Derivatization of Phytochelatins from *Silene vulgaris*, Induced upon Exposure to Arsenate and Cadmium: Comparison of Derivatization with Ellman's Reagent and Monobromobimane

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Phytochelatins (PCs) are a family of thiol-rich peptides, with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, with $n = 2-11$, induced in plants upon exposure to excessive amounts of heavy metals and some metalloids, such as arsenic. Two types of PC analyses are currently used, i.e., acid extraction and separation on HPLC with either precolumn derivatization (pH 8.2) with monobromobimane (mBBr) or postcolumn derivatization (pH 7.8) with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid), DTNB]. Although both methods were satisfactory for analysis of Cd-induced PCs, formation of $(RS)_3-As$ complexes during extraction of As-induced PCs rendered the DTNB method useless. This paper shows that precolumn derivatization with mBBr, during which the $(RS)_3-As$ complexes are disrupted, provides a qualitative and quantitative analysis of both Cd- and As-induced PCs. In addition, derivatization efficiencies of both methods for the oligomers with $n = 2-4$ (PC_{2-4}) are compared. Derivatization efficiency decreased from 71.8% and 81.4% for mBBr and DTNB derivatization, respectively, for PC_2 to 27.4% and 50.2% for PC_4. This decrease is most likely due to steric hindrance. Correction of measured thiol concentration is therefore advised for better quantification of PC concentrations in plant material.

Keywords: *Silene vulgaris; phytochelatins; heavy metal; arsenic; cadmium; Ellman's reagent; monobromobimane*

INTRODUCTION

Phytochelatins (PCs) are a family of small, thiol-rich peptides, produced in plants upon exposure to excessive amounts of heavy metals. They have the common structure $(\gamma\text{-Glu-Cys})_n$Gly, where $n = 2-11$ (Grill et al., 1985), although in maize, other grasses, and leguminous species, Gly can be substituted by Glu, Ser, and β-Ala, respectively (Grill et al., 1986a; Klapheck et al., 1994; Meuwly et al., 1995). The metalloid arsenic can induce PC synthesis as well in cell suspension cultures of *Rauvolfia serpentina* (Grill et al., 1987) and *Schizosaccharomyces pombe* (Grill et al., 1986b), root cultures of *Rubia tinctorum* (Maitani et al., 1996), and in roots of *Silene vulgaris* (Sneller et al., 1999).

Two methods are commonly used for measuring PCs: (1) separation with RP-HPLC, using postcolumn derivatization with Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)] (Habeeb, 1972; Grill et al., 1987; De Knecht et al., 1994) and (2) derivatization with monobromobimane (mBBr), prior to separation with RP-HPLC (Newton et al., 1981; Ahner et al., 1994; Rijstenbil and Wijnholds, 1996).

DTNB derivatization provides a cheap and fast method for the analysis of thiols. However, the minimum concentration for detection is fairly high, about 0.3 nmol of SH per injection. Separation of As-induced PCs by HPLC with postcolumn derivatization by DTNB proved to be unsatisfactory, due to the formation of stable complexes of GSH and other thiols with As(III), which occurs under acid to mildly alkaline conditions (Jocelyn, 1972; Scott et al., 1993; Delnomdedieu et al., 1994):

$$6H^+ + AsO_4^{3-} + 3RS^- \rightarrow (RS)_3-As + 3H_2O$$

The mBBr derivatization is more costly, but it is very sensitive, with a minimum concentration for detection of 0.3 pmol of SH per injection (Ahner et al., 1994). Another advantage of the mBBr derivatization is the extreme stability of the derivates: over 20 months no loss of fluorescence was observed for mBBr derivatives of cysteine and GSH (Fahey and Newton, 1987). In this paper, the derivatization efficiency of three main PCs occurring in metal-exposed plant material (PC_2, PC_3, and PC_4) of both methods was quantified by analysis of cysteine concentrations of purified PCs. For this purpose, both methods were then compared with amino acid analysis. This paper shows that the mBBr method is suitable for the determination of both Cd- and As-induced PCs in plant material.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest purity available. DTPA, DTNB, NAC, mBBr, and GSH were obtained from Sigma. TFA, formic acid, and acetic acid were obtained from Riedel−de Haen. Acetonitrile, potassium phosphate,

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methanol, ethanol, water, hydrogen peroxide, and sodium acetate were purchased from Baker. EDTA (titriplex III) was obtained from Merck; HEPPS and MSA were obtained from Fluka. Triethylamine, HCl, phenol, PITC, and the amino acid standard (standard H, no. 20088) were obtained from Pierce.

Plant Material and Culture Conditions. Seedlings of *Silene vulgaris* (Moench) Garcke (Amsterdam population) were hydroponically grown in a solution that contained 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $NH_4H_2PO_4$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.1 μM $CuSO_4 \cdot 5H_2O$, 0.1 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 20 μM Fe(Na)EDTA. After 14 days of preculture, the seedlings were transferred to fresh nutrient solution, buffered with 2 mM MES [pH 5.5 (KOH)], and exposed to 30 μM Na_2HAsO_4. The plants were put in a growth chamber (20/15 °C, light intensity (14 h d^{-1}) 200 μE m^{-2} s^{-1}, RH 75%). After 4 days the roots were rinsed with demineralized water and subsequently frozen in liquid nitrogen, lyophilized, and stored under vacuum until PC analysis. Cd-induced PCs were obtained by exposing *Silene vulgaris* seedlings to 40 μM $CdSO_4$ for 4 days, as described by De Knecht et al. (1994).

Extraction of PCs. Nonprotein thiols were extracted by homogenization of 20 mg of lyophilized root material in 2 mL of ice-cold 6.3 mM DTPA and 0.1% (v/v) TFA with a mortar, pestle, and quartz sand. The homogenate was centrifuged at 10000*g* in an Eppendorf centrifuge at 4 °C. The supernatant was filtered through a Costar Spin-X centrifuge tube with a nylon filter (0.22 μm).

Purification of PCs. Three main PCs occurring in metal-exposed plants (PC_2, PC_3, and PC_4) were purified from Cd-exposed root material by fast-performance liquid chromatography (FPLC). The filtered PC extract (0.5 mL) was eluted with a linear gradient of water with 0.1% (v/v) TFA and 0−25% (v/v) acentonitrile with 0.1% (v/v) TFA in a PEP−RPC column (HR 5/5, Pharmacia) (De Knecht et al., 1994). Purified PCs were divided into three fractions. An aliquot of 100 μL was mixed with 30 μL of 100 μM NAC as an internal standard, for testing of the derivatization with mBBr. For analysis of the derivatization with DTNB, 200 μL of the pooled fraction was mixed with 100 μL of 100 μM NAC. Waters Pico-Tag amino acid analysis was performed with a 1.0 mL sample. All samples were lyophilized prior to PC and amino analysis. Calibration curves of glutathione were used in all measurements.

Derivatization of PCs with mBBr. The lyophilized sample for mBBr was dissolved in 300 μL of 6.3 mM DTPA with 0.1% (v/v) TFA. The derivatization procedure was adapted from Rijstenbil and Wijnholds (1996): 450 μL of 200 mM HEPPS buffer, pH 8.2, containing 6.3 mM DTPA was mixed with 10 μL of 25 mM mBBr. To this mixture was added 250 μL of the PC sample, and derivatization was carried out for 5, 15, 30, and 60 min at 4, 22, and 45 °C in the dark, to optimize the derivatization. The reaction was stopped by adding 300 μL of 1 M MSA. The samples were stored in the dark at 4 °C until HPLC analysis.

PCs were separated on a Nova-Pak C_{18} analytical column (60 Å, 4 μm, 3.9 × 300 mm; Waters catalog no. 11695), kept at 37 °C with a column oven. Before injection, the column was equilibrated in 12% (v/v) methanol and 88% (v/v) water, both containing 0.1% (v/v) TFA, at a flow of 0.5 mL min^{-1}. Twenty-five microliters of derivatized sample was injected and run in a slightly concave gradient of 12−25% (v/v) methanol in 15 min, then a linear gradient from 25% to 35% (v/v) methanol from 15 to 29 min. Next, a linear gradient was used from 35% to 50% (v/v) methanol, from 29 to 50 min after injection. The column was then cleaned with 100% (v/v) methanol and reequilibrated in 12% (v/v) methanol. Fluorescence was monitored by a Waters 464 fluorescence detector. Excitation wavelength was 380 nm; emission wavelength was 470 nm. Total analysis time was 70 min. The analytical data were integrated by use of the Waters Millennium software.

Derivatization of PCs with DTNB. The lyophilized fractions for DTNB derivatization were dissolved in 100 μL of 6.3 mM DTPA and 0.1% (v/v) TFA. Derivatization with DTNB was carried out according to De Knecht et al. (1994). PCs were separated on a Waters Nova-Pak C_{18} column (catalog no. 36920) at 37 °C, using a linear gradient of acetonitrile and water, both acidified with 0.1% (v/v) TFA. The column effluent was derivatized with DTNB at pH 7.8, in a Waters RXN 1000 coil, with an Eldex postcolumn pump. Absorbance at 412 nm was monitored by a Waters 996 PDA detector. Total analysis time was 40 min.

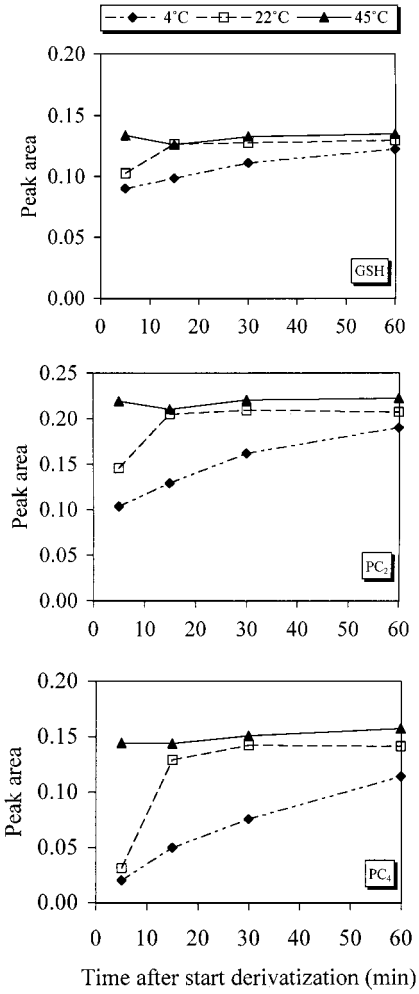

Figure 1. Optimization of the mBBr derivatization of several thiols (GSH, PC_2, and PC_4) at increasing time and temperature.

Amino Acid Analysis. Sample preparation for the amino acid analysis was carried out by the Pico Tag method as described by De Knecht et al. (1994), in which the purified PCs were subsequently peroxidized, hydrolyzed, and derivatized with PITC. The derivatized amino acids were then separated on a Waters Pico Tag HPLC column, thermostated at 37 °C (3.9 × 150 mm; Waters catalog no. 88131). The amino acid standard was used to identify the different amino acids.

RESULTS

Derivatization of PCs with mBBr. Derivatization of phytochelatins with mBBr was maximal within 30 min at 45 °C (Figure 1). However, mBBr hydrolysis bypeaks (Newton et al., 1981), identified in a sample without thiols, continued to increase after this time. To ensure maximal derivatization of longer-chain PCs, 30 min was chosen as standard derivatization time. The use of DTPA instead of the commonly used EDTA was described by Fahey and Newton (1987). The detection limit proved to be 0.3 pmol of SH per injection. GSH proved to be suitable for calibration [$y = 1\ 271\ 267x -$

Table 1. Results of Amino Acid Analysis of GSH and PCs from Roots of *Silene vulgaris*, Expressed as Cys/Gly Ratio[a]

compd	Cys/Gly ratio	compd	Cys/Gly ratio
GSH	1.09 (0.01)	PC_3	3.08 (0.09)
PC_2	1.95 (0.04)	PC_4	3.85 (0.07)

[a] Amino acids other than Cys, Glu, and Gly were not detected. Values are means (± SE) of four separate experiments.

Table 2. Efficiencies of mBBr and DTNB Derivatization of Thiol Groups of Three Main PCs[a]

	derivatization method	
	mBBr (%)	DTNB (%)
PC_2	71 (0.5)	83 (2.3)
PC_3	58 (1.4)	64 (1.9)
PC_4	27 (0.6)	49 (1.1)

[a] Efficiencies are expressed as percentage of amino acid analysis cysteine concentrations. Amino acid analysis results are set at 100%. Data are means (±SE) from seven separate experiments.

42 223, $R^2 = 0.998$, SE of the slope 61 577, SE of the intercept 12 897, SE of the regression coefficient 0.001 ($n = 7$)].

Derivatization with DTNB. Derivatization of PCs with DTNB was nearly maximal after 1.2 min at 37 °C (Rauser, 1991). Residence time in the RXN 1000 reaction coil was 1.33 min. Detection limit proved to be 0.25 nmol of SH per injection. GSH proved to be suitable for calibration [$y = 54\,115x + 8139$, $R^2 = 0.997$, SE of the slope 1139, SE of the intercept 3554, SE of the regression coefficient 0.003 ($n = 4$)].

Derivatization Efficiencies of mBBr and DTNB. Rechromatography of the purified PCs resulted in a chromatogram with a single PC peak, showing that the PCs were indeed purified well. Amino acid analysis results of PCs from Cd-exposed plant material showing that PC_2, PC_3, and PC_4 are present are given in Table 1.

Derivatization efficiency is defined as the ratio of the estimated thiol concentration as determined by one of the derivatization methods to the cysteine concentration as determined by amino acid analysis (Table 2). Cysteine concentrations as determined by amino acid analysis always exceeded those determined by the DTNB or mBBr derivatization. As chain length increased, derivatization efficiency decreased. This decrease was much stronger in the case of mBBr than in the case of DTNB.

Dilution of the samples (5 or 10 times) did not alter the derivatization efficiencies of both methods (data not shown).

Derivatization of As-Induced PCs. Separation of As-induced PCs, by postcolumn derivatization with DTNB, proved to be unsatisfactory. In contrast to the Cd−PCs (Figure 2B), the As−PCs (Figure 2A) were not separated. In addition, each time the same As-exposed sample was run, a different chromatogram was obtained (data not shown).

When As−PCs and Cd−PCs were derivatized with mBBr, both As−PCs (Figure 3A) and Cd−PCs (Figure 3C) were clearly separated. Furthermore, the chromatogram was reproducible, both with successive extractions and with successive injections.

The calculated concentrations of several thiols from arsenate-exposed roots differed between the DTNB and mBBr methods (Figures 2A and 3A, Table 3). Concentrations, based on a calibration curve of GSH, were compared, after correction for derivatization efficiencies, as shown in Table 2. Since longer PCs than PC_2 were not separated by the DTNB method, quantification of concentrations of these PCs was not possible. The decrease in recovery is higher for PC_2 than for the monothiols GSH and NAC.

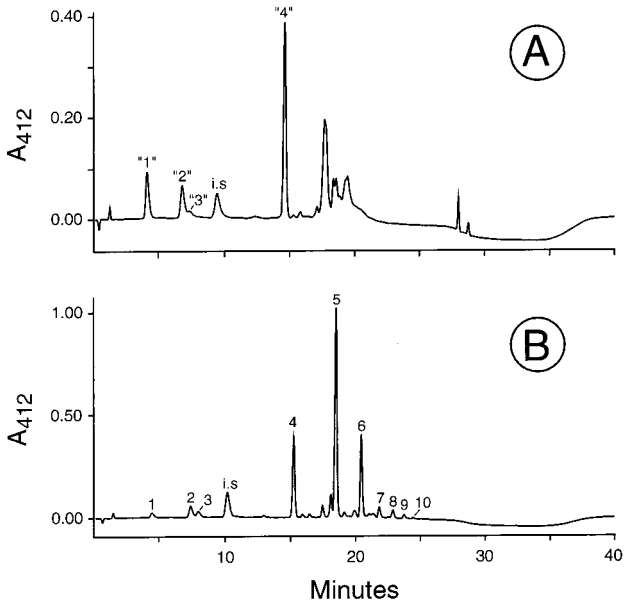

Figure 2. Chromatograms of phytochelatins from *Silene vulgaris* root material, exposed to AsO_4^{3-} (A) and Cd (B), using derivatization with DTNB. The peaks in panel A cannot be named with certainty (see text for explanation). The peaks in panel B are (1) cysteine, (2) glutathione, (3) γ-Glu-Cys, (4) PC_2, (5) PC_3, and (6) PC_4. Peaks 7−10 have not been identified but are most probably PC_{5-8}. Peaks without number have not been identified. i.s. = internal standard (N-acetyl-L-cysteine).

DISCUSSION

Derivatization Efficiency. Purified phytochelatins are not commercially available. It is therefore necessary to quantify them in a real sample with calibration by another thiol, such as GSH. For this procedure, however, the derivatization efficiencies must be known (Table 2). The exact magnitude of these figures may depend on reaction circumstances. The decrease in derivatization efficiencies with increasing chain length cannot be explained by insufficient reaction time (Figure 1), nor can it be explained by different cysteine content or availability of the different PCs (Table 1). Steric hindrance may provide a better explanation for this phenomenon. A larger molecule would encounter more steric hindrance in binding. Therefore, the steric hindrance would be stronger for mBBr derivatization, in which a larger molecule is attached to the PCs, than for derivatization with DTNB. Another possibility may be that due to a changed molar absorptivity or quantum yield of fluorescence, the intrinsic fluorescence is decreasing with an increasing molecular mass of the analytes (Fahey et al., 1981). However, the results from the DTNB derivatization support the steric hindrance hypothesis. DTNB provides an indirect measurement of derivatization: only half of the DTNB molecule, a TNB anion, is attached to the thiol group of the PC. The remaining free TNB anion is detected (Habeeb, 1972. Derivatization with DTNB decreases with increasing chain length as well, which supports steric hindrance. This idea is currently under investigation by MS techniques.

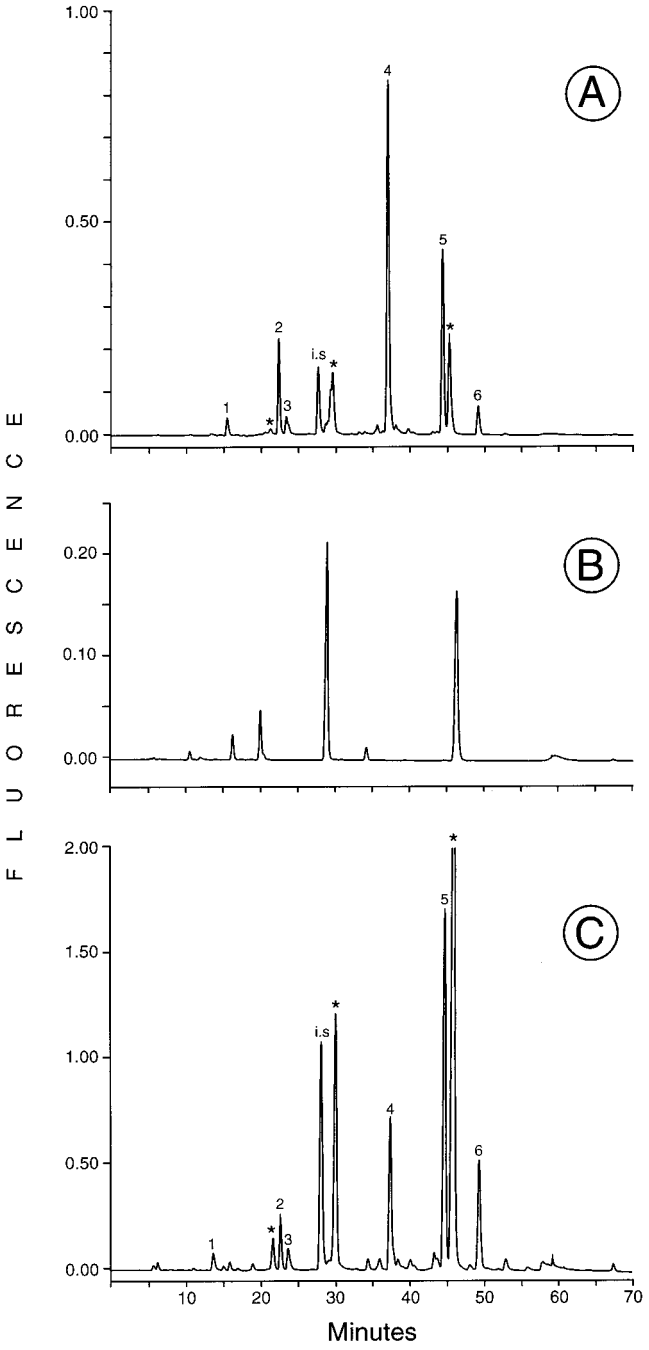

Figure 3. Chromatograms of phytochelatins from *Silene vulgaris* root material, exposed to AsO_4^{3-} (A) and Cd (C), using derivatization with mBBr. In panel B, a sample without thiols is shown to identify the mBBr hydrolysis peaks. Peak numbers correspond with those in Figure 2. Peaks with an asterisk are nonthiol mBBr hydrolysis peaks. i.s. = internal standard (*N*-acetyl-L-cysteine).

Separation of As-Induced PCs. Grill et al. (1986b, 1987), Maitani et al. (1996), and Sneller et al. (1999) have reported the induction of phytochelatins (PCs), heavy-metal-detoxifying thiol compounds, in plant cells after exposure to As(V) and As(III).

Under acid to mildly alkaline conditions, GSH and other thiols form complexes with As(III) (Jocelyn, 1972; Scott et al., 1993; Delnomdedieu et al., 1994). These complexes are stable over a broad pH range. The complex with glutathione dissociates at pH values exceeding 7−7.5, As(III) being oxidized to As(V), releasing reduced GSH (Delnomdedieu et al., 1994). During acid extraction of PCs, $(RS)_3$−As complexes will be formed or maintained in the sample, due to the low pH. In postcolumn derivatization with DTNB, the sample is injected onto the HPLC column and separated under acid conditions. In this way the complexes, rather than the individual PCs, are separated. After separation, the pH in the postcolumn coil is raised to 7.8. The complexes are then dissociated and the individual PCs are derivatized by DTNB. This is illustrated by Figure 2, in which the As−PCs are not separated, in contrast to the Cd−PCs.

Table 3. Relative Recovery (Concentration) of Different Thiols from Roots of Arsenate-Exposed *Silene vulgaris*[a]

	relative recovery	
thiol compd	mBBr	DTNB
N-acetyl-L-cysteine	1.00	0.94
GSH	1.00	0.81
PC_2	1.00	0.65

[a] Values were obtained by precolumn derivatization with mBBr and postcolumn derivatization with DTNB, after correction for derivatization efficiencies. Calculations were based on a calibration curve of GSH. Recoveries with mBBr were set at 1.00.

Since the affinity of dithiols for As(III) is higher than the affinity of monothiols for As(III) (Webb, 1966; Jocelyn, 1972), As(III) will bind more strongly to PCs than to GSH. This has also been found for Hg, Pb, Cu, and Cd (Matsumoto et al., 1990; Mehra and Mulchandani, 1995; Mehra et al., 1995, 1996). In addition, for Hg, Pb, and Cd, affinity increases with PC chain length (Matsumoto et al., 1990; Mehra et al., 1995, 1996). The complexes that we find with DTNB derivatization of As-induced PCs will thus preferentially be formed with longer-chain PCs. However, since the concentration of longer PCs is lower than that of shorter PCs, also shorter PCs and GSH will be incorporated in the complexes. Thus, a large number of different complexes will be formed, resulting in the chromatogram as shown in Figure 2A.

When As−PCs and Cd−PCs are derivatized with mBBr, the precolumn derivatization at pH 8.2 will ensure that no $(RS)_3$−As complexes will be present. Therefore, this pH was chosen for the derivatization. The thiol groups react with mBBr, and after the derivatization reaction is stopped by lowering the pH, the thiol groups will no longer be accessible for As(III) to form complexes. Next, the derivates are injected onto the HPLC column and both As− and Cd−PCs are separated correctly (Figure 3A,C). Of course, other derivatization methods at high pH could be used, but at high pH thiol groups will oxidize rather quickly. The use of mBBr ensures the fastest derivatization process (Blau and Halket, 1993) and thus the smallest chance for oxidation. In addition, it is a very sensitive method (detection limit 0.3 pmol of SH per injection (Ahner et al., 1994; this paper). Another solution to prevent the formation of $(RS)_3$−As complexes could be extraction with $NaBH_4$ (Grill et al., 1987; Maitani et al., 1996), in which the As will form the volatile As hydride, which will then escape from the solution. Lowering the pH again will, in that case, not give rise to complexes, since As is no longer present in the sample. However, the chance of oxidation of the thiol groups of the PCs will be increased when this highly alkaline extraction method is used.

The decrease in recovery as shown by Table 3 is higher for PC_2 than for the monothiols GSH and NAC,

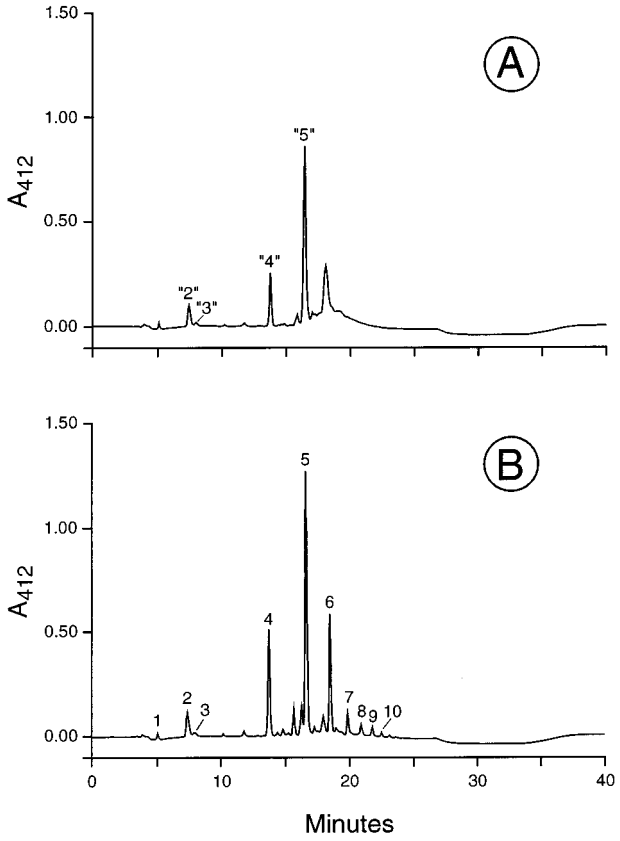

Figure 4. Chromatograms of Cd-induced PCs from *Silene vulgaris* root material. To this extract, As(III) (A) or As(V) (B) was added, prior to derivatization with DTNB. Peak numbers correspond with those in Figure 2.

which agrees with the results reported in the literature that binding of As to dithiols is stronger than binding of As to monothiols (Jocelyn, 1972; Scott et al., 1993; Delnomdedieu et al., 1994).

The formation of $(RS)_3$−As complexes also occurs after adding 500 μM (final concentration) As(III) as AsI_3 to a Cd−PC sample (Figure 4A) and postcolumn derivatization with DTNB. Peak height of GSH was reduced by 10%, peak height of PC_2 by 43%. Reduction of peak heights could not be calculated for longer PCs, because they were not separated with postcolumn derivatization. Addition of As(V) as AsO_4 to the Cd−PC sample did not alter the chromatogram (Figure 4B). In addition, adding As(III) to a GSH solution at a low pH gives rise to a peak that coelutes with PC_2. This is the $(GS)_3$−As complex (data not shown). So in Table 3 the recovery of PC_2 may actually still be overestimated due to coelution of $(GS)_3$−As with PC_2.

From the results presented here, it is concluded that mBBr precolumn derivatization of As-exposed plant samples provides a correct and sensitive measurement of As-induced PCs. The results show that the thiol content of PC_{2-4} and most likely even more of still longer chain PCs is underestimated with both the DTNB and mBBr methods. This underestimation is larger for the mBBr method. Therefore, we suggest that derivatization efficiencies should be included in calculations of phytochelatin concentrations of plant material.

ABBREVIATIONS USED

DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5′-dithiobis[2-nitrobenzoic acid]; EDTA (titriplex III), (ethylenedinitrilo)tetraacetic acid disodium salt dihydrate; FPLC, fast-performance liquid chromatography; GSH, glutathione; RP-HPLC, reversed-phase high-performance liquid chromatography; HEPPS, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid; mBBr, monobromobimane; MES, 2-(*N*-morpholino)ethanesulfonic acid; MSA, methanesulfonic acid; NAC, *N*-acetyl-L-cysteine; PC, phytochelatin; PITC, phenylisothiocyanate; TFA, trifluoroacetic acid.

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