts) (0.1 mL) was added to the reaction mixture consisting of 0.1 M potassium phosphate buffer pH 8.5 (0.5 mL), isopropanol (0.5 mL) and 60 mM 1,2-benzenedithiol in 2-propanol (0.1 mL). The mixture was incubated for 60 min at 65 °C to accomplish the cyclocondensation reaction. The content of 1,3-benzenedithiole-2-thione (BDTT) was determined by Agilent 1200 system (Agilent Technologies, USA) coupled with diode-array detector (DAD). The reaction mixture (30 µL) was injected onto Kinetex PFP 100A column (150 mm × 4.6 mm, 5 µm) (Phenomenex, USA). The mobile phase contained 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B); the flow rate was set at 1 mL/min. The gradient program for elution of BDTT was as follows: initial increase from 60% to 100% B within 12 min and then column equilibration for 5 min at 60% B. Chromatograms were recorded at 365 nm. The calibration curves used for quantification of total ITC in plant samples were generated by the integration of the area of absorption peak of BDTT determined during analyses of serial dilutions of PEITC standard (0.1–1 µmol/mL) mixed and incubated with BDT.

2.6. Chromatographic analysis of ITC-NAC conjugates

All analyses were performed with an Agilent 1200 HPLC, using a 150 mm × 4.6 mm id., 5 µm, Kinetex PFP 100A (Phenomenex, USA) column protected by a guard cartridge of the same material, connected to Agilent 1200 diode array detector (DAD) and to 6130 quadrupole mass spectrometer (Agilent Technologies, USA) with electrospray ionization in positive ion mode. The solvents were: 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B), with a flow rate of 1 mL/min. The linear gradient for solvent B was as follows: 0 min, 5%; 15 min, 40%; 20 min, 65%; 25 min 100%; followed by 4 min of column equilibration with 5% B. An injection volume of 10 µL was used. The analytes were traced at 272 nm. After the DAD detector, the eluent flow was guided to 6130 quadrupole mass spectrometer (Agilent Technologies, USA) with electrospray ionization ion source (ESI). Ionization in the ESI mode was performed, using a drying gas temperature of 350 °C, a drying gas flow of 12 L/min, a nebulizer pressure of 50 psi and a capillary voltage of 3000 V. Identification of dithiocarbamates was performed in positive ionization modes by identifying the parent ion $[M + H]^+$.

2.7. Derivatization of ITCs with NAC

The derivatization solution was prepared by dissolving of NAC and NaHCO₃ in water to obtain final concentration 0.2 M of each. Then, 500 µL of derivatization solution was combined with 500 µL of either isopropanolic solution of ITC standards or SPE plant extracts. The reaction mixtures were incubated for 1 h at 50 °C to accomplish the derivatization. After incubation, the samples were chilled and injected into HPLC system.

2.8. Analyses of plant samples

To prepare water extracts, 400 mg of lyophilized plant material was mixed with 10 mL of water and incubated for 3 h at 37 °C to enable myrosinase catalyzed conversion of GLs into ITCs to complete. Then, the samples were centrifuged (5000 rpm, 15 min, 4 °C; Eppendorf, Microcentrifuge 5415 R, Germany). Collected supernatants were subjected to SPE procedure described in details in Section 2.6. The ITCs in obtained eluates were derivatized in

parallel with NAC or BDT according to the procedures described in Sections 2.7 and 2.5, respectively. The derivatization products, that is NAC-ITCs or BDTT, were determined with the use of HPLC under conditions described in Sections 2.6 and 2.5, respectively.

The peaks of NAC-ITC were identified by comparison of retention times and UV and mass spectra with those of authentic reference substances (when available), while for the rest tentative identification was performed on the basis of UV and MS spectra and literature data documenting the presence of specific parent GLs in plant tissue studied. The quantification of the analytes for which standards were available was performed with external calibration curves, whereas for other identified analytes, the measured chromatographic area was substitute into the calibration equation of the reference standards with similar structure.

3. Results and discussion

3.1. ITC purification from complex matrix

There are two major challenges that may prevent reliable determination of ITCs by chromatographic techniques: low concentration of these phytochemicals in complex plant or another matrix, as well as high content of other compounds that may interfere during analysis. In our earlier study on indolic components, which similarly to ITCs can be formed as a result of glucosinolate (GL) degradation, we have successfully overcome such problems by applying solid-phase extraction (SPE) for purification and concentration of required analytes prior to HPLC determination (Pilipczuk, Dawidowska, Kusznierevicz, Namieśnik, & Bartoszek, 2015). In the case of GL derivatives, the plant extracts for determination of indoles and ITCs are prepared in the same way. This strengthened the hypothesis of SPE technique suitability for ITC purification. To verify our presumptions, 10 mL of water solution containing one of ITC standards at a concentration of 0.1 µmol/mL (MITC, SFN, EITC, AITC, 3-MTPITC, PITC, 4-MTBITC, BITC or PEITC) was loaded onto the Bakerbond C18 column preconditioned according to manufacturer's recommendations with 3 mL of methanol and then 3 mL of water. Each standard analyte was eluted with 1.0 mL of one of tested solvents, which included commonly used methanol, ethanol or isopropanol. The concentration of ITC moieties in the eluates was determined by Zhang's method as described in Section 2.5 of Materials and Methods. Isopropanol used as the eluting medium ensured the most efficient extraction of ITCs from the sorbent with recoveries of analytes falling in the range of 86–106%, while ethanol in the range 76–97%. Methanol gave less satisfactory results; the recoveries were in the range of 62–81%. For the purpose of further experiments, isopropanol was selected as an eluting medium also for another reason described in the next section, i.e. high yield of ITC derivatization achieved in its presence.

3.2. Development of HPLC-DAD-MS conditions

Zhang et al. (1996) proposed mentioned earlier derivatization of ITCs to stable 1,3-benzenedithiole-2-thione (BDTT), which displays high absorption at 365 nm ensuring sensitive quantitative spectrophotometric detection. Unfortunately, their method gives only quantitative information about the total content of ITCs in the samples. In many cases, such information is sufficient, but taking into account diversified biological activity of ITCs, including those present in food, the qualitative composition seems at least equally important. Therefore, the attempts have been repeatedly undertaken to develop HPLC method enabling both qualitative and quantitative determination of these compounds. We decided to convert ITCs into the chemical form retaining structure of the ITC side chain and known to be more suitable for HPLC analysis than parent

compounds. The proposed way of derivatization follows the natural route via which these compounds are metabolized to be released from the organism (Janobi et al., 2006). This metabolic process is initiated by ITC reaction with cysteine built into GSH. The initial conjugate is then transformed into ITC-NAC dithiocarbamate on the mercapturic acid pathway constituting the final stage of phase II detoxification. It can be presumed that naturally occurring ITC detoxification pathway should rely on the efficient and relatively rapid chemical process. Dithiocarbamates derived from common plant ITCs proved very stable, did not degrade when heated nor when exposed to extreme pH (data not shown). Moreover, the reaction of PITC with amino acids has been long known to proceed with good yield, in e.g. protein sequencing by Edman's degradation, even though in the latter case, it involves binding with amino group which is less nucleophilic than -SH group.

However, before exploiting derivatization to dithiocarbamates as a part of analytical protocol of ITC determination, it was necessary to examine the efficiency of chromatographic separation of ITC-NACs containing different side chains. This part of the study was carried out with the aid of synthetic ITC-NAC conjugates: AITC-NAC, PITC-NAC, BITC-NAC and PEITC-NAC. The conditions of synthesis of ITC-NAC conjugates, their MS and NMR spectra are given in [Supplementary Material](#). These data confirmed both the high purity of synthesized dithiocarbamates, as well as their chemical structures. The methodology of chromatographic separation of dithiocarbamates was initially inspired by reports concerning PEITC-NAC determinations in human urine published by Vermeulen, Zwanenburg, Chittenden, and Verhagen (2003), but the final conditions were substantially modified. The four synthetic ITC derivatives were separated using a Kinetex PFP 100A column and water/acetonitrile gradient elution. The addition of formic acid to mobile phase turned out to improve greatly chromatographic separation providing sharper peak shapes and better peak symmetry for all ITC-NAC conjugates tested. The optimized parameters of gradient elution (given in Section 2.6 of Materials and Methods) ensured proper separation of the analytes with divergent chemical structures. The UV spectra were collected in the range of 220–400 nm ([Fig. S-12; Supplementary Material](#)). Since all tested compounds showed high response at 272 nm, this wavelength was finally selected for their monitoring during HPLC analysis. Additionally, the identity of peaks was confirmed by mass spectrometry ([Fig. S-12; Supplementary Material](#)).

3.3. ITC derivatization to ITC-NAC dithiocarbamates

The initial stage of derivatization method development embraced testing of the impact of reaction conditions on the yield of binding of purified ITCs to NAC. In order to shift the reaction equilibrium towards the formation of ITC-NAC conjugates, we decided to apply the large excess of NAC (0.2 M) in relation to the typical content of ITCs (0.02 mM) that can be expected in water extracts obtained from *Brassicaceae* plants (Piekarska et al., 2014). In addition, we included sodium bicarbonate (0.2 M) into the reaction mixture, so as NAC occurs in the form of sodium salt, which is easily soluble in water. Such an approach was previously reported to increase the rate of dithiocarbamate formation (Vermeulen et al., 2003).

This step of the method development focused also on the evaluation of the impact of the type of organic solvent present in the reaction mixture on the derivatization reaction kinetics and efficiency. In the [Fig. 1A–C](#), the progress of the derivatization of nine ITCs to NAC-ITC in reaction mixture containing different solvents monitored at ambient temperature with 30 min intervals is presented. After reaction, the ITC-NAC conjugates were analyzed using HPLC-DAD technique (see Section 2.6). The obtained results indicate that target products are formed faster in reaction mixture con-

taining ethanol than methanol ([Fig. 1A and B](#)). However, the highest concentrations of NAC-ITCs were observed in the presence of isopropanol ([Fig. 1C](#)). Therefore, isopropanol was selected as the most suitable component of the reaction mixture. This finding indicates that isopropanolic extracts obtained during described earlier SPE procedure can be directly used for the derivatization process.

In the case of isopropanol, the decrease in ITC concentration during derivatization could be described by a pseudo-first order reaction (Eqs. (1) and (2)). Since NAC was added in great excess (200 fold), its concentration was assumed to remain constant throughout the reaction course.

$$d[\text{ITC}]_t/dt = -k \times [\text{ITC}]_t \quad (1)$$

$$[\text{ITC}]_t = [\text{ITC}]_0 \times \exp(-k \times t) \quad (2)$$

where k is the pseudo-first order constant (min^{-1}), while $[\text{ITC}]_t$ and $[\text{ITC}]_0$ are the concentrations of ITC at time t and 0, respectively. The values of k for all ITCs tested were determined from Eq. (2) by plotting $\ln[\text{ITC}]_t$ as a function of reaction time t ([Fig. S-11, Supplementary Material](#)). The calculated k values equaled to $1.84 \times 10^{-2} \text{ min}^{-1}$ for MITC, and $1.59 \times 10^{-2} \text{ min}^{-1}$ for SFN, $1.72 \times 10^{-2} \text{ min}^{-1}$ for EITC, $3.83 \times 10^{-2} \text{ min}^{-1}$ for AITC, $1.80 \times 10^{-2} \text{ min}^{-1}$ for 3-MTPITC, $3.17 \times 10^{-2} \text{ min}^{-1}$ for PITC, $1.59 \times 10^{-2} \text{ min}^{-1}$ for 4-MTBITC, $4.27 \times 10^{-2} \text{ min}^{-1}$ for BITC and $1.42 \times 10^{-2} \text{ min}^{-1}$ for PEITC. Under the conditions applied (i.e., excess of NAC, ambient temperature), the minimum time needed for derivatization of all analytes to reach the steady state was 500 min ([Fig. 1C](#)). There were no significant variations in the peak area noted for ITC-NAC conjugates after this time.

The reaction carried out at ambient temperature for over 8 h seemed rather too long a period for a routine analytical protocol, so we investigated the possibility of reduction of the time needed for ITC derivatization by elevating the temperature of reaction mixture to 40 °C, 50 °C or 60 °C. In the [Fig. 1D–F](#), the influence of different reaction temperatures on the yield of reaction of EITC, 4-MTBITC and BITC carried out in a mixture containing isopropanol is presented. The increase of temperature significantly shortened the time needed to reach the steady state of ITC-NAC conjugate formation. At 40 °C, the minimum time needed to react ITCs with NAC was about 50 min, while at 50 °C, it dropped to about 30 min. The increase of the temperature to 60 °C did not result in higher rate of reaction, but to make matters worse, reduced the yield of some ITC-NACs. In the light of these results, we propose the optimal derivatization temperature to be 50 °C and reaction time 60 min. The extension of minimal reaction time needed for ITC derivatization under selected temperature was indicated to ensure conversion of all ITCs present in plant extract to dithiocarbamates. Summarizing, the 50% isopropanol was chosen as the best medium for the derivatization of ITCs with NAC. This finding indicates that isopropanolic extract obtained from SPE step can be directly used for derivatization procedure and should be mixed with derivatization mixture in ratio 1:1 (v/v).

3.4. Validation of analytical procedure for determination of NAC-ITC

3.4.1. Linearity range and calibration curves

Calibration data were determined for all the analytes under investigation, using the chromatographic and derivatization conditions described in Section 2.6 and 2.7. The calibration curves were generated by carrying out a series of separations of standard mixtures containing the ITCs within the ranges of 0.005–0.1 $\mu\text{mol/mL}$ and 0.1–1.0 $\mu\text{mol/mL}$ after pre-column derivatization. Three independent measurements were carried out for each calibration point. Calibration lines based on six calibration points were prepared by plotting concentration versus chromatographic peak area of the analyte and applying the least squares method. The results of



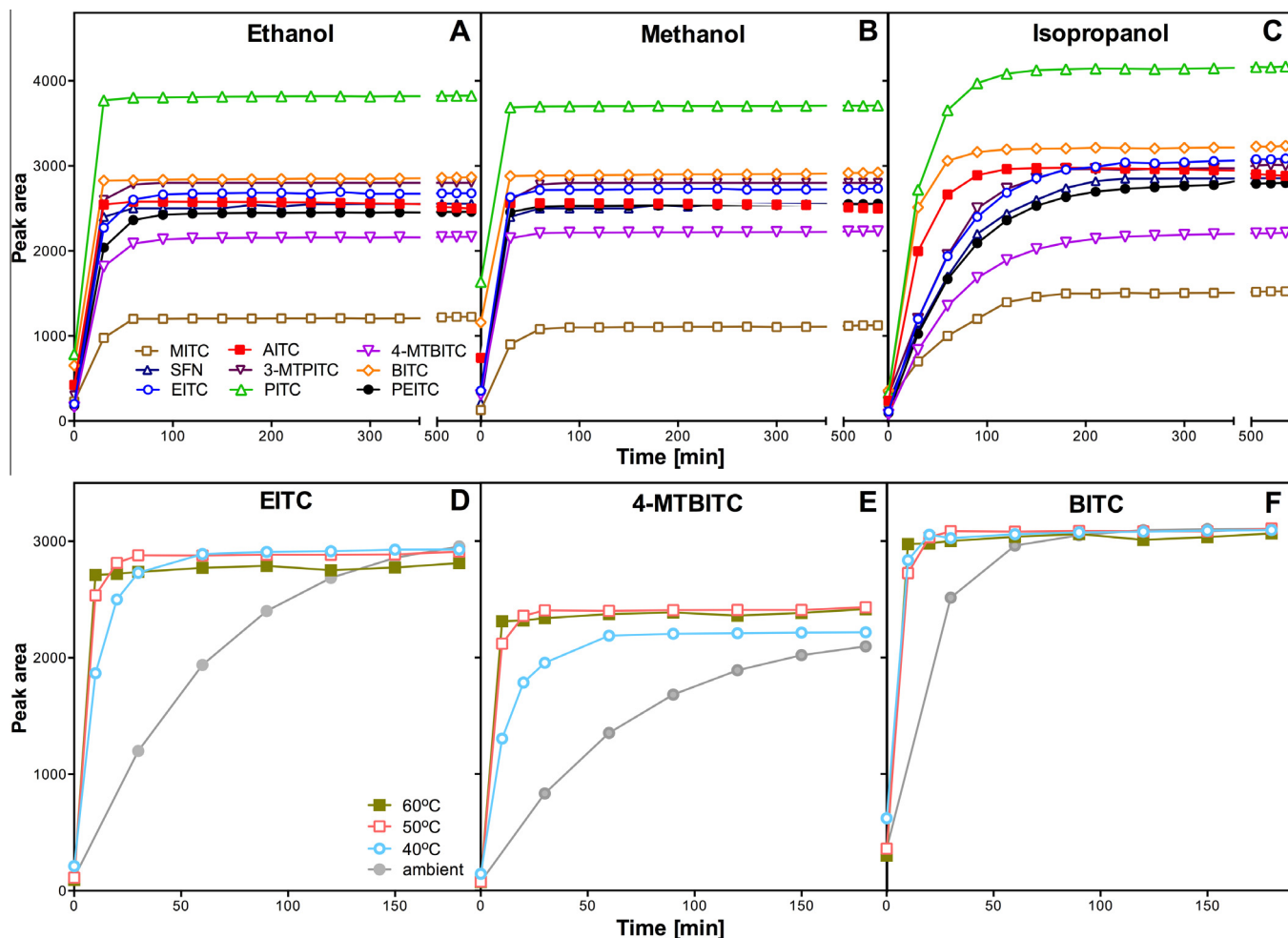


Fig. 1. Kinetics of derivatization of ITCs to ITC-NAC conjugates in reaction mixture containing 0.1 M NAC, 0.1 M NaHCO₃ and 0.5 mM ITC in water: alcohol = 1:1. Panels A, B, C – reaction carried out at ambient temperature in different solvents as indicated. Panels D, E, F – reaction carried out in isopropanol at indicated temperatures for three ITCs, whose rates of conversion to ITC-NAC conjugates at ambient temperature differed substantially. Points are results of single determination.

Table 1

Calibration data for the determination of isothiocyanates (ITCs) analyzed by the developed HPLC-DAD method.

Analyte	Concentration range [μmol/mL]	Calibration curve ^a	R ²	LOD [nmol/mL]	LOQ (nmol/mL)	Repeatability (n = 3) ^b	RSD [%]	
							Intra-day precision (n = 6)	Inter-day precision (n = 9)
MITC	0.005–0.1	y = 1364x + 8.04	0.9965	4.93	14.79	3.49	2.33 ^c –4.10 ^d	1.76 ^c –3.64 ^d
	0.1–1.0	y = 1411x + 4.76	0.9990					
SFN	0.005–0.1	y = 4032x + 5.45	0.9996	1.72	5.16	1.77	1.90 ^c –3.79 ^d	2.71 ^c –4.05 ^d
	0.1–1.0	y = 3392x – 7.52	0.9995					
EITC	0.005–0.1	y = 2581x – 0.36	0.9975	4.12	12.35	3.09	2.94 ^c –3.87 ^d	2.07 ^c –3.36 ^d
	0.1–1.0	y = 1968x + 15.64	0.9968					
AITC	0.005–0.1	y = 2091x – 1.13	0.9917	4.17	12.50	4.76	3.43 ^c –5.18 ^d	4.14 ^d –4.82 ^c
	0.1–1.0	y = 2255x + 2.27	0.9914					
3-MTPITC	0.005–0.1	y = 4475x + 1.15	0.9993	2.13	6.39	2.35	1.65 ^c –1.84 ^d	0.62 ^d –1.19 ^c
	0.1–1.0	y = 3025x – 12.37	0.9991					
PITC	0.005–0.1	y = 3214x + 0.20	0.9965	4.89	14.67	3.52	3.12 ^c –4.03 ^d	3.70 ^d –5.84 ^c
	0.1–1.0	y = 3179x + 59.38	0.9936					
4-MTBITC	0.005–0.1	y = 2092x + 0.28	0.9986	3.14	9.41	2.83	2.40 ^c –3.88 ^d	3.34 ^c –3.95 ^d
	0.1–1.0	y = 1825x – 0.77	0.9990					
BITC	0.005–0.1	y = 2348x + 2.43	0.9970	4.50	13.51	4.21	3.73 ^d –4.36 ^c	2.61 ^d –3.59 ^c
	0.1–1.0	y = 2485x + 14.92	0.9955					
PEITC	0.005–0.1	y = 2345x + 4.15	0.9921	3.63	10.89	4.96	3.25 ^d –5.27 ^c	4.83 ^d –5.10 ^c
	0.1–1.0	y = 2467x + 23.42	0.9946					

MITC – methyl isothiocyanate, SFN – sulforaphane, EITC – ethyl isothiocyanate, AITC – allyl isothiocyanate, 3-MTPITC – 3-methyl-thiopropyl isothiocyanate, PITC – phenyl isothiocyanate, 4-MTBITC – 4-methylthiobutyl isothiocyanate, BITC – benzyl isothiocyanate, PEITC – phenethyl isothiocyanate.

^{c,d} Precision determined for two different sample matrices of papaya and white cabbage, respectively, spiked with ITC standards.

^a y: peak area of ITC standard; x: concentration [μmol/mL].

^b Repeatability of the method determined based on the analysis of calibration solutions containing the mixture of ITC standards.

regression analysis are listed in Table 1. The linearities of the calibration curves are reflected by the coefficient of determination (R^2). Statistical evaluation of the calibration data confirmed a high linearity expressed by values of determination coefficient ranging from 0.9914 for AITC to 0.9996 for SFN.

3.4.2. Limits of detection and quantification

The calibration curves obtained for standard solutions within the lower concentration range (0.005–0.1 $\mu\text{mol/mL}$) were used to evaluate the limits of detection (LODs) of developed SPE/HPLC-DAD-MS method for determination of ITCs. The LOD values were calculated based on the residual standard deviation of the calibration curve ($s_{x,y}$) and standard deviation of the intercept (s_a). $\text{LOD}_{x,y}$ and LOD_a values were determined as $\text{LOD} = 3.3s/b$, where s is the standard deviation and b is the slope, and then averaged to obtain a final LOD. The calculated LODs were checked using the following requirements: (i) $10\text{LOD} > C_{\text{min}}$, (ii) $\text{LOD} < C_{\text{min}}$. The limits of quantification (LOQs) were calculated as tripled LOD value. The values of detection and quantification limits for analyzed ITCs were summarized in Table 1. All calculated LOD values fulfilled the above requirements. The LODs of elaborated analytical procedure ranged from 1.72 nmol/mL for SFN to 4.93 nmol/mL for MITC, while the LOQ values were within the range of 5.16–14.79 nmol/mL. These data suggest that low quantities of all studied ITC compounds can be determined by the developed analytical procedure with NAC derivatization. The former chromatographic methodology incorporating derivatization with mercaptoethanol for simultaneous determination of ITCs was characterized by a high sensitivity comparable with our results (Wilson et al., 2011). A slight exception was observed only for PEITC and BITC, for which our procedure gave 2-fold higher and 4-fold lower LOD value (6.7 and 5.9 ng), respectively. Moreover, the method developed by Wilson et al. (2011) showed the lowest LOD for BITC (1.5 ng);

Table 2 Composition and content [$\mu\text{mol/g}$ d.w.] of isothiocyanates in plant samples compiled with chromatographic and spectrometric data of NAC-ITC conjugates registered during HPLC-DAD-MS analyses.

Peak no	Rt [min]	MS (+)	Isothiocyanates			Content [$\mu\text{mol/g}$ d.w. \pm SD (n = 3)]					
			Chemical name	Mw	Parent GLS	Broccoli BF	White cabbage	Radish	Garden cress	Horse-radish	Papaya
1 ^a	6.3	327	3-(Methylsulfinyl)propyl	163	Glucobriferin	nd	3.803 \pm 0.192	nd	nd	1.161 \pm 0.028	nd
2 ^a	6.4	293	2(R/S)-2-Hydroxy-3-butenyl	129	Epi/progoitrin	0.652 \pm 0.013	nd	nd	nd	nd	nd
3	6.5	237	Methyl	73	Glucocapparin	nd	nd	nd	nd	nd	nd
4 ^a	7.2	341	4-(Methylsulfinyl)butyl	177	Glucoraphanin	4.078 \pm 0.210	1.255 \pm 0.103	nd	nd	nd	nd
5 ^a	7.4	339	4-(Methylsulfinyl)-3-butenyl	175	Glucoraphenin	nd	0.856 \pm 0.073	0.204 \pm 0.017	nd	nd	nd
6	8.2	251	Ethyl	87	Glucopidiiin	nd	nd	nd	nd	nd	nd
7 ^b	9.4	263	2-Propenyl	99	Sinigrin	nd	2.860 \pm 0.004	nd	nd	14.888 \pm 1.020	nd
8 ^a	11.1	383	6-(Methylsulfinyl)hexyl	219	Glucosesperin	nd	nd	nd	nd	0.403 \pm 0.023	nd
9 ^c	11.7	311	3-(Methylthio)propyl	147	Glucobriferin	nd	0.165 \pm 0.001	nd	nd	nd	nd
10 ^d	12.0	279	1-Methylpropyl	115	Glucocochlearin	nd	nd	nd	nd	0.213 \pm 0.008	nd
11	12.6	299	Phenyl	135	–	nd	nd	nd	nd	nd	nd
12 ^e	13.2	325	4-(Methylthio)butyl	161	Glucorucin	nd	0.136 \pm 0.022	nd	nd	nd	nd
13 ^e	13.9	323	4-(Methylthio)butenyl	159	Glucoraphasatin	nd	nd	0.268 \pm 0.027	nd	nd	nd
14 ^f	14.1	313	Benzyl	149	Glucotropaeolin	nd	nd	nd	0.695 \pm 0.025	nd	0.072 \pm 0.007
15 ^g	15.4	327	2-Phenylethyl	163	Glucunasturtiin	nd	nd	nd	nd	0.238 \pm 0.012	nd

The concentration of ITC was calculated with the use of calibration curves of available standards:

- ^a SFN.
- ^b AITC.
- ^c 3-MTPTIC.
- ^d EITC.
- ^e 4-MTBITC.
- ^f BITC.
- ^g PEITC, nd – not detected.

which slightly increased for AITC, MITC, SFN, PITC and PEITC to reach 2.1, 2.8, 2.9, 7.8 and 14.2 ng, respectively. The analytical procedure proposed by German researchers for HPLC determination of selected ITCs as stable *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester derivatives in biological samples was more sensitive for SFN and 4-MTBITC (LOD; 0.5 and 1.0 nmol/mL) (Budnowski et al., 2013).

3.4.3. Repeatability of the method

The repeatability of the developed analytical procedure was determined based on the data obtained for the calibration solutions. This validation parameter was expressed as the relative standard deviation (RSD) of the chromatographic peak areas. Three replications were averaged for each regression point and the results were used to calculate the total RSD using the following equation:

$$RSD = \sqrt{\frac{1}{n} \cdot \sum_{i=1}^n RSD_i^2} \quad (3)$$

where RSD_i is the coefficient of variation for the i -th calibration solution, and n is the number of calibration points. The repeatability of the results obtained by the developed analytical procedure are listed in Table 1. This method ensured the highest repeatability for SFN (RSD = 1.77%) and the lowest for PEITC (RSD = 4.96%). In general, the obtained RSD values less than 5% indicated a very good repeatability of the results.

3.4.4. Intermediate precision

To check the invariability of the derivatization process and to test the stability of chromatographic system, the intermediate precision was assessed by carrying out a series of runs for two different sample matrices, white cabbage and papaya, spiked with all ITC

standards used at one concentration level of 82.1, 70.5, 87.7, 60.5, 75.1, 88.8, 79.1, 80.4 and 73.5 nmol/mL for MITC, SFN, EITC, AITC, 3-MTPITC, PITC, 4-MTBITC, BITC and PEITC, respectively. Before HPLC analysis, the analytes were isolated using SPE technique and derivatized with NAC under optimal conditions. This study comprised six independent measurements of the same plant sample in a single day and nine analyses performed on three different days over one month period (three independent runs during the day) for the intra-day and inter-day assay, respectively. The results of these determinations are summarized in [Table 1](#). The intra-day precision expressed as RSD of the content of single ITCs corresponding to peak area varied between 1.84–5.18% and 1.65–5.27%, while the inter-day precision was in the range of 0.62–4.83% and 1.19–5.84% for spiked white cabbage and papaya samples, respectively. The short-term and long-term studies have shown that the developed method is characterized

by the highest precision for 3-MTPITC and the lowest precision for PEITC.

3.4.5. Recovery test

In order to verify the efficiency of the optimized SPE procedure and stability of NAC derivatives, recovery tests were carried out. Recoveries of ITCs were determined by spiking two different plant samples (white cabbage or papaya) with known amounts of ITC standards at two concentration levels as shown in [Table S-1 \(Supplementary Material\)](#). After addition of authentic standards to equal amounts of sample, the analytes generated by autolysis of GLs were purified, concentrated and isolated using SPE under optimal conditions and then derivatized with NAC. Every sample was analyzed in triplicate. The results of recovery studies are presented in [Table S-1 \(Supplementary Material\)](#). Satisfactory recoveries (83.3–103.7%), with RSD values below 5.4%, were obtained for all

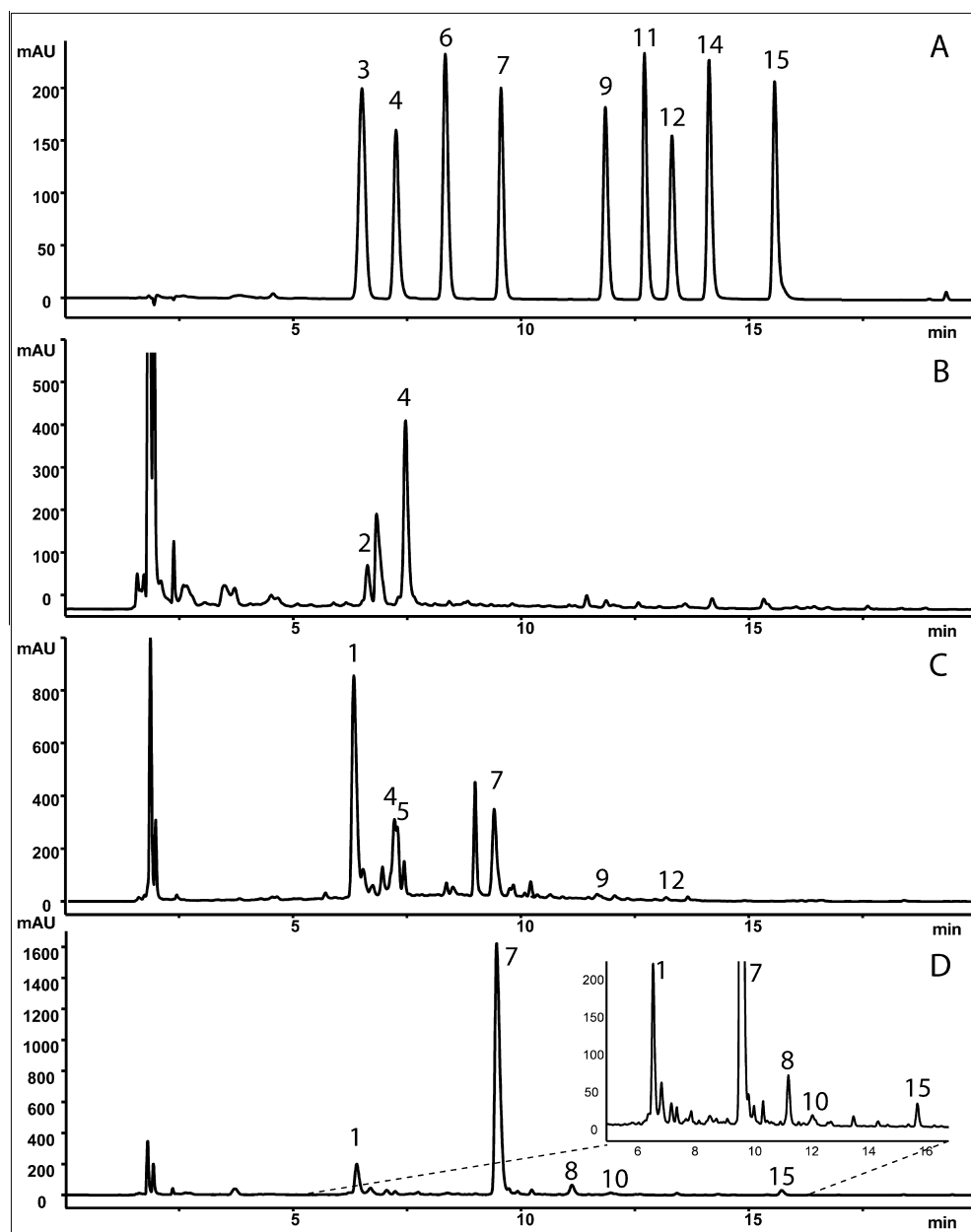


Fig. 2. Sample HPLC-DAD chromatograms ($\lambda = 272$ nm) of ITC-NAC conjugates formed as a result of derivatization of ITCs present in mixture of ITC standards (A), broccoli extract (B), white cabbage extract (C) or horseradish extract (D). The conjugation reaction was carried out for 60 min at 50 °C for mixtures containing 0.1 M NAC, 0.1 M NaHCO₃ in water and 0.5 mL of 0.5 mM of each ITC standard or 0.5 mL of plant extract in isopropanol. The numbers of peaks of detected ITC-NAC conjugates correspond to analytes listed in [Table 2](#).

ITCs, regardless of the spiking level. These results fulfill currently accepted requirements for the analytical procedures wherein the recovery should range from 70% to 120% depending on matrix complexity (Chmiel, Abogado, & Wardencki, 2014).

The analytical procedure applied by Wilson et al. (2011) for determination of ITCs as mercaptoethanol derivatives in dichloromethane extracts of white cabbage was characterized by similar recovery values ranging from 90 to 101% for all studied ITCs. In addition, it is worth noting that the use of different sample matrices simultaneously demonstrated the versatile applicability of the proposed method for the determination of ITCs in samples of different origin.

3.5. Plant samples analysis

The optimized conditions of purification, derivatization and chromatographic separation of ITCs were applied for the qualitative and quantitative analysis of these components in broccoli BF, white cabbage, radish, garden cress, horseradish and papaya. First step of the evaluated procedure includes hydrolysis of GLs present in plant material by the endogenous myrosinase. For this purpose, lyophilized plant material was mixed with water and incubated for 3 h at 37 °C to enable myrosinase catalyzed conversion of GLs into ITCs to complete. These parameters of incubation were set according to earlier studies; the progress of enzymatic reaction was monitored by measuring pH of the reaction mixture. GLs hydrolysis by myrosinase releases H⁺ ions, so until the reaction progresses, pH of a sample decreases. Therefore, when pH level remained stable for at least 20 min, the hydrolysis of GLs was regarded as completed. Our results indicate that for all plant material studied, 3 h of incubation is enough to finish hydrolysis. Each plant sample was analyzed in triplicate and the results obtained are presented in Table 2. The identification of separated NAC-ITC conjugates was done on the basis of UV and MS spectra. The UV spectra of NAC-ITCs in most cases displayed similar shape with three maxima of absorption at about 220, 250 and 270 nm (Fig. S-12, Supplementary Material). The mass spectra of NAC-ITCs collected during analysis of plant extracts revealed fragmentation patterns characterized by a major molecular ion peak [M+H]⁺. The other most abundant fragment ions were sodium adducts [M+Na]⁺ and N-acetyl-L-cysteine (fragment m/z 164) (Fig. S-12, Supplementary Material). On the basis of chromatographic and spectrometric data of available standards, 6 compounds were identified. Further 6 structures were deduced according to peaks' UV and MS spectra and literature data. Among these 12 compounds, there were 7 structures of ITCs, which in side chains contained sulfur atom in

two states of oxidation (4 as methylsulfinylalkyl- and 3 as methylthioalkyl-), 3 aliphatic, one aliphatic with hydroxyl group, and 2 aromatic (Table 2).

The exemplary chromatograms obtained for standards, broccoli, cabbage and horseradish samples are shown in Fig. 2. Not surprisingly, the highest content of SFN, one of the most studied ITCs that exhibits anticarcinogenic and antiinflammatory properties, was detected in broccoli BF (4.1 μmol/g d.w.). According to the literature data, the content of SFN in this plant can vary from 0.1 to 6.8 μmol/g d.w. (Oliviero, Verkerk, Vermeulen, & Dekker, 2014), thus its concentration determined by HPLC-DAD method following derivatization to ITC-NAC conjugates is within an expected range. The SFN abundance depends on the broccoli cultivar, but is also related to the activity of epithiospecifier protein. The latter can negatively redirect myrosinase-catalyzed hydrolysis of alkenyl glucosinolate glucoraphanin toward epithionitrile (Jones, Frisina, Winkler, Imsic, & Tomkins, 2010; Matusheski et al., 2006).

White cabbage and horseradish contain sinigrin whose content ranges from 0.6 to 4.3 μmol/g d.w. (Kusznierewicz et al., 2008, 2012) and from 2 to 258 μmol/g d.w. (Xian & Kushad, 2004), respectively. This glucosinolate may be converted to AITC, allyl cyanide and, in the presence of an epithiospecifier protein, into 1-cyano-2,3-epithiopropene (Rungapamestry, Duncan, Fuller, & Ratcliffe, 2006). Therefore, the concentration of AITC in brassica plants producing sinigrin may be highly differentiated. Our analysis indicated AITC level in cabbage samples to equal to about 3 μmol/g d.w. In the case of horseradish tested, the content of AITC was five times higher (about 15 μmol/g d.w.). In cabbage samples, besides AITC, the significant content of iberin (3.8 μmol/g d.w.), sulforaphane (1.3 μmol/g d.w.) and sulforaphene (0.9 μmol/g d.w.) was also detected. In the case of horseradish, the presence of iberin (1.2 μmol/g d.w.), 1-methylpropyl ITC and PEITC in similar concentration (about 0.2 μmol/g d.w.) were observed (Table 2, Fig. 2). The PEITC is a product of hydrolysis of gluconastrutiin and is known to prevent the initiation phase of carcinogenesis and also to inhibit the progression of tumorigenesis. PEITC is under clinical trials for leukemia and lung cancer and is perceived as a future anti-cancer agent (Gupta, Wright, Kim, & Srivastava, 2014).

In radish roots only two ITCs, i.e. sulforaphene (0.2 μmol/g d.w.) and 4-methylthiobutenyl (0.3 μmol/g d.w.), were determined. These are products of hydrolysis of respectively glucoraphenin and glucoraphasatin, the GLs whose presence is typical for *Raphanus sativus* (Barillari et al., 2007; Montaut, Barillari, Iori, & Rollin, 2010).

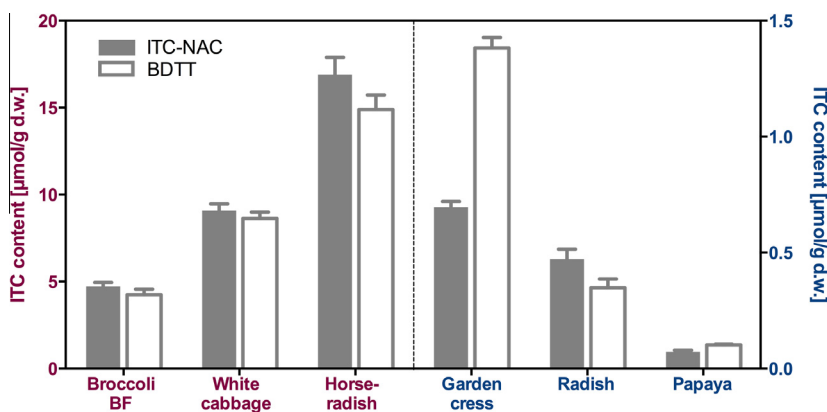


Fig. 3. The total contents of ITCs (μmol/g d.w.) in plant samples determined either as ITC-NAC conjugates by the NAC derivatization method (grey bars) or as BDTT by Zhang's method (white bars). Two scales are applied in the graph depending on ITC content in samples analyzed. The conditions of derivatization reactions were as described under Materials and Methods. The values are means ± SD of 3–6 independent determinations.

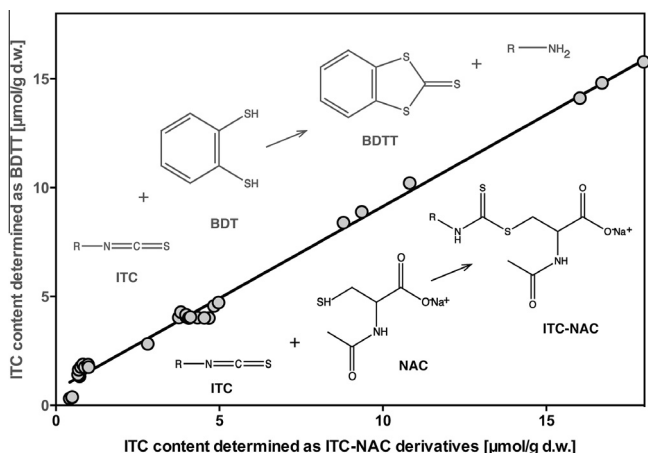


Fig. 4. The correlation between total ITC contents determined as ITC-NAC conjugates and as BDTT for individual plant extracts. Pearson correlation coefficient is $r = 0.996$.

In garden cress and papaya fruit, as expected BITC, the product of hydrolysis of glucotropaeolin, was detected and its content determined by our method was 0.7 and 0.1 $\mu\text{mol/g d.w.}$, respectively. BITC similarly to PEITC has been found to exhibit anticarcinogenic activity in laboratory animals and might also be chemoprotective in humans (Lai et al., 2010).

In Fig. 3, the sum of all ITCs present in plant samples studied determined according the proposed procedure as NAC-ITC conjugates were assembled along with the total ITC content determined as BDTT according Zhang method (Zhang et al., 1996). The results obtained by both methods gave similar total contents of ITCs in most of the samples. Only in the case of garden cress, the level of total ITC content was about twice higher in Zhang method than in the procedure developed. This could have been caused by some contaminants reacting with 1,2-benzenedithiol. Nonetheless, the results of two methods were highly correlated as shown by Pearson coefficient $r = 0.996$ (Fig. 4).

4. Conclusions

ITCs play an important role in plant self-defense against pests and are also known to favorably influence functioning of the human organism. Up to date, there is limited number of liquid chromatographic methods for routine analysis of these compounds despite their importance in food processing, supplement production, as well as in chemical industry. The quantitative determination of total ITCs may be done by LC after their derivatization with 1,2-benzenedithiol to stable 1,3-benzenedithione-2-thione. The method proposed in this paper enables not only quantitative, but also qualitative determination of ITCs in the samples and involves only three steps: (i) isolation and purification of compounds of interest using SPE technique, (ii) derivatization of analytes to dithiocarbamates with readily accessible non-toxic NAC and (iii) chromatographic separation coupled with UV (or/and) MS detection of ITC-NAC conjugates. All steps are simple and not labour- or time-consuming. Moreover, the extract used for the determination of ITCs may be also suitable for the determination of indoles, a group of phytohormones, as well as degradation products of indolic glucosinolates known for their anticarcinogenic activity. Thus, the proposed approach allows obtaining a number of outcomes from a small amount of plant material in a short time needed for sample preparation. The method was validated for 8 commercially available, occurring in nature and structurally different ITCs. The successful application of the method for

determination of ITCs in broccoli, white cabbage, horseradish, radish, garden cress and papaya suggests that it may be of interest not only to researchers, but also for pharma or food industry.

Funding

This work was developed within the framework of the ERA-NET SUSFOOD Sunniva project, "Sustainable food production through quality optimized raw material production and processing technologies for premium quality vegetable products and generated by-products", and supported by the National Centre for Research and Development (NCBiR), Poland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.125>.

References

- Agerbirk, N., De Nicola, G. R., Olsen, C. E., Müller, C., & Iori, R. (2015). Derivatization of isothiocyanates and their reactive adducts for chromatographic analysis. *Phytochemistry*, 118, 109–115. <http://dx.doi.org/10.1016/j.phytochem.2015.06.004>.
- Aires, A., Mota, V. R., Saavedra, M. J., Monteiro, A. A., Simões, M., Rosa, E. A. S., & Bennett, R. N. (2009). Initial in vitro evaluations of the antibacterial activities of glucosinolate enzymatic hydrolysis products against plant pathogenic bacteria. *Journal of Applied Microbiology*, 106(6), 2096–2105. <http://dx.doi.org/10.1111/j.1365-2672.2009.04181.x>.
- Aissani, N., Tedeschi, P., Maietti, A., Brandolini, V., Garau, V. L., & Caboni, P. (2013). Nematicidal activity of allyl isothiocyanate from horseradish (*Armoracia rusticana*) roots against *Meloidogyne incognita*. *Journal of Agricultural and Food Chemistry*, 61(20), 4723–4727. <http://dx.doi.org/10.1021/jf4008949>.
- Barillari, J., Iori, R., Broccoli, M., Pozzetti, L., Canistro, D., Sapone, A., ... Paolini, M. (2007). Glucoraphasatin and glucoraphenin, a redox pair of glucosinolates of brassicaceae, differently affect metabolizing enzymes in rats. *Journal of Agricultural and Food Chemistry*, 55(14), 5505–5511. <http://dx.doi.org/10.1021/jf070558r>.
- Bhat, V., Allan, K. M., & Rawal, V. H. (2011). Total synthesis of N-methylwielwitindolinone D isonitrile. *Journal of the American Chemical Society*, 133(15), 5798–5801. <http://dx.doi.org/10.1021/Ja201834u>.
- Budnowski, J., Hanschen, F. S., Lehmann, C., Haack, M., Brigelius-Flohé, R., Kroh, L. W., ... Hanske, L. (2013). A derivatization method for the simultaneous detection of glucosinolates and isothiocyanates in biological samples. *Analytical Biochemistry*, 441(2), 199–207. <http://dx.doi.org/10.1016/j.ab.2013.07.002>.
- Chen, C., & Ho, C. (1998). Thermal degradation of allyl isothiocyanate in aqueous solution. *Journal of Agricultural and Food Chemistry*, 46(1), 220–223.
- Chiang, W. C. K., Pusateri, D. J., & Leitz, R. E. (1998). Gas chromatography/mass spectrometry method for the determination of sulforaphane and sulforaphane nitrile in broccoli. *Journal of Agricultural and Food Chemistry*, 46(97), 1018–1021. <http://dx.doi.org/10.1021/jf970572b>.
- Chmiel, T., Abogado, D., & Wardencki, W. (2014). Optimization of capillary isotachopheric method for determination of major macroelements in blue honeysuckle berries (*Lonicera caerulea* L.) and related products. *Analytical and Bioanalytical Chemistry*, 406(20), 4965–4986. <http://dx.doi.org/10.1007/s00216-014-7879-4>.
- Gupta, P., Wright, S. E., Kim, S. H., & Srivastava, S. K. (2014). Phenethyl isothiocyanate: A comprehensive review of anti-cancer mechanisms. *Biochimica et Biophysica Acta – Reviews on Cancer*, 1846(2), 405–424. <http://dx.doi.org/10.1016/j.bbcan.2014.08.003>.
- Hanschel, F. S., Platz, S., Mewis, I., Schreiner, M., Rohn, S., & Kroh, L. W. (2012). Thermally induced degradation of sulfur-containing aliphatic glucosinolates in broccoli sprouts (*Brassica oleracea* var. *Italica*) and model systems. *Journal of Agricultural and Food Chemistry*, 60(9), 2231–2241. <http://dx.doi.org/10.1021/jf204830p>.
- Higdon, J. V., Delage, B., Williams, D. E., & Dashwood, R. H. (2009). Cruciferous vegetables and human cancer risk: Epidemiologic evidence and mechanistic basis. *Pharmacological Research*, 55(3), 224–236. <http://dx.doi.org/10.1016/j.phrs.2007.01.009.Cruciferous>.
- Janobi, A. A., Al Mithen, R. F., Gasper, A. V., Shaw, P. N., Middleton, R. J., Ortori, C. A., & Barrett, D. A. (2006). Quantitative measurement of sulforaphane, iberin and their mercapturic acid pathway metabolites in human plasma and urine using liquid chromatography-tandem electrospray ionisation mass spectrometry. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 844(2), 223–234. <http://dx.doi.org/10.1016/j.jchromb.2006.07.007>.
- Jones, R. B., Frisina, C. L., Winkler, S., Imsic, M., & Tomkins, R. B. (2010). Cooking method significantly effects glucosinolate content and sulforaphane production

- in broccoli florets. *Food Chemistry*, 123(2), 237–242. <http://dx.doi.org/10.1016/j.foodchem.2010.04.016>.
- Kusznierewicz, B., Bączek-Kwinta, R., Bartoszek, A., Piekarska, A., Huk, A., Manikowska, A., ... Konieczka, P. (2012). The dose-dependent influence of zinc and cadmium contamination of soil on their uptake and glucosinolate content in white cabbage (*Brassica oleracea* var. *capitata* f. *alba*). *Environmental Toxicology and Chemistry*, 31(11), 2482–2489. <http://dx.doi.org/10.1002/etc.1977>.
- Kusznierewicz, B., Bartoszek, A., Wolska, L., Drzewiecki, J., Gorinstein, S., & Namieśnik, J. (2008). Partial characterization of white cabbages (*Brassica oleracea* var. *capitata* f. *alba*) from different regions by glucosinolates, bioactive compounds, total antioxidant activities and proteins. *LWT – Food Science and Technology*, 41(1), 1–9. <http://dx.doi.org/10.1016/j.lwt.2007.02.007>.
- Lai, K.-C., Huang, A.-C., Hsu, S.-C., Kuo, C.-L., Yang, J.-S., Wu, S.-H., & Chung, J.-G. (2010). Benzyl isothiocyanate (BITC) inhibits migration and invasion of human colon cancer HT29 cells by inhibiting matrix metalloproteinase-2/-9 and urokinase plasminogen (uPA) through PKC and MAPK signaling pathway. *Journal of Agricultural and Food Chemistry*, 58, 2935–2942. <http://dx.doi.org/10.1021/jf9036694>.
- Li, N., Tu, M. S., Jiang, B., Wang, X., & Tu, S. J. (2013). Domino [3+2+1] heterocyclization of isothiocyanates with aryl amidines leading to polysubstituted 1,3,5-triazine derivatives. *Tetrahedron Letters*, 54(13), 1743–1746. <http://dx.doi.org/10.1016/j.tetlet.2013.01.086>.
- Mathiselvam, M., Loganathan, D., & Varghese, B. (2013). Synthesis and characterization of thiourea- and urea-linked glycolipids as low-molecular-weight hydrogelators. *RSC Advances*, 3(34), 14528. <http://dx.doi.org/10.1039/c3ra42041h>.
- Matich, A. J., McKenzie, M. J., Lill, R. E., Brummell, D. A., McGhie, T. K., Chen, R. K. Y., & Rowan, D. D. (2012). Selenoglucosinolates and their metabolites produced in *Brassica* spp. fertilised with sodium selenate. *Phytochemistry*, 75, 140–152. <http://dx.doi.org/10.1016/j.phytochem.2011.11.021>.
- Matusheski, N. V., Swarup, R., Juvik, J. A., Mithen, R., Bennett, M., & Jeffery, E. H. (2006). Epithiospecifier protein from broccoli (*Brassica oleracea* L. ssp. *italica*) inhibits formation of the anticancer agent sulforaphane. *Journal of Agricultural and Food Chemistry*, 54(6), 2069–2076. <http://dx.doi.org/10.1021/jf0525277>.
- Mithen, R. F., Armah, C., & Traka, M. (2011). Vegetables, whole grains, and their derivatives in cancer prevention. In *Cancer* (pp. 1–30). <http://dx.doi.org/10.1007/978-90-481-9800-9>.
- Montaut, S., Barillari, J., Iori, R., & Rollin, P. (2010). Glucoraphasatin: Chemistry, occurrence, and biological properties. *Phytochemistry*, 71(1), 6–12. <http://dx.doi.org/10.1016/j.phytochem.2009.09.021>.
- Navarro, S. L., Li, F., & Lampe, J. W. (2011). Mechanisms of action of isothiocyanates in cancer chemoprevention: An update. *Food & Function*, 2(10), 579–587. <http://dx.doi.org/10.1039/c1fo10114e>.
- Oliviero, T., Verkerk, R., Vermeulen, M., & Dekker, M. (2014). In vivo formation and bioavailability of isothiocyanates from glucosinolates in broccoli as affected by processing conditions. *Molecular Nutrition and Food Research*, 58(7), 1447–1456. <http://dx.doi.org/10.1002/mnfr.201300894>.
- Pandurangan, K., Kitchen, J. A., McCabe, T., & Gunnaugsson, T. (2013). Hydrogen bonding interactions and supramolecular networks of pyridine-aryl based thiosemicarbazides and their Zn(II) complexes. *CrystEngComm*, 15(7), 1421. <http://dx.doi.org/10.1039/c2ce26718g>.
- Piekarska, A., Kołodziejcki, D., Pilipczuk, T., Bodnar, M., Konieczka, P., Kusznierewicz, B., ... Bartoszek, A. (2014). The influence of selenium addition during germination of *Brassica* seeds on health-promoting potential of sprouts. *International Journal of Food Sciences and Nutrition*, 65(6), 692–702. <http://dx.doi.org/10.3109/09637486.2014.917148>.
- Pilipczuk, T., Dawidowska, N., Kusznierewicz, B., Namieśnik, J., & Bartoszek, A. (2015). Simultaneous determination of indolic compounds in plant extracts by solid-phase extraction and high-performance liquid chromatography with UV and fluorescence detection. *Food Analytical Methods*, 8(9), 2169–2177. <http://dx.doi.org/10.1007/s12161-015-0106-x>.
- Pilipczuk, T., Piekarska, A., Kusznierewicz, B., Bartoszek, A., & Namieśnik, J. (2013). Biofumigacja jako przyjazna środowisku technologia ochrony roślin. *Analityka Nauka i Praktyka*, 1, 36–46.
- Psurski, M., Piguła, M., Ciekot, J., Winiarski, Ł., Wietrzyk, J., & Oleksyszyn, J. (2012). Convenient syntheses of novel 1-isothiocyano-alkylphosphonate diphenyl ester derivatives with potential biological activity. *Tetrahedron Letters*, 53(44), 5845–5847. <http://dx.doi.org/10.1016/j.tetlet.2012.08.037>.
- Rungapamestry, V., Duncan, A. J., Fuller, Z., & Ratcliffe, B. (2006). Changes in glucosinolate concentrations, myrosinase activity, and production of metabolites of glucosinolates in cabbage (*Brassica oleracea* var. *capitata*) cooked for different durations. *Journal of Agricultural and Food Chemistry*, 54(20), 7628–7634. <http://dx.doi.org/10.1021/jf0607314>.
- Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., & Talalay, P. (2001). Chemoprotective glucosinolates and ssouthiocyanates of broccoli sprouts. *Cancer Epidemiology Biomarkers & Prevention*, 10(5), 501–508.
- Smith, C. D., Baxendale, I. R., Tranmer, G. K., Baumann, M., Smith, S. C., Lewthwaite, R. A., & Ley, S. V. (2007). Tagged phosphine reagents to assist reaction work-up by phase-switched scavenging using a modular flow reactor. *Organic & Biomolecular Chemistry*, 5(10), 1562–1568. <http://dx.doi.org/10.1039/b703033a>.
- Trott, D., Lepage, J., & Hebert, V. R. (2012). Assessing natural isothiocyanate air emissions after field incorporation of mustard cover crop. *Bulletin of Environmental Contamination and Toxicology*, 88(3), 482–485. <http://dx.doi.org/10.1007/s00128-011-0506-6>.
- Vermeulen, M., Zwanenburg, B., Chittenden, G. J. F., & Verhagen, H. (2003). Synthesis of isothiocyanate-derived mercapturic acids. *European Journal of Medicinal Chemistry*, 38(7–8), 729–737. [http://dx.doi.org/10.1016/S0223-5234\(03\)00141-7](http://dx.doi.org/10.1016/S0223-5234(03)00141-7).
- Wesseling, C., McConnell, R., Partanen, T., & Hogstedt, C. (1997). Agricultural pesticide use in developing countries: Health effects and research needs. *International Journal of Health Services*, 27, 273–308.
- Wilson, E. A., Ennahar, S., Zhao, M., Bergaentzle, M., Marchioni, E., & Bindler, F. (2011). Simultaneous determination of various isothiocyanates by RP-LC following precolumn derivatization with mercaptoethanol. *Chromatographia*, 73(SUPPL. 1). <http://dx.doi.org/10.1007/s10337-010-1878-1>.
- Wittstock, U., & Burow, M. (2007). Tipping the scales – specifier proteins in glucosinolate hydrolysis. *IUBMB Life*, 59(12), 744–751. <http://dx.doi.org/10.1080/15216540701736277>.
- Xian, L., & Kushad, M. M. (2004). Correlation of glucosinolate content to myrosinase activity in horseradish (*Armoracia rusticana*). *Journal of Agricultural and Food Chemistry*, 52(23), 6950–6955. <http://dx.doi.org/10.1021/jf0401827>.
- Zhang, Y. S., Kolm, R. H., Mannervik, B., & Talalay, P. (1995). Reversible conjugation of isothiocyanates with glutathione catalyzed by human glutathione transferases. *Biochemical and Biophysical Research Communications*, 206(2), 748–755. <http://dx.doi.org/10.1006/bbrc.1995.1106>.
- Zhang, Y., Wade, K. L., Prestera, T., & Talalay, P. (1996). Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Analytical Biochemistry*, 239(2), 160–167. <http://dx.doi.org/10.1006/abio.1996.0311>.