Food Chemistry 214 (2017) 587–596

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

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

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ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form 19 July 2016
Accepted 20 July 2016
Available online 21 July 2016

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

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 μL of isopropanolic eluate obtained by SPE combined with 500 μL of derivatizing reagent (0.2 M NAC and 0.2 M $NaHCO_3$ 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 ($R^2 \geqslant 0.991$) 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.

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 (Kusznierewicz et al., 2012; Pilipczuk, Piekarska, Kusznierewicz, 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),

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http://dx.doi.org/10.1016/j.foodchem.2016.07.125
0308-8146/

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 termoionic-specific detector (TSD) (Trott, Lepage, & Hebert, 2012), flame ionization detector (FID) (Hanschen 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 worldwidely 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 MΩ/cm). All other reagents were of analytical grade. Bakerbond SPE Octadecyl C_{18} (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 (λ = 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.