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# Simultaneous Determination of Indolic Compounds in Plant Extracts by Solid-Phase Extraction and High-Performance Liquid Chromatography with UV and Fluorescence Detection

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**Abstract** A high-performance liquid chromatographic method with UV and fluorescence detection (HPLC-DAD-FLD) was developed for simultaneous determination of indolic compounds in plant material. Indole-3-carbinol (I3C), indole-3-acetic acid (I3AA), indole-3-acetonitrile (I3ACN), and 3,3′-diindolylmethane (DIM) were used as representative compounds that cover a wide spectrum of indole structures occurring in nature. For concentration and purification of the analytes, a solid-phase extraction (SPE) pretreatment was used. The separation utilized a Zorbax XDB-C8 reversed-phase column, acetonitrile-water containing 0.01 % formic acid gradient and UV (280 nm) in series with fluorescence (ex. 280 nm; em. 360 nm) detection. Good linearities of calibration curves were found within the ranges of 0.1–100 nmol/mL for I3C and DIM, 0.4–100 nmol/mL for I3AA and I3ACN for UV detection and 0.003–10 nmol/mL for I3C, 0.003–5 nmol/mL for DIM, and 0.02–10 nmol/mL for I3AA and I3ACN for fluorescence detection. The lowest detection limits (LOD) for UV detection were 0.03 nmol/mL for I3C, 0.1 nmol/mL for I3AA and I3ACN, and 0.04 nmol/mL for DIM, while for fluorescence detection were 0.001 nmol/mL for I3C and DIM, and 0.006 nmol/mL for I3AA and I3ACN. The usefulness of HPLC-DAD-FLD method with SPE pretreatment is illustrated by the analysis of indolic compounds in extracts from different parts of *Brassica* vegetables.

**Keywords** Indolic compounds · HPLC-DAD-FLD · SPE · Savoy cabbage · Brussels sprouts · Auxins

## Introduction

Indolic compounds are widespread throughout the plant kingdom. Plants can synthesize indoles by several independent biosynthetic pathways. Most of them start from tryptophan resulting in such secondary metabolites as indole-3-pyruvic acid, tryptamine, indole-3-acetaldoxime, indole-3-acetamide, or indolic glucosinolates (Kobayashi et al. 1993; Mashiguchi et al. 2011; Sugawara et al. 2009). There are also pathways independent of tryptophan in which biosynthesis of indole-3-acetic acid (I3AA) begins with indole-3-glycerol phosphate (Ouyang et al. 2000; Zhang et al. 2008) or indole (Mano and Nemoto 2012; Sitbon et al. 2000).

Diversified structures and physicochemical properties of indoles are behind their varied biological activities. Indolic phytohormones belonging to the auxins, such as indole-3-butyric acid, 4-chloroindole-3-acetic acid, and indole-3-acetic acid (I3AA), contain both hydrophilic acidic moiety (carboxyl group) and hydrophobic moiety (indole ring) in their structures (Barkawi et al. 2008). Another type of indoles is represented by indolic β-thioglucosides—glucosinolates (GLs)—a defense-related secondary plant metabolites that include glucobrassicin and its derivatives: 1-methoxyglucobrassicin (Pedras and Hossain 2011), 4-hydroxyglucobrassicin (West et al. 2004), 4-

methoxyglucobrassicin (Kim and Ishii 2006), and 1,4-dimethoxyglucobrassicin (Agerbirk et al. 2001). These phytochemicals following plant tissue disruption are degraded by endogenous enzyme myrosinase and other protein factors into new aglycon indolic compounds, which also differ in their physicochemical properties from precursor GLs. Glucobrassicin breakdown product—indole-3-carbinol (I3C) which possesses C=NH group—has different polarity than its dimer 3,3′-diindolylmethane (DIM) or its derivative *N*-methoxyindole-3-carbinol with *N*-methoxy group (He 1999). It can be presumed that the mentioned above modifications in the structure of indole ring determine differences in anticarcinogenic (Acharya et al. 2010; Kim et al. 2013; Neave et al. 2005; Wang et al. 2013), antioxidative (Shertzer et al. 1986), antimicrobial (Sung and Lee 2007b), or antifungal (Sung and Lee 2007a) properties. For example, I3C and DIM are well known for their multiple therapeutic and preventive effects on prostate (Zhang et al. 2014), colon, or breast (Maruthanila et al. 2014) cancers. Both compounds per se inhibit the growth of different cancer cell lines in vitro and in vivo, e.g., hepatocellular carcinoma cells (Wang et al. 2015), while I3AA requires combination with horseradish peroxidase or activation with UVB/Vis light to induce apoptosis of melanoma cells (Kim et al. 2009). I3ACN does not inhibit cancer cell growth but exhibits inhibitory effect against Gram-negative bacteria. I3C has no effect on this type of bacteria, even at a high dose (Aires et al. 2009), but has inhibitory activity against Gram-positive bacteria such as *Staphylococcus aureus* or *Escherichia coli* (Monte et al. 2014). These data pointing to various structure dependent health-promoting properties of some plant indoles have been confirmed in numerous studies and emphasize nutritional importance of indolic food components.

The significance of indoles in agriculture, as well as in human nutrition, has encouraged the development of several analytical methods for determination of these substances in plant samples (Durgbanshi et al. 2005; Hou et al. 2008). Unfortunately, plant indoles are difficult to analyze because they naturally occur in very low amounts in plants that, to make matters worse, are very rich in interfering components. To cope with this problem, before indoles can be reliably measured, the plant extracts must undergo several purification steps. For example, liquid-liquid extraction of I3AA metabolites was proposed by Liu et al. (2002). It occurred, however, that when the plant samples were analyzed, the interference from the impurities such as pigments allowed to identify only one analyte, so the additional purification step was necessary. In a different method, plant hormone analysis was preceded by HPLC column purification (Kowalczyk and Sandberg 2001). In this case, samples were injected into the LC/MS system and analytes were adsorbed on a C18 precolumn. After washing procedure, precolumn was connected to analytical column, and the analysis was performed in which as an internal standard heavy-labeled compounds had to be used. Moreover, purification on the C18 column was preceded by two liquid-liquid extractions, evaporation of the solvents and pH adjustment. When GC-MS technique is applied, yet, another additional step, such as derivatization with diazomethane (Barkawi et al. 2008) or methyl chloroformate (Liu et al. 2012) or trimethylsilyldiazomethane (Schmelz et al. 2003), is required. In the latter protocol, methylated derivatives must be additionally heated. After evaporation, the compounds of interest are collected on a polymeric adsorbent and then eluted into the vials (Schmelz et al. 2003). Purification of I3AA and its conjugates with amino acids is possible on an immunoaffinity column with immobilized polyspecific rabbit polyclonal antibodies against I3AA (Pencik et al. 2009). In this approach, sample extract is injected onto the immunoaffinity column, then washed with buffer, eluted, and analyzed by HPLC coupled with MS/MS detector. However, when applied to crude extracts, cross-reactivity or inhibition of antibodies may impair quantification results. These time-consuming methods were modified, and for example, liquid-liquid extraction of phytohormones was replaced by time-efficient single-drop liquid-liquid-liquid microextraction (Bai et al. 2012).

As can be concluded from the above survey of literature, a number of very specific techniques have been proposed for the determination of individual indolic compounds in plants. Nonetheless, there is no single fast and reliable method, which would enable quantitative and qualitative analysis of a wide spectrum of indolic compounds that can be expected to occur at highly variable amounts in the same plant sample. The main objectives of the present study were to simplify the procedures of indole purification from plant extracts and to develop a rapid and sensitive liquid chromatography method enabling the simultaneous separation of structurally divergent analytes. Spectrophotometric detection with diode array detector (DAD) is proposed to pinpoint a broad spectrum of even not identified indoles taking advantage of their characteristic spectral properties, while the use of fluorometric detection ensures high sensitivity of determinations. The applicability of the developed analytic procedure was verified for model indoles I3C, I3AA, I3ACN, and DIM, which cover a wide range of structural and physicochemical variations observed for this group of phytochemicals.

## Materials and Methods

### Chemicals and Reagents

The standard indolic compounds were purchased from Sigma (Steinheim, Germany), indole-3-carbinol (I3C; ≤100 %) and 3,3′-diindolylmethane (DIM; ≥98 %), or Merck (Darmstadt, Germany), indole-3-acetic acid (I3AA; ≥99 %) and indole-3-acetonitrile (I3ACN; ≥98 %). HPLC-grade solvents

(acetonitrile and methanol) and analytical-grade formic acid were obtained from Merck (Darmstadt, Germany). Bakerbond SPE Octadecyl $C_{18}$ (500 mg, 3 mL) and Bakerbond $C_{18}$ Polar Plus® (500 mg, 3 mL) cartridges were obtained from J.T. Baker (Greisheim, Germany).

### Plant Material

Seeds of *Brassica* plants produced by PNOS (Ożarowice Mazowieckie, Poland) were placed in germination plates and then transferred to the phytotron with controlled temperature (25 °C) and photoperiod (16 h of light, 8 h in the dark). During germination, the seeds were sprinkled with water twice a day. After 7 days, sprouts were harvested, washed to remove seed husks, and kept freeze-dried until investigation.

Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*) and savoy cabbage (*Brassica oleracea* L. var. *sabauda* L.) were harvested from organic plantation in Czapielsk (Poland); white cabbage (*Brassica oleracea* var. *capitata* f. *alba*) was grown in the vicinity of Gdańsk University of Technology, Gdańsk (Poland). The fruit papaya (*Carica papaya* L.) was obtained from a local store. Freshly collected vegetables were lyophilized, ground, and stored at 4 °C until investigation. The seeds were ground right before use.

### Extraction and Purification Procedures

The lyophilized samples were ground to a fine powder. Then, 0.2 g of plant material was mixed with 5 mL of sodium phosphate buffer (0.01 M; pH 7.4) and incubated for 3 h at 37 °C to enable full enzymatic degradation of glucosinolates (GLs). The reaction mixture pH was monitored, and when its level became stable for at least 20 min, the hydrolysis of GLs was regarded as complete. Subsequently, the samples were centrifuged (4 °C; 4000 rpm; 10 min; Eppendorf, Microcentrifuge 5415 R, Germany) and supernatants passed through a Bakerbond SPE $C_{18}$ 500-mg cartridges preconditioned with 3 mL of methanol and 6 mL of water. Stationary phase was washed with 1 mL of sodium phosphate buffer (0.01 M, pH 6.0). Finally, the retained indoles were eluted with 1 mL of methanol. The optimization of the purification conditions is described in the "Results and Discussion" section.

### HPLC Analyses

An Agilent 1200 Series HPLC-DAD-FLD system (Agilent Technologies, USA) was employed in the study. All modules were controlled by HPLC ChemStation data acquisition software. The column employed was Zorbax Eclipse XDB-C8 (150×4.60 mm, 3.5 μm) fitted with a suitable precolumn. The mobile phase consisted of (A) 0.01 % formic acid in water and (B) acetonitrile; the flow rate was set at 1 mL/min and the injection volume of extracts was 20 μL. The mobile phase gradient was programmed as follows: initially, 20 % B increased linearly to 44 % B over 15.4 min, then to 100 % B until 22 min, and finally was kept at 100 % B until 25 min. Finally, the system was equilibrated for 7 min. Indoles were monitored by UV detection at 280 nm (Agilent 1200 series diode array detector) and/or fluorescence detection at 280/360 (ex./em.) (Agilent 1260 fluorescence detector). The contents of indolic compounds in different samples, for which standards were available, were calculated from the calibration curves generated by the integration of areas of absorption peaks determined during analysis of serial dilutions of I3C, I3AA, I3ACN, or DIM.

## Results and Discussion

In order to achieve the optimum chromatographic conditions for analysis of indoles, variable parameters including mobile phase composition, injection volume, mobile phase flow-rate, and detection wavelength were tested. Only about 10 % of organic molecules have fluorophore structures. One of such groups embraces indolic compounds, which are therefore often used in experiments as fluorescence probes (Borsarelli et al. 2001; Callis 1997). This property of indole ring makes it possible to employ fluorescence detectors during chromatographic analyses, which offers high selectivity, combined with superior limits of detection (LOD) compared to UV detectors. For the four indolic standards selected for the current study, the optima for excitation and emission wavelengths were determined using combining a diode array detector with a fluorescence detector in series. This enables monitoring UV-VIS and fluorescence spectra in a single run. Such data sets were used to select the optimum fluorescence excitation and emission wavelengths for indolic compounds. Based on these results, the excitation and emission wavelength suitable for the simultaneous detection of all of tested analytes was set at 280 and 360 nm, respectively. The four indole derivatives were separated using a C8 column with water/acetonitrile gradient elution. With the addition of formic acid, chromatographic separation was improved greatly providing sharper peak shapes and better peak symmetry of the indolic standards. Figure 1 shows the representative chromatogram obtained during HPLC resolution of the standard substances with retention times of 6.16, 9.24, 14.95, and 21.22 min for I3C, I3AA, I3ACN, and DIM, respectively.

### Optimization of the Procedure of Extraction and Purification

In the developed method, solid-phase extraction (SPE) has been proposed as an alternative to popular liquid-liquid extraction (LLE) of indoles because of simplicity and easy automation. Reversed-phase sorbents (C18 in our study) are commonly used in SPE when aqueous samples are involved.

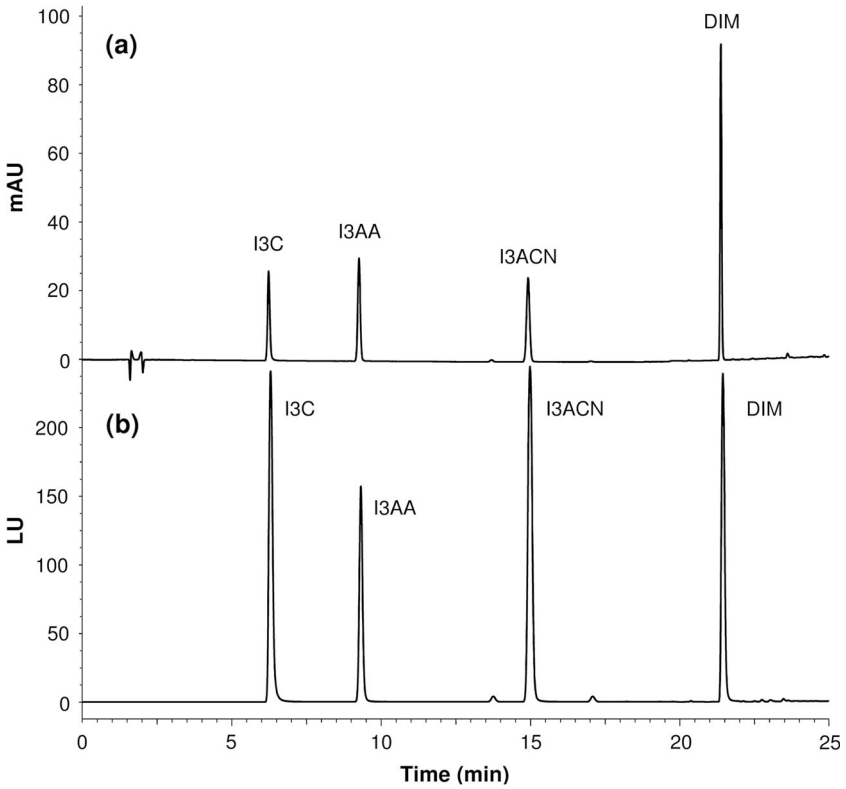


**Fig. 1** The representative HPLC chromatograms obtained for a mixture of standards of indolic compounds occurring most abundantly in *Brassica* plants followed by DAD ($\lambda$=280 nm) (*a*) or FLD (ex./em.—280/360 nm) (*b*). *I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane. The concentration of indoles in a mixture solution was 25 nmol $mL^{-1}$

To determine the most suitable medium for washing indole-containing sample retained on SPE cartridges, a series of methanol solutions of different concentrations were tested. In our study, 1 mL of mixed standard solution of I3C, I3AA, I3ACN, and DIM was loaded onto preconditioned $C_{18}$ cartridges. The concentration of the mixed standard solution was 10 nmol $mL^{-1}$ for I3C, I3AA, and I3ACN and 1 nmol $mL^{-1}$ for DIM. In order to eliminate the contaminating interferents as much as possible, the cartridges were washed by 1 mL of water, 1 mL of sodium phosphate buffer (0.01 M, pH 6.0), or 1 mL of 10, 20, 30, and 40 % methanol solutions and the acidified ones (0.1 % formic acid) of corresponding concentrations, respectively. The indoles were eluted with 1 mL of methanol. Results presented in Fig. 2 indicate that the subsequent recoveries of the indolic compounds from a stationary phase that was washed with methanol were better than those observed for acidified solutions of methanol. Moreover, acidified washing solutions caused decomposition of I3C, and new peak on chromatograms was observed (results not shown). Therefore, for further analyses, non-acidified washing medium was selected.

Because of the low recoveries of I3C and I3AA during SPE washing step, another stationary phase—Bakerbond $C_{18}$ Polar plus®—was tested. The obtained results presented in Fig. 2c indicate that such a type of cartridges did not improve recoveries of analytes studied and even additionally caused significant loses of DIM during washing procedure. Among six different washing solutions, the one containing sodium phosphate buffer (0.01 M, pH 6.0) ensured the highest retention of all four standards on the bed. Considering the above, for further analyses, Bakerbond $C_{18}$ cartridges and sodium phosphate buffer (0.01 M, pH 6.0) for washing the cartridges were chosen.

In order to completely elute the adsorbed indoles from stationary phase while leaving the strongly bound impurities behind, a series of methanol solutions of high concentrations was tested (Fig. 3). Aliquots (1 mL) of mixed standard solution of the I3C, I3AA, I3ACN (10 nmol $mL^{-1}$), and DIM (1 nmol $mL^{-1}$) were loaded onto preconditioned four Bakerbond $C_{18}$ columns and washed with 1 mL of sodium phosphate buffer (0.01 M, pH 6.0). Then, the eluting operation was carried out using 1 mL of 70, 80, 90, or 100 % methanol solutions. The results showed that 90 % methanol is sufficient to elute I3C, I3AA, and I3ACN, but in the case of DIM, 100 % methanol was required. Therefore, 100 % methanol was used as the eluting medium in further experiments.

## Method Evaluation

The linearity of detector response for each analyte was evaluated by using a series of standard solutions. For each standard solution, measurement was repeated three times. The calibration curves were constructed based on peak area ratio versus concentration of the analyte. The precisions of HPLC-DAD-FLD were examined by comparing the results of three times repeated analysis of the same mixed standard solution of

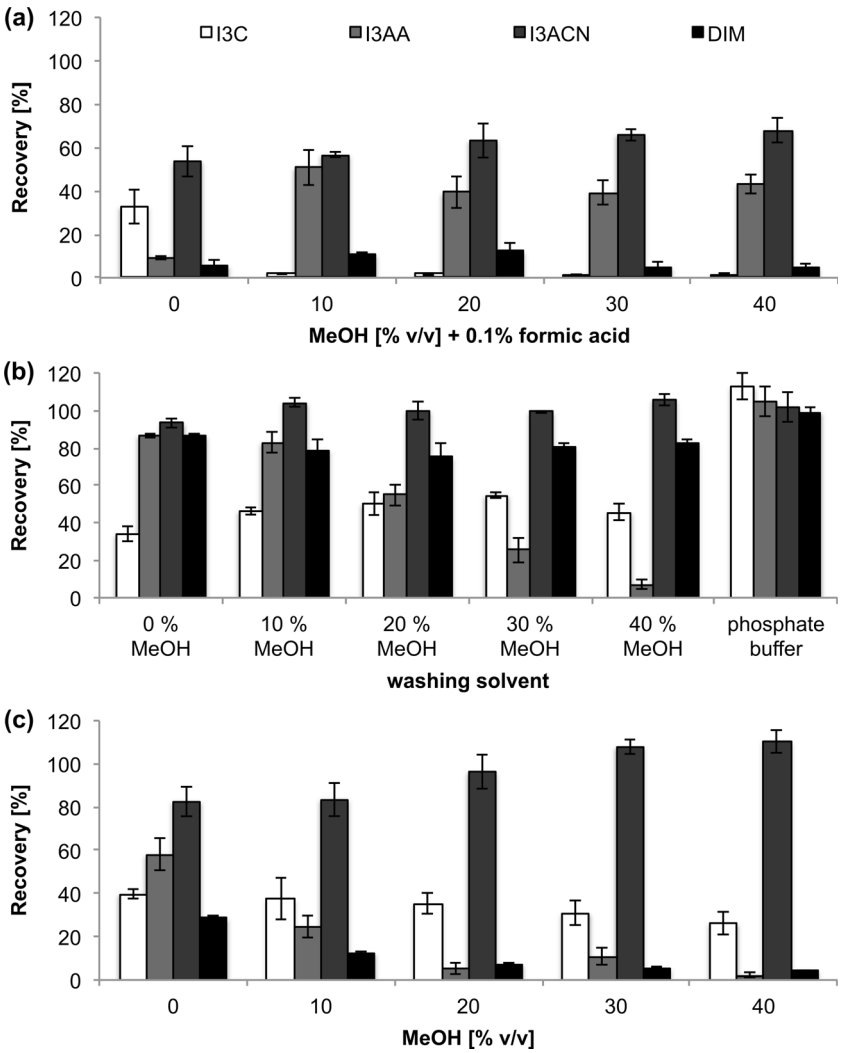


**Fig. 2** The comparison of retention efficiency of different indolic standard compounds on stationary phases: **a**, **b** Bakerbond $C_{18}$ or **c** Bakerbond $C_{18}$ Polar Plus. The washing solutions contained either MeOH+0.1 % formic acid in water (**a**) or MeOH in water (**b**, **c**) or 0.01 M phosphate buffer, pH 6.0 (**b**). *I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane. The results are means ± SD of two independent determinations

indoles. Table 1 gives the summary of linear range, calibration curve, and detection limit for all analyzed indoles.

For FLD detector, good linearities were found in the ranges of 0.003–10 nmol $mL^{-1}$ for I3C, 0.003–5 nmol $mL^{-1}$ for DIM, 0.02–10 nmol $mL^{-1}$ for I3AA and I3ACN. Spectrophotometric detector gave linear signal response in the range of 0.1–100 nmol $mL^{-1}$ for I3C and DIM or 0.4–100 nmol $mL^{-1}$ for I3AA and I3ACN. The relative standard deviations of retention times and peak areas were all under 0.21 % for FLD detector and under 0.52 % for UV detector. The detection limits based on signal-to-noise ratio of three to one were lower for I3C and DIM than for I3AA and I3ACN. The LOD and LOQ for analytes were about 17–40 times lower in the case of fluorescence detection and they were in the range of 0.001–0.006 and 0.003–0.02 nmol $mL^{-1}$, respectively. Other laboratories also observed higher sensitivity of fluorescence detection. For example, the values of LOD for I3AA were determined in the case of fluorescence detection at the level of 0.05 nmol $mL^{-1}$ (Szkop and Bielawski 2013) and for MS/MS detection at the level of 13 nmol $mL^{-1}$ (Hou et al. 2008). The Anderton et al. (2003) reported LOD for I3C and DIM equal to 0.20 and 0.12 nmol $mL^{-1}$ when the UV detector was used.

In order to examine the recovery efficiency of indolic analytes by the developed method, papaya extracts were spiked with four standard indoles at a different concentration

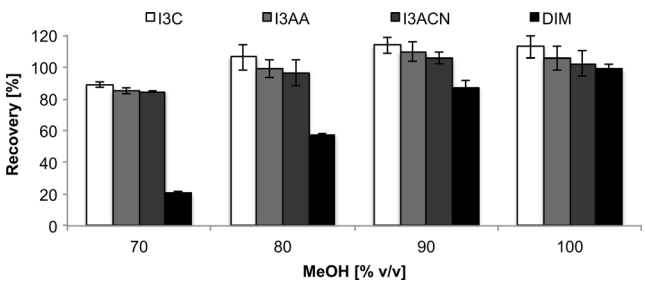


**Fig. 3** The influence of MeOH in water proportion in elution solvent on the recovery of indolic standard compounds from Bakerbond $C_{18}$ SPE column. *I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane. The results are means ± SD of two independent determinations

**Table 1** Calibration data for standard indoles determined by HPLC-DAD-FLD method using Zorbax Eclipse XDB-$C_8$ (150×4.60 mm, 3.5 μm) column

| Detector | Analyte | Concentration range (nmol $mL^{-1}$) | Calibration curve[a] | LOD (nmol $mL^{-1}$) | LOQ (nmol $mL^{-1}$) | RSD (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | Retention time | Peak area |
| FLD | I3C | 0.003–10 | $y$=152.59x+0.0033 | 0.001 | 0.003 | 0.06 | 0.15 |
| | I3AA | 0.02–10 | $y$=56.95x+0.0556 | 0.006 | 0.02 | 0.04 | 0.21 |
| | I3ACN | 0.02–10 | $y$=128.44x+0.0395 | 0.006 | 0.02 | 0.02 | 0.16 |
| | DIM | 0.003–5 | $y$=195.88x+0.0607 | 0.001 | 0.003 | 0.01 | 0.03 |
| UV | I3C | 0.1–100 | $y$=5.2813x+0.0391 | 0.03 | 0.1 | 0.05 | 0.26 |
| | I3AA | 0.4–100 | $y$=6.1444x+0.0094 | 0.1 | 0.3 | 0.04 | 0.52 |
| | I3ACN | 0.4–100 | $y$=5.9937x+0.0015 | 0.1 | 0.3 | 0.02 | 0.11 |
| | DIM | 0.1–100 | $y$=12.7430x+0.0044 | 0.04 | 0.1 | 0.01 | 0.20 |

*I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane, *LOD* limit of detection, *LOQ* limit of quantification, *RSD (%)* relative standard deviation

[a] $y$ is the peak area ratio of standard, $x$ is the concentration (nmol $mL^{-1}$), for each calibration curve correlation coefficient $R^2$ >0.999

level (0.003, 50, 100 nmol $mL^{-1}$ for I3C and DIM or 0.02, 50, 100 nmol $mL^{-1}$ for I3AA and I3ACN) and then extracted by the optimized extraction method. Every sample was analyzed in triplicate. The recoveries summarized in Table 2 were always higher than 94.5 % for I3C, 92.9 % for I3AA, 95.1 % for I3ACN, and 93.2 % for DIM considering three levels of spiking. Additionally, the stability of indoles was investigated by analyzing the plant extract spiked with standards of indolic compounds. The I3C, I3AA, I3ACN, and DIM turned out to be stable at −20 °C for at least 2 days (data not shown).

## Quantitative Analysis of Indoles in Plant Material

The HPLC-DAD-FLD method developed was applied to analyze I3C, I3AA, I3ACN, and DIM in plant material derived from different parts of savoy cabbage and brussels sprouts. In Fig. 4, the exemplary chromatogram obtained for cabbage sample is shown. In Table 3, the obtained levels of four indolic compounds are presented. The content of individual indoles differed significantly among various parts of *Brassica* plants. In the case of I3C and its dimer DIM, the highest content was observed in roots of both plants studied. The amount of I3C was 181 and 171 nmol $g^{-1}$ dw in the case of savoy cabbage and brussels sprouts, respectively. It is in accord with the observations that the highest concentration of the precursor indolic glucosinolates occurs in this organ of plant (Rosa 1997; Kirkegaard and Sarwar 1998). However, Lee et al. (2010) reported similar levels of I3C (630±210 nmol $g^{-1}$ dw) after autolysis in edible part of Chinese cabbage and lower amount of this analyte in mustard (130±40 nmol $g^{-1}$ dw). The high level of indoles in roots makes this usually discarded part of *Brassica* plants an attractive raw material for functional food and supplement production.

**Table 2** Recoveries obtained by HPLC-DAD-FLD analysis of papaya extract spiked with standard solutions of four indoles

| Analyte | Recovery (%)[a] | | |
|---|---|---|---|
| | 0.003[b] (0.02)[c] nmol $mL^{-1}$ | 50 nmol $mL^{-1}$ | 100 nmol $mL^{-1}$ |
| I3C | 94.5±6.3 | 113.8±1.4 | 113.6±7.3 |
| I3AA | 92.9±7.3 | 104.8±2.7 | 103.4±0.4 |
| I3ACN | 95.1±8.9 | 102.4±3.6 | 99.0±0.8 |
| DIM | 93.2±4.0 | 100.5±6.1 | 103.2±1.7 |

*I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane

[a] Results are mean±RSD (%) for three independent samples

[b] The concentration of I3C and DIM

[c] The concentration of I3AA and I3ACN (in parenthesis)

The data assembled in Table 3 reveal also other interesting interdependencies. The contents of I3C and DIM are significantly correlated in all samples studied (Pearson coefficient $r$=0.999 and $r$=0.891 in the case of savoy cabbage and brussels sprouts, respectively). This suggests that all DIM forms from I3C and conditions in all parts of the plants under study are promoting the condensation reaction to a similar extent. Different situation was described by Ciska et al. (2009) who observed lower I3C and higher DIM amounts in cooked fermented cabbage where the content of these compounds was respectively 0.85 and 1.70 nmol $g^{-1}$ fw. These results are in agreement with the observations by Bradlow and Zeligs (2010) who suggested alleviated conversion of I3C into DIM under acidic conditions.

In contrast to DIM, no correlation between I3C and I3AA contents is seen (Pearson coefficient $r$=−0.172 and $r$=0.236 for savoy cabbage and brussels sprouts, respectively). This is not surprising as the physiologically most active auxin in plants, that is I3AA, in the case of brassicas can be formed both as a product of degradation of indolic glucosinolates via

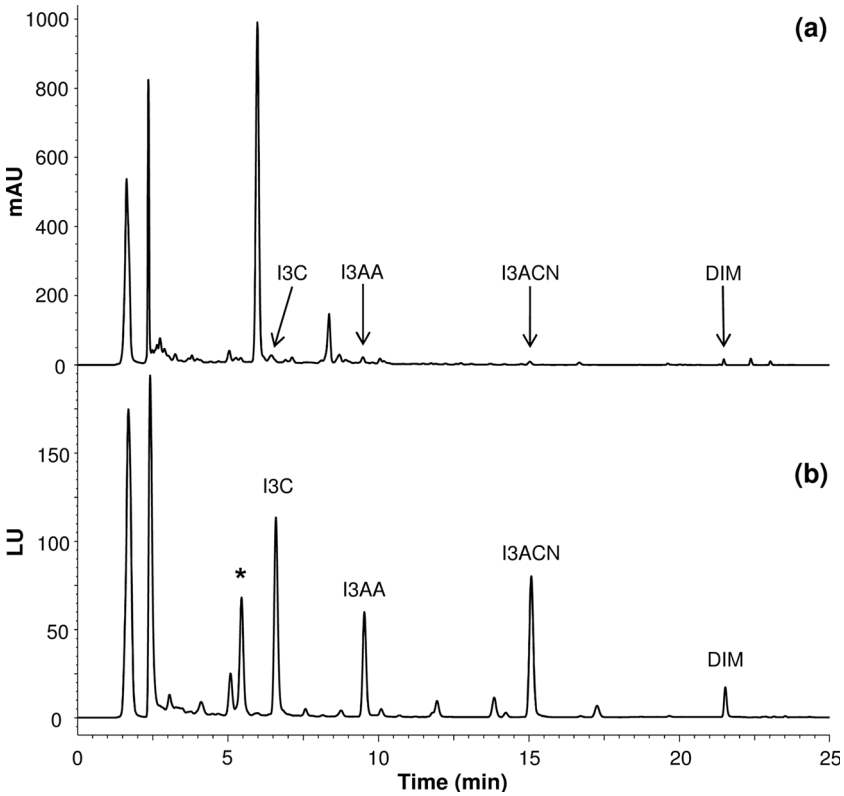


**Fig. 4** The representative HPLC chromatograms of white cabbage extract followed by DAD ($\lambda$= 280 nm) (*a*) or FLD (ex./em.— 280/360 nm) (*b*). *I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane, * unknown indolic compound, *LU* luminescence units, *mAU* milli-arbitrary units

I3ACN or be synthetized in a different pathway (De Vos et al. 2008). Apparently, both pathways are active in samples analyzed. The highest concentration of I3AA and I3ACN in sprouts can be explained by their high potential to fast growth. Interestingly, high concentration of I3AA is also present in stalk of brussels sprouts. It seems that I3AA auxin is needed to induce "sprouting" of heads from the stalk. As can be seen, the possibility of detection of several indole metabolites simultaneously may lead to an array of conclusions with regard to plant physiology.

## Conclusions

In this study, we have developed the HPLC-DAD-FLD method that enables simultaneous determination of structurally different indolic compounds in plant extract. In addition, the SPE method has been proposed as an alternative to popular liquid-liquid extraction of indoles, because of its simplicity and easy automation. The obvious advantage of this analytical procedure is the reduction of amounts of solvents used and also the efficiency of removal of interferences from extracts, while

**Table 3** The content of four indoles in *Brassicaceae* samples determined by HPLC-FLD method

| Sample | | Contents nmol $g^{-1}$ dw (mean±SD)[a] | | | |
|---|---|---|---|---|---|
| | | I3C | I3AA | I3ACN | DIM |
| Savoy cabbage | Roots | 181.2±4.6 | 12.90±0.50 | 0.98±0.03 | 0.2660±0.0058 |
| | Seed | 31.5±1.2 | 7.50±0.25 | 0.32±0.01 | 0.0853±0.0021 |
| | Sprouts | 2.3±0.1 | 18.69±0.741 | 155.93±7.37 | 0.0501±0.0022 |
| Brussels sprout | Roots | 171.5±6.7 | 11.52±0.34 | 1.32±0.03 | 0.4073±0.0180 |
| | Seed | 1.2±0.1 | 3.00±0.03 | 0.56±0.02 | 0.0660±0.0030 |
| | Sprouts | 2.5±0.1 | 18.41±0.83 | 172,62±8.56 | 0.1531±0.0080 |
| | Stalk | 77.9±2.5 | 18.86±1.05 | 12.30±0.05 | 0.1182±0.0028 |
| | Leaves (heads) | 24.1±1.2 | 2.92±0.10 | 0.64±0.02 | 0.1020±0.0057 |

*I3C* indole-3-carbinol, *I3AA* indole-3-acetic acid, *I3ACN* indole-3-acetonitrile, *DIM* 3,3′-diindolylmethane

[a] The results are mean ± SD for three independent samples

concentrating indoles, which is particularly important for the analytes occurring at the low level. The developed and optimized chromatographic conditions can be applied for routine analyses of plant extracts. The incorporation of fluorescence detection improved the sensitivity of detection, so the limit of detection (LOD) of indoles has become 40 times lower than in the case of commonly used UV-VIS-only detection. The satisfactory results of analyses of *Brassica* extracts demonstrated that this method may be suitable for the determination of indolic compounds in agriculture and plant physiology studies, as well as in food and food supplement industry.

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