Chromatographia (2011) 73 (Suppl 1):S137–S142
DOI 10.1007/s10337-010-1878-1

FULL SHORT COMMUNICATION

Simultaneous Determination of Various Isothiocyanates by RP-LC Following Precolumn Derivatization with Mercaptoethanol

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Received: 8 October 2010 / Revised: 16 November 2010 / Accepted: 23 November 2010 / Published online: 8 January 2011

Abstract Numerous isothiocyanates (ITCs) are poorly soluble in water which causes their precipitation in aqueous mobile phases used in reversed phase liquid chromatography (RP-LC), thus impacting the accuracy of the quantification. By comparing the amounts of ITCs injected and released from the column, losses could be estimated at 5–32% depending on polarities and concentrations. Results could be dramatically improved in terms of separation and quantification using RP-LC with a mercaptoethanol precolumn derivatization aimed at avoiding ITCs precipitation. The cancer chemoprotective allyl-ITC and sulforaphane were found in cabbage extracts at 1.2 and 2.7 μg g^{-1} fresh weight, respectively.

Keywords Column liquid chromatography · UV-detection · Mass spectrometry · Isothiocyanates · Solubility · Mercaptoethanol

Introduction

Biological studies have shown that the consumption of cruciferous vegetables, like cabbage, is associated with a reduced risk of degenerative diseases such as cancer. This chemo-protective action is often attributed to isothiocyanates (ITCs) which are enzymatic hydrolysis products of glucosinolates (GLS) [1]. These sulfur-containing glycosides are a group of secondary plant metabolites present in cruciferous vegetables belonging to the Brassicaceae family. When the plant tissues are damaged by chopping, crushing or chewing, GLSs are brought into contact with the endogenous plant enzyme myrosinase [2]. The types and amounts of hydrolysis products formed depend on the parent GLS that have either aliphatic or aromatic side chains and on reaction conditions. GLS are hydrolyzed into thiocyanates, nitriles, oxazolidinethiones and ITCs which constitute the main group of enzymatic hydrolysis products [3].

In recent years, ITCs have been studied for their potential anticarcinogenic effect. This cancer protective effect has been attributed to the ability of these molecules to inhibit phase I enzymes, responsible for the bioactivation of carcinogens, and to activate phase II detoxification enzymes [4, 5]. In addition, a recent study showed the role of ITCs in the inhibition of the growth of cancer cells through the induction of apoptosis [6]. In this way, ITCs can prevent the development of various human cancers, such as colon, oesophagus, rectum, bladder and mammary cancers [7, 8].

Due to the growing interest in their potential protective effects, dietary ITCs attracted much attention from analysts. Several analytical methods using gas chromatography (GC) [9–11] and liquid chromatography (normal phase (NP-LC) [12] and reversed phase (RP-LC) [13, 14]) have been developed. RP-LC methods based on a C18 are generally the methods of choice when it comes to analyzing specific ITCs molecular species. However, only a limited number of ITCs could be simultaneously investigated using a single chromatographic method. In fact, given that ITCs show various physicochemical properties,

Presented at: 16th International Symposium on Separation Science, Recent Advancements in Chromatography and Capillary Electromigration Techniques. Rome, Italy, 6–10 September, 2010.

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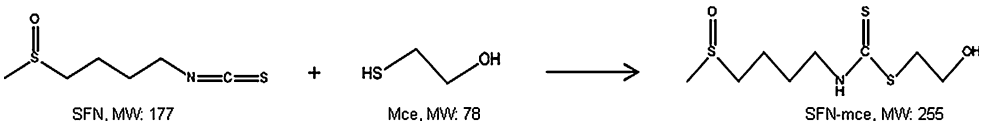

Fig. 1 Derivatization of ITCs with mercaptoethanol leading to the formation of ITC derivatives (ITC-mce), example of SFN

especially in terms of polarity and volatility, the analysis of a wider range of compounds often calls for the use of two methods in conjunction, e.g. GC and LC for volatile and nonvolatile ITCs, respectively, or NP-LC and RP-LC for polar and nonpolar compounds, respectively. When it comes to polarity, ITCs range from the water soluble sulforaphane (SFN) to compounds that are highly hydrophobic, such as heptyl-ITC (HITC) [15]. This may cause some ITCs to precipitate in the aqueous mobile phases, thus impacting on the accuracy of quantifications and also resulting in technical problems such as clogging of the HPLC system and pressure increase.

In this paper, an RP-LC analytical method was developed to separate, identify and quantify ITCs with various physicochemical properties. This method consisted of a mercaptoethanol (mce) precolumn derivatization aimed at avoiding precipitation during RP-LC [16]. The amount of ITCs injected was compared with those collected after separation with and without precolumn derivatization. Quantification was done by using a previously developed assay where ITCs are allowed to react quantitatively with 1,2-benzenedithiol (BDT) forming a stable cyclic dithiol thione product [1, 17, 18]. ITCs and their derivatives could be detected and identified using their UV and mass spectra.

Experimental

Chemicals and Reagent

Chemicals used were of LC grade. ITCs with various polarities were chosen for this study: D,L-SFN (97%) was purchased from Enzo Life Sciences (Villeurbanne, France); allyl-ITC (AITC) (95%), benzyl-ITC (BITC) (98%), methyl-ITC (MITC) (97%), phenyl-ITC (PITC) (99%), phenylethyl-ITC (PEITC) (99%), and propyl-ITC (proITC) (98%) were obtained from Sigma-Aldrich (Schnelldorf, Germany); HITC (97%) was purchased from Acros organics (Geel, Belgium).

1,2-benzenedithiol (96%), formic acid (98%), acetonitrile (ACN), and methanol were obtained from Sigma-Aldrich. Triethylamine (99%) and 2-mercaptoethanol (mce) (99%) were purchased from Acros Organics. Dichloromethane and ethanol were obtained from VWR (Briare, France). 1,3-benzodithiole-2-thione (BD2T) was prepared and characterized previously in our laboratory according to Kristensen et al. [17]. Ultrapure water was produced by a Synergy UV purification system Millipore (Molsheim, France). Other chemicals were of analytical grade and were as follows: dichloromethane (for extraction) and anhydrous sodium sulfate from SDS (Vaidereuil, France); sodium dihydrogen phosphate and disodium hydrogen phosphate from Merck (Darmstadt, Germany).

ITC stock solutions (2 mg mL^{-1}) were prepared in ethanol. Working solutions at various concentrations were prepared by diluting stock solutions in ACN or in dichloromethane. Stock and working solutions were stored at 5 °C for no more than 2 weeks. Before injection, all solutions were filtered through a 0.45 µm membrane filter (Macherey–Nagel, Hoerdt, France).

Determination of ITCs Losses during RP-LC

An LC system made of two Prostar 210 solvent delivery systems, a Prostar 410 autosampler and a Prostar 330 Photodiode Array (PDA) UV/vis detector (Varian, les Ulis, France) was used in this study. A mixture (100 µl) of eight ITCs at 80 µg mL^{-1} each (alternatively at 1,000 µg mL^{-1}) in ACN was injected and separated on a Hypersil C18 (5 µm) 4.6 × 250 mm column (Interchim, Montluçon, France). Fractions of 0.8 mL containing ITCs were collected after PDA detection.

The amounts of ITCs, injected and collected, were quantified according to Kristensen et al. [17], by using a cyclocondensation reaction of ITCs with BDT, which led to a single and stable product, the BD2T [18]. A standard curve of BD2T was constructed using a series of solutions with concentrations ranging from 0.03 to 0.81 mmol L^{-1}. Quantities of ITCs were expressed as BD2T equivalent in mmol. Each determination was done three times.

Analysis of ITCs after Mercaptoethanol Derivatization

One milliliter of a solution of ITCs (each at 50 µg mL^{-1}) was mixed with 500 µL of a reagent containing 20 mM triethylamine and 200 mM mce in dichloromethane (Fig. 1). The mixture was incubated at 30 °C for 60 min under constant stirring, and then dried under a stream of nitrogen. The residue containing ITC derivatives (ITC-mce) was dissolved in 1 mL of ACN/water (1:1) (*v*/*v*), and 10 µL of the obtained solution was injected onto a Nucleosil C18 analytical (5 µm) 4.6 × 150 mm column (Macherey–Nagel (Hoerdt, France) with the corresponding guard column (5 µm) 4.6 × 10 mm. The PDA detector

was set at 271 nm. Besides, LC–MS analysis was performed on a 1200L Triple Quadrupole mass spectrometer (Varian, Les Ulis, France) equipped with an atmospheric-pressure chemical ionization (APCI) interface operating in positive and negative modes. The mass range was set between 50 and 300 m/z. The conditions of the APCI source were as follows: high purity nitrogen, produced by a nitrogen generator (Domnick Hunter, Villefranche-sur-Saône, France), was used as nebulizing gas (45.8 psi, in positive mode), drying gas (18 psi, 250 °C) and auxiliary gas (31.8 psi, 400 °C); needle voltage, 600 V; Ion source pressure, 3 mTorr; collision cell pressure, 0.138 mTorr. In negative mode, air was used as nebulizing gas, and the switch was reset automatically.

Analysis of ITCs in White Cabbage

Extraction of ITCs in cabbage was conducted as described by Liang et al. [19], with several modifications. Cabbage was purchased from a local producer in the north-east of France.

In summary, 100 g of fresh food matrix were grinded in liquid nitrogen using a cryogenic grinder (6870 Freezer/Mill, Spex CertiPrep, Stanmore, Great Britain) with 2 steps of 2 min at a rate of 24 impacts s^{-1}. The obtained frozen powder was stored at −20 °C until use. For extraction, 10 g of this powder were vortexed with 10 mL of ultrapure water and left to autolyse at room temperature for 1 h under shaking to ensure that all glucosinolates were hydrolyzed. Isothiocyanates were extracted four times with 5 mL of dichloromethane. The four organic extracts were combined and dehydrated with the addition of 250 mg of anhydrous sodium sulfate. The dichloromethanic fraction was reduced down to 2 mL in a rotary evaporator (30 °C, 600 mPa). ITC extracts were derivatized and analyzed by LC-UV–MS as described above. All experiments were performed in triplicates.

Quantification and Calibration

To assess linearity, six point calibration plots (1–50 μg L^{-1}, $n = 3$) were drawn for each of the eight derivatized ITCs by plotting peak areas against concentrations. The limits of detection (LOD) and quantification (LOQ) were experimentally defined, after ten injections, as the concentration of ITC derivatives, which produced a chromatographic peak with signal to noise ratio (S/N) >3 and 10, respectively. The accuracy of the method was determined using cabbage extracts spiked with a known amount of ITCs within the working range. The analysis was performed, in triplicate, to determine the amount of ITCs present in cabbage samples. The intra-day and inter-day precision of the method was assessed by measurement of relative standard deviation (RSD, %) of results.

Results and Discussion

Loss in ITCs during RP-LC

RP-LC with C18 columns and aqueous mobile phases with up to 80% water are the most widely used methods for the determination of ITCs [13, 14, 20]. Using a similar method, a good separation of all ITCs could be achieved, the least separated peaks corresponding to BITC and PEITC, had a resolution (Rs) of 1.75 (Fig. 2a). However, due to their poor solubility in water, precipitation of ITCs in the LC system could be observed. The loss of each ITC (IL) was estimated by comparing the amounts injected and collected after separation. ITC solutions at two concentrations (80 and 1,000 μg mL^{-1}) were tested in order emphasize the impact of the concentration on the quantification data. Except for SFN, the most polar and hydrophilic compound (water solubility 8 g L^{-1}) [15], losses were observed at both concentrations, but were higher at 1,000 μg mL^{-1} than at 80 μg mL^{-1}. HITC, MITC, BITC and PITC, presented the highest IL values (26, 29, 32, and 24% at 1,000 μg mL^{-1}, respectively). Three of these compounds have low water solubility values (0.035, 0.11 and 0.13 g L^{-1}, respectively) [15]. However, MITC which is a polar compound (water solubility 3.9 g L^{-1}) was poorly soluble in the mobile phase at the working concentrations. With regard to AITC, proITC and PEITC, losses were lower (19, 17, and 14%, at 1,000 μg mL^{-1}, respectively). These results showed that mobile phases containing a high proportion of water (80%), by causing precipitation of ITCs in the LC system, could dramatically impact the accuracy of quantitative assessments.

RP-LC with Mercaptoethanol Precolumn Derivatization

The grafting of hydroxyl group (Fig. 1) makes ITC derivatives more polar and thus more likely to be soluble in aqueous mobile phases than their corresponding precursors. The optimal conditions for the reaction of derivatization were determined by using eight ITC standards. Following derivatization, the problems linked to the precipitation of ITCs in the mobile phase did not appear, even after several injections. The eight ITC-mce derivatives (Fig. 2b) were eluted in the order of their precursors' polarities, except for MITC-mce and SFN-mce whose polarities were switched by comparison to their respective precursors. By comparing the chromatograms in Fig. 2a (ITC 1,000 μg mL^{-1}) and Fig. 2b (ITC-mce 50 μg mL^{-1}), it can be noticed that the UV signals of ITC derivatives were more important than the UV signal of their precursors, which shows a high sensitivity of the mce-derivatization method.

In order to confirm peak identities and molecular mass, ITC-mce was analyzed by LC–MS, a method widely used for neutral compounds [21]. In the negative mode, the

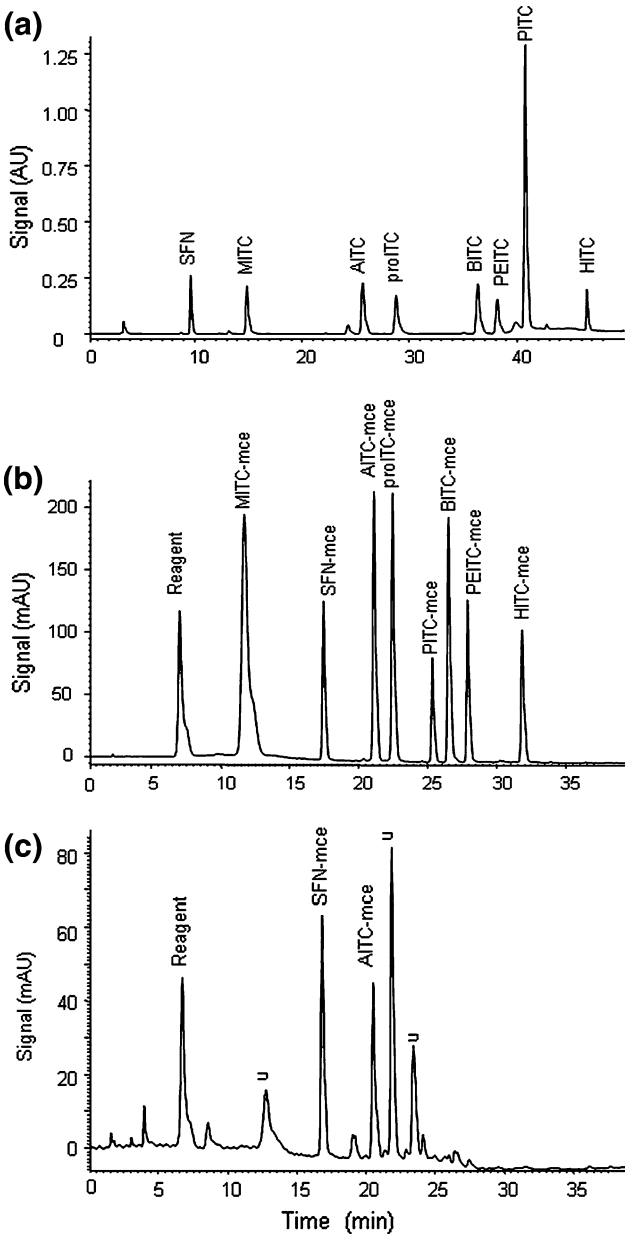

Fig. 2 Representative Chromatograms: **a** eight ITC standards (1,000 μg mL^{-1} each) separated on a Hypersil C18 column and a mobile phase consisted of water (A) and ACN (B). The gradient was as follows: 0–8 min, 20–40% B (*v*/*v*); 8–20 min, 40–50% B; 20–35 min, 50–60% B; 35–43 min, 60–100% B; 43–49 min, 100% B; 49–50 min, 100–20% B. The flow rate was 0.8 mL min^{-1}. UV detection was carried out at 245 nm; **b** eight ITC standard derivatives (50 μg mL^{-1}) and **c** white cabbage extract analyzed on a Nucleosil C18. The separation was achieved at a flow rate of 0.8 mL min^{-1} using a water (A) and ACN (B) gradient as follows: 0–10 min, 10% B (*v*/*v*); 10–40 min, 10–100% B; 40–45 min, 100% B. UV detection was carried out at 271 nm. At the end of each run, columns were allowed to equilibrate for 10 min before the following injection. *u* unidentified

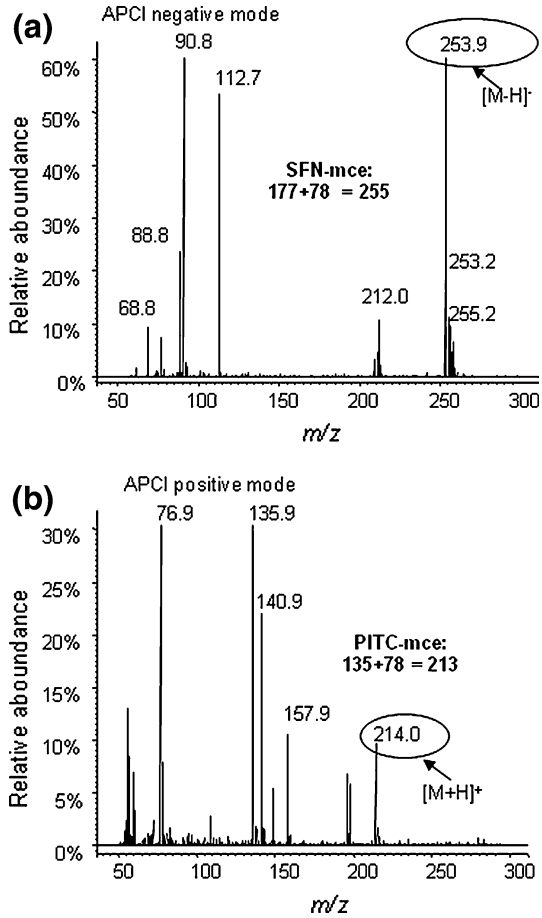

Fig. 3 **a**, **b** Mass spectra of SFN and PITC derivatives obtained by using APCI in positive and negative modes

derivatives of MITC, SFN (Fig. 3a), AITC, proITC, BITC, PEITC and HITC showed an $[M-H]^-$ molecular ion (Table 1). For the PITC-mce, a response was obtained only in positive mode (Fig. 3b). Another $[M+H]^+$ ion was observed at *m/z* 135.9 and corresponded to a fragment of PITC without mce. The comparison of the molecular mass of ITC-mce and the obtained *m/z* values allowed, therefore, the confirmation of their structures. As all tested ITCs easily reacted with mce, it is very likely that other R-NCS compounds could react in the same way.

Application of the Method

The derivatization method was used to investigate ITCs contained in white cabbage among the eight ITC standards available (Fig. 2c). With regard to the specificity, Fig. 2b, and c shows three peaks of compounds from cabbage with retention times that coincided with those of tested ITC, no other major interfering peaks are present. For the quantification, eight calibration curves were constructed by plotting the mean areas against ITC concentrations. The results indicated that there is a good correlation (r^2) between ITC peak area and concentration (Table 1). The resulting regression equations were used to determine the concentration of ITCs identified in cabbage. The LOD and LOQ of the method, shown in Table 1, suggest that low

Table 1 Results from identification and quantification of ITC derivatives

Identification				Quantification					
ITC derivatives	Molecular mass (M)	Ionization mode	*m/z* detected on the spectra	r^2	LOD (ng)	LOQ (ng)	Recovery (%)	Intra-day (RSD%)	Inter-day (RSD%)
MITC-mce	151	Negative	150	0.9996	2.8	9.4	99	2.5	0.1
SFN-mce	255	Negative	254	0.9995	2.9	9.7	101	1.1	1.2
AITC-mce	177	Negative	176	0.9999	2.1	7.1	97	1.6	1.3
proITC-mce	179	Negative	178	1	4.4	14.7	93	0.9	3.1
PITC-mce	213	Positive	214	0.9948	7.8	26.0	98	1.1	2.5
BITC-mce	227	Negative	226	0.9994	1.5	5.1	99	2.5	3.4
PEITC-mce	241	Negative	240	0.9994	14.2	47.7	101	0.9	1.2
HITC-mce	235	Negative	234	0.9998	6.5	21.7	90	0.6	2.6

quantities of all compounds can be determined. The results from recovery determinations indicated that there were no significant differences between the amounts of the ITCs added to samples and the amounts recovered (Table 1). With regard to the stability determined in an ACN/water solvent, solutions of ITC-mce derivatives were analyzed every week during 1 month and the peak areas obtained were compared. No decrease in the amounts of derivatives could be observed.

Compounds were identified by comparing their retention times, UV and mass spectra with those of ITC-mce derivatives previously obtained as described above. Based on these data, the chemoprotective AITC and SFN were identified and quantified in cabbage at 1.2 and 2.7 μg g^{-1} of fresh weight, respectively. A third compound whose retention time is close to that of proITC, was however found to be structurally different. Similar findings were mentioned in previous studies [19, 21]. Using LC–UV, Liang et al. [19] identified SFN in 18 varieties of Chinese cabbage, and estimated its contents in the range of 0.7–5.3 μg g^{-1} of fresh weight. Karcher et al. [22], by using laser-induced fluorescence capillary electrophoresis, identified SFN, AITC and Iberin in white cabbage grown in Oklahoma. It is noteworthy, however, that differences between studies in terms of the ITCs identified and of the amounts detected are quite common and may be attributed to differences in sample extraction methods, to genetic variability among different varieties of cabbage, and to the climate and cultivation conditions of cabbage [23, 24].

Conclusion

The accurate determination of ITCs in cruciferous vegetables is increasingly important given the growing interest surrounding these compounds. The widely used method today, which consists of RP-LC on C18 using gradient of ACN in water, did not allow accurate quantification of ITCs, due to precipitation in the presence of water. This study showed that a sound alternative could lie in the precolumn derivatization of ITCs so as to avoid the poor water solubility issue and increase the accuracy of RP-LC determinations. A previously described method based on a derivatization with mce was used. The generated ITC derivatives were shown to be more polar and more soluble in water, which allowed the accurate analysis of their corresponding ITCs using an aqueous mobile phase. The method proved sensitive with fairly low LOD and LOQ values allowing a determination in cabbage, and suggesting that the use of this technique could be extended to the analysis of other cruciferous vegetables.

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