



Separation and quantification of monothiols and phytochelatins from a wide variety of cell cultures and tissues of trees and other plants using high performance liquid chromatography

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ABSTRACT

The HPLC method presented here for the quantification of metal-binding thiols is considerably shorter than most previously published methods. It is a sensitive and highly reproducible method that separates monobromobimane tagged monothiols (cysteine, glutathione, γ -glutamylcysteine) along with polythiols (PC₂, PC₃, PC₄ and PC₅) within 23 min from a wide variety of samples. Total run time of the method is 35 min. Detection limits for thiols is 33 fmol for 10 μ L injection. This method will be applicable to study the metal detoxification mechanisms for a wide variety of cell cultures and tissues of plants and trees including algae, Arabidopsis, crambe, rice, and red spruce.

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1. Introduction

Potential metal-binding ligands in plants and animals include amino acids and organic acids, and two classes of cysteine-rich peptides; phytochelatins [PCs, γ -(Glu-Cys)_{*n*}-Gly or (γ -EC) *n*-Gly] and metallothioneins [1,2]. Phytochelatins are found in plants ranging from algae to trees. The number of γ -EC moieties in PCs varies from 2 to 11. Thiol-rich PCs (also known as class III metallothioneins-like proteins) are involved in both metal homeostasis and protection of plants from metal toxicity by serving as metal chelators [3]. PCs are enzymatically synthesized by PC synthase [4] via consecutive transfer of the γ -EC moiety to glutathione [GSH, γ -(Glu-Cys)-Gly]. The monothiols, Cys and GSH, are actively involved in PC synthesis as well as in metal sequestration in plants. As an antioxidant and PC precursor, GSH and its metabolism also play an important role in plant responses and adaptation to various stress conditions. Consequently, there has been tremendous interest in studying changes in the levels of cellular thiols.

Various analytical approaches, such as capillary electrophoresis equipped with either laser-induced fluorescence detection [5] or photodiode array detection [6]; electrochemical methods [7,8]; high-performance liquid chromatography (HPLC) [9–15] and mass spectrometry [16–19] with fluorescence detections have been used to detect biologically active thiol compounds in organisms at different phylogenetic levels ranging from phytoplankton to humans. Each method mentioned here has advantages and disadvantages as described in the review by Kawakami et al. [15]. However, there is little available information related to the determination of thiol compounds in foliage of forest trees [20–22]. Thangavel et al. [23] described a short HPLC method that was used for the analyses of monothiols and PC₂ in red spruce (*Picea rubens* Sarg.) cell suspension cultures exposed to Cd or Zn stress. The present study optimized this HPLC method to make it more versatile and to improve the reproducibility of retention times by changing both the solvent composition and the gradient profile. The updated method can also quantify additional thiols (Cys, PC₃, PC₄ and PC₅) not described in our earlier method. This method is more sensitive than other established methods for PC analyses [24,25]; it has a shorter total run time and was used with a variety of tissues from algae to higher plants.

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2. Experimental

2.1. Chemicals

All chemicals used in the analyses were of the highest purity available [$>99\%$ except for γ -glutamylcysteine (95%), PC₂ ($>98\%$), PC₃, PC₄ and PC₅ ($>95\%$)]. Trifluoroacetic acid (TFA), methanesulfonic acid (MSA), 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), *N*-acetyl-L-cysteine (NAC), glutathione, γ -glutamylcysteine were purchased from Sigma, St. Louis, MO, USA; diethylenetriamine-pentaacetic acid (DTPA) and L-cysteine (Cys) were obtained from Fluka, Milwaukee, WI, USA; monobromobimane (mBBR) was purchased from Molecular Probes, Inc., Eugene, OR, USA; custom ordered phytochelatins (PC₂, PC₃, PC₄ and PC₅) [PC_{*n*}, (γ -Glu-Cys)_{*n*}-Gly, where *n* = 2–5] were obtained from Anaspec, San Jose, CA, USA. HPLC-grade acetonitrile (ACN) (Burdick & Jackson, Muskegon, MI, USA), methanol (EMD Chemicals Inc., Gibbstown, NJ, USA) and all solvents were filtered with a 0.2 μ m nylon filter (Nylaflo; Pall Corp., Ann Arbor, MI, USA). Water was purified by a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Sample preparation

Cell suspension cultures, algal cultures, root, shoot, foliar and wood tissue samples of different species were used for analyses of thiol compounds including Cys, GSH, γ -EC, and PCs. Protocols for collection of samples for thiol derivatization varied with species and cell type.

2.2.1. Cell suspension and algal cultures

The maintenance of cell suspension cultures of red spruce [*P. rubens* Sarg. (cell line RS 61.1, 03–92)] and details of metal additions to the cells are described in Thangavel et al. [23]. Poplar (*Populus nigra* $\times$ *maximowiczii*) cultures were maintained as described in Page et al. [26]. Marine green algal cultures (*Dunaliella tertiolecta*) were maintained as described in Jahnke and White [27]. Thirty mL of algal cultures were exposed to 100 μ M Cd for 24 h before collection. The cells were harvested by vacuum filtration using Miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA). Filtered cells were washed twice with an equal volume of deionized water. Approximately 0.1 g of cells grown in suspension cultures were collected for analyses.

2.2.2. Root, shoot, foliar and wood tissues from different plant species

Foliar samples of mature red spruce (*P. rubens*) and sugar maple (*Acer saccharum*) trees growing at high elevation at Hubbard Brook Experimental Forest, NH, USA, were collected and processed according to the procedure described in Minocha et al. [28].

Using an increment hammer, two to three wood plugs (4 mm in diameter and 1.5 cm in length, without bark) were pooled per sample from balsam fir (*Abies balsamea*) trees growing at the Penobscot Experimental Forest, ME, USA.

Cuttings of willow shrub clones *Salix eriocephala* (00x-026-082, an arsenate sensitive clone) and *Salix purpurea* (99113-012, a clone moderately tolerant to arsenate) were grown without phosphorus supplementation in hydroponic cultures and exposed to 250 μ M sodium arsenate dibasic heptahydrate for three weeks according to the detailed procedure described in Purdy and Smart [29].

Seedlings of crambe (*Crambe abyssinica*) were grown in half-strength MS liquid media for seven days with constant shaking and then exposed to 250 μ M sodium arsenate for 24 h. For rice, 10-day-old rice seedlings grown in vermiculite were transferred to hydroponics system containing half-strength Hoagland solution,

acclimatized for another 10 days and then seedlings were exposed to 150 μ M sodium arsenate for 24 h. Both crambe and rice seedlings were grown in growth chambers at 22 and 26 °C, respectively, with a light regime of 16h/8h light/dark periods. The harvested samples were thoroughly washed with deionised water and kept in liquid nitrogen before shipment to Minocha laboratory.

Arabidopsis (*Arabidopsis thaliana*, ecotype Columbia) plants were grown in 4 parts Pro-Mix (Scotts Company, Marysville, OH, USA) soil-less medium supplemented with 2 parts Perlite, and watered by capillary action using 1/4–1/2 strength Miracle-Gro (Scotts Company, Marysville, OH, USA) fertilizer solution every third day. Twenty-day-old plants were kept under 18 h photoperiod (80 μ mol m^{−2} s^{−1} fluorescent light) at 21 °C. These plants were grown and treated with 250 μ M Cd dissolved in water for five days before the collection of leaf samples according to the method described in Rice [30].

Approximately 0.2 g of shoot or foliar samples and approximately 0.3–0.5 g of wood tissues were collected for analyses.

2.3. Extraction of thiols

Acid extraction is necessary to avoid oxidation of thiols and to allow the precipitation of proteins and other undesirable molecules that could interfere in the derivatization reaction. Trichloroacetic acid, sulfosalicylic acid, trifluoroacetic acid, and hydrochloric acid have all been used for the extraction of non-protein thiols [6]. In the present study, 6.3 mM DTPA containing 0.1% (v/v) TFA was used as an extraction buffer according to the method of Sneller et al. [12].

After collection, all cell/tissue samples were immediately placed in individual pre-weighed microfuge tubes containing 1 mL of extraction buffer (6.3 mM DTPA containing 0.1%, v/v, TFA). The only exceptions were crambe and rice where extraction buffer was added to previously frozen samples. Field samples were kept on ice during transportation to the laboratory. Samples were stored at −20 °C until the time of analyses. Samples were frozen (−20 °C) and thawed three times prior to analyses to release cellular contents according to the procedure described in Minocha et al. [31]. The supernatant was collected by centrifugation at 13,000 $\times$ g for 10 min and used for subsequent analyses.

2.4. Preparation of standard and reactant solutions

Ten microliter aliquots of 8 mM stock solution of each standard (Cys, GSH, γ -EC, NAC, PC₂, PC₃, PC₄, and PC₅) were prepared using deionized water and stored in the dark at −20 °C. With the exception of NAC, appropriate portions of each stock were mixed together and further diluted with extraction buffer (6.3 mM DTPA with 0.1%, v/v, TFA) to create a series of seven working standards (S1–S7) with concentrations of Cys, GSH and γ -EC at 1, 2, 3, 4, 5, 7.5 and 10 pmol μ L^{−1} injected and PC₂–PC₅ at 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2.0 pmol μ L^{−1} injected. Internal standard, NAC was used at a final concentration of 5.0 pmol μ L^{−1} injected. Twenty millimolar TCEP solution was made in HEPPS buffer and 50 mM mBBR solution was made in ACN. The mBBR solution was covered with aluminum foil and kept in the dark before use. Final dilutions of standards and solutions of TCEP and mBBR were made on the day of derivatization.

2.5. Derivatization of thiol compounds

The derivatization of thiol compounds with mBBR was based on the methods of Rijstenbil and Wijnholds and Sneller et al. [11,12], as described by Thangavel et al. [23]. Briefly, 615 μ L of 200 mM HEPPS buffer (6.3 mM DTPA, pH 8.2) was mixed with 25 μ L of 20 mM TCEP that was used as a disulfur reductant. To this mixture, 250 μ L mix of standards or sample extract was added. Ten microliters of 0.5 mM

Table 1
Solvent gradient profile for the separation of thiol compounds using monobromobimane (mBBBr) derivatization

Step	Time (min)	Flow (mL min ⁻¹)	Solvent A (by volume) [ACN + 0.1% TFA (%)]	Solvent B (by volume) [89.9% water + 10% ACN + 0.1% TFA (%)]	Curve ^a	Cumulative time (min)
1	0.5	1.0	0.0	100	0	0.5
2	11.2	1.0	10.6	89.4	1	11.7
3	13.6	1.0	21.1	78.9	1	25.3
4	5.0	2.5	100.0	0.0	0	30.3
5	4.0	2.5	0.0	100	0	34.3
6	0.5	1.0	0.0	100	0	34.8

^a 1, Linear; 0, step.

NAC was also added as an internal standard. This reaction mix was pre-incubated at 45 °C for 10 min in a water bath in order to fully convert the disulfide bonds to sulfhydryls so that the thiols were present in a reduced state before mBBBr derivatization. The derivatization was then carried out by incubating the samples in the dark in a water bath for 30 min at 45 °C after the addition of 10 µL of 50 mM mBBBr. The reaction was terminated by the addition of 100 µL of 1 M MSA. The derivatized samples were filtered with 0.45 µm nylon syringe filters (Pall-Gelman Labs, Ann Arbor, MI, USA) and stored at -20 °C for up to a week for HPLC analyses. Some samples were stored at -20 °C for a period of up to 2 years to test the stability of derivatized samples.

2.6. Recovery assays

Recovery assays were conducted to determine the extent of binding of free thiols with other charged compounds in sample extracts before mBBBr derivatization could occur. Such binding may interfere with the accuracy of quantification. Since the extent of binding varied with sample type, assays were conducted with different species and tissue types. Samples were derivatized with or without the addition of known amounts of standards mix (10, 15, 20 and 25 pmol of Cys, GSH, γ-EC, NAC and 1, 2, 3, 4 and 5 pmol of PC₂–PC₅ per 5 µL injection) according to the derivatization protocol described above. The percent recovery of the known amount of an added standard indicated the extent of binding. These assays were run in duplicate and the experiment was conducted twice.

2.7. Instrumentation

The HPLC system consisted of a PerkinElmer (Wellesley, MA, USA) Series 200 pump, fluorescence detector, degasser and autosampler, fitted with a 200 µL loop. The columns included; a Phenomenex Synergi-Hydro-RP C₁₈ column (4 µm particle size, 100 mm × 4.6 mm) and a C₁₈ Securityguard™ (5 µm, 4 mm × 3 mm) cartridge guard column (Phenomenex, Torrance, CA, USA). The temperature of the column oven was maintained at 40 °C with a column heater (Bio-Rad Laboratories, Hercules, CA, USA). A C₁₈ Scavenger (10 µm, 3.3 mm × 4.6 mm) cartridge column (PerkinElmer) was placed in line between the pump and autosampler to further purify the solvents before they entered the analytical column. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. Data were integrated using TotalChrom HPLC software (PerkinElmer, Version 6.2.1).

2.8. Chromatographic conditions

Thiol compounds were separated by using solvents (A) 99.9% ACN and (B) 89.9% water + 10% ACN, both containing 0.1% TFA by volume. The gradient profile is described in Table 1. A linear gradient of mobile phase A from 0 to 10.6% was run for 11.2 min at

1 mL min⁻¹ to elute monothiols. Further, the linear gradient of solvent A was raised from 10.6 to 21.1% in 13.6 min at 1 mL min⁻¹. After this step, the column was washed with 100% of solvent A for 5 min at a flow rate of 2.5 mL min⁻¹. The column was equilibrated with 100% of solvent B for a total of 4.5 min; 4 min at 2.5 mL min⁻¹ and then 0.5 min at 1.0 mL min⁻¹ to match the initial run conditions. Total run time for each sample was 34.8 min including column cleaning and re-equilibration. The injection volume was 5–10 µL depending upon the type of tissue being analyzed.

2.9. Column maintenance

At the end of each batch of sample runs (50–80 injections), a cleaning method was used before shutting down the system. This cleaning method consisted of 3 steps: Step 1 (Solvent A); Step 2 (Solvent C consisted of distilled and deionized water containing 10% ACN) and Step 3 (45% Solvent A and 55% Solvent C), each for 15 min at a flow rate of 1 mL min⁻¹ in the order given here. Before beginning a new batch of sample injections, the column was manually reversed and flushed using a two step wash method: Solvent A for 35 min followed by 10% Solvent C for 5 min, both at a flow rate of 1 mL min⁻¹. The column was equilibrated with 100% Solvent B (buffer) at 1 mL min⁻¹ for 20 min. The next batch of samples was then run using the column in the same direction in which it was cleaned and equilibrated. Therefore, on a routine basis, the analytical column was used in both orientations for sample analyses. The guard column was replaced every two weeks depending on the total number of sample runs. The life of the analytical column was prolonged considerably by following these protocols.

2.10. Peak identifications

In order to identify peaks arising from the derivatization of reagents, a blank reaction mix was run in which the volume of the sample was replaced with extraction buffer. Identification of unknown peaks was made by comparing the profiles of blank (extraction buffer) and individual standards or standards mix with known retention times. In cases where a known peak co-eluted with other unknown compounds or retention times shifted, samples were spiked with a known single standard or a mix of standards to identify the actual thiol compounds. Multiple analyses were performed using blanks and standards to determine detection and quantitation limits, response linearity and reproducibility of the protocol.

3. Results and discussion

Two well known approaches for the quantification of phytochelatins using HPLC are: (i) post-column derivatization with Ellman's reagent [DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid)] [4,32] and (ii) pre-column derivatization with mBBBr [11,23,33]. In this study, the latter approach was used for the identification and quantification of thiols. The proposed method consisted of

Table 2Linear ranges and r^2 values for quantification of thiol compounds

Component name	Retention time (min)	Linear range tested (pmol)	r^2		Slope ($\times 10^2$)		Intercept ($\times 10^2$)	
			Mean	S.E.	Mean	S.E.	Mean	S.E.
Cys	3.47	5–100	0.998	0.001	86.02	1.36	1.20	0.72
GSH	6.26	5–100	0.995	0.002	116.54	8.10	−6.20	2.93
γ -EC	6.78	5–100	0.998	0.001	75.29	1.25	2.52	0.64
PC ₂	13.88	1–100	0.997	0.001	146.78	7.46	−0.46	0.55
PC ₃	17.65	1–50	0.997	0.001	156.05	8.79	−0.36	0.50
PC ₄	20.36	1–25	0.995	0.002	141.17	4.60	−2.87	0.52
PC ₅	22.54	1–25	0.984	0.003	100.94	12.66	−6.24	0.84

Standard curves were run with 6–7 points.

three main steps after the thiols were extracted: (i) reduction, (ii) pre-column derivatization and (iii) separation of mBBR derivatized thiols using HPLC followed by fluorescence detection.

3.1. Reduction of thiols

The reactions of mBBR with thiols are pH dependent and an alkaline buffer has to be used for better derivatization [34]. In this study, HEPES buffer (pH 8.2) was used for tagging thiol compounds present in samples. Due to the presence of oxidative species in environmental samples, a reducing reagent (TCEP) was used for converting disulfide bonds to release sulfhydryl groups before derivatization of total thiols [13]. 2-Mercaptoethanol, a reducing agent, was shown to interfere with the derivatization of thiols in

the marine microalga, *Tetraselmis suecica* [6]. Other studies have shown that dithiothreitol (DTT) TCEP reacts with the derivatizing reagents and results in the production of additional chromatographic peaks that interfere with the peaks of interest [11,35]. For the present study, the thiol reductant TCEP was used. In comparison with DTT, it produced fewer blank reagent peaks (37 peaks for DTT as compared to 25 for TCEP). In addition, TCEP is a faster and stronger reductant as compared to DTT at a pH of approximately 8.0 [36].

3.2. Derivatization with mBBR

Though pre-column derivatization with mBBR is costly, it is a very sensitive method for the detection of thiol compounds and

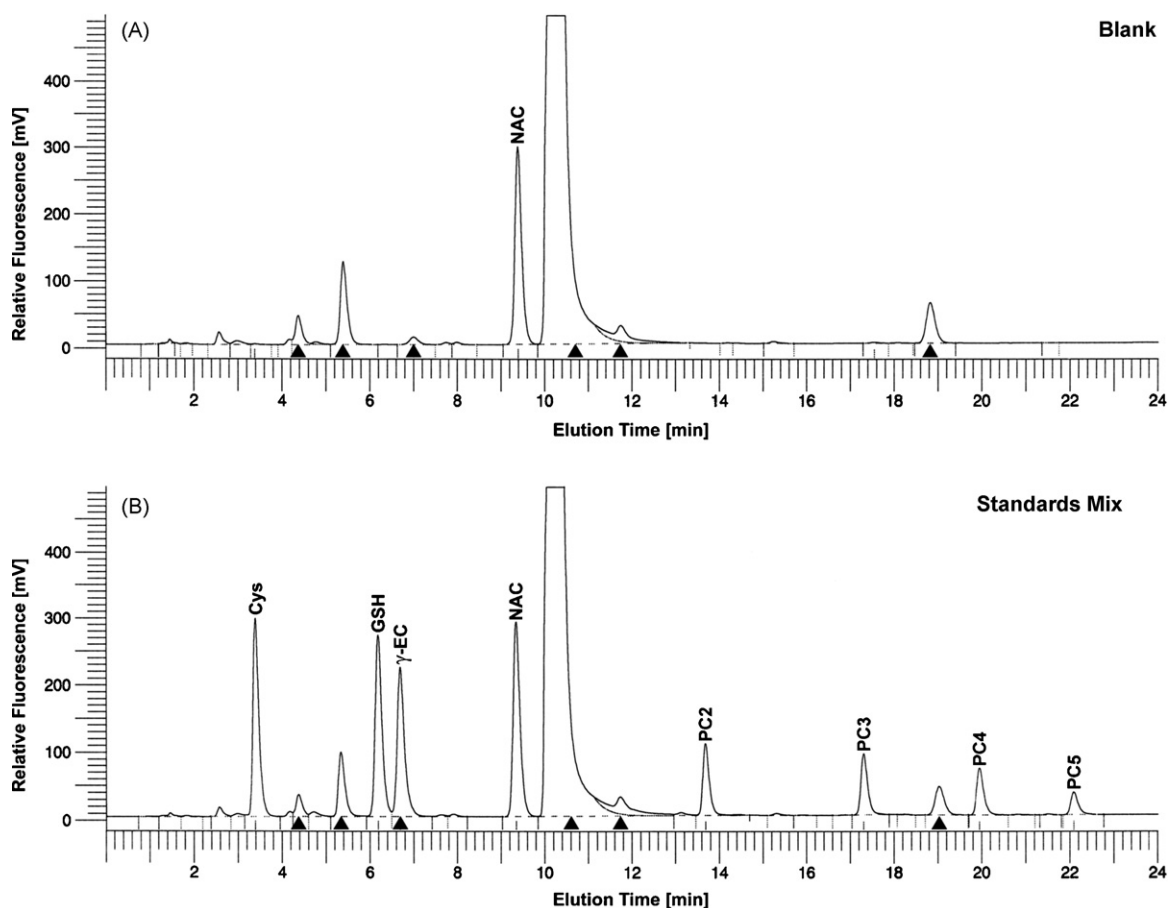


Fig. 1. Separation profiles of (A) reaction blank run with extraction buffer, (B) eight different mBBR derivatized thiol standards including an internal standard using the gradient profile described in Table 1. The standards mix contained the equivalent of 50 pmol for Cys, GSH, γ -EC, 10 pmol for PC₂–PC₅ and 50 pmol for the internal standard, NAC per 10 μ L injection. Run conditions are as described in the text. The '▲' symbol indicates "blank" peaks originating from the derivatization reaction in the absence of thiols and '+▲' symbol whenever present in a figure indicates the co-elution of unknown and/or known thiols with the blank peak.

Table 3
Average retention time in minutes for monothiols and phytochelatin standards

RT (min)	GSH	γ-EC	PC ₂	PC ₃	PC ₄	PC ₅	γ-EC
Mean	6.26	6.78	9.51	13.88	17.65	20.36	22.54
S.E.	0.05	0.04	0.06	0.04	0.07	0.10	0.12
n size	13	13	13	13	13	13	10

the derivatives are also relatively stable. We observed only minimal degradation (4% in the case of NAC and PC₂ and 7% for GSH) of mBBR derivatives after 21 months of storage of control, Cd- or Zn-treated red spruce cells stored at −20 °C. As mentioned previ-

ously by Fahey and Newton [37], these derivatives are sensitive to repeat freezing and thawing, and light. Their study also showed that the mBBR derivatives of Cys and GSH standards very stable with no loss of fluorescence for more than 20 months in storage at

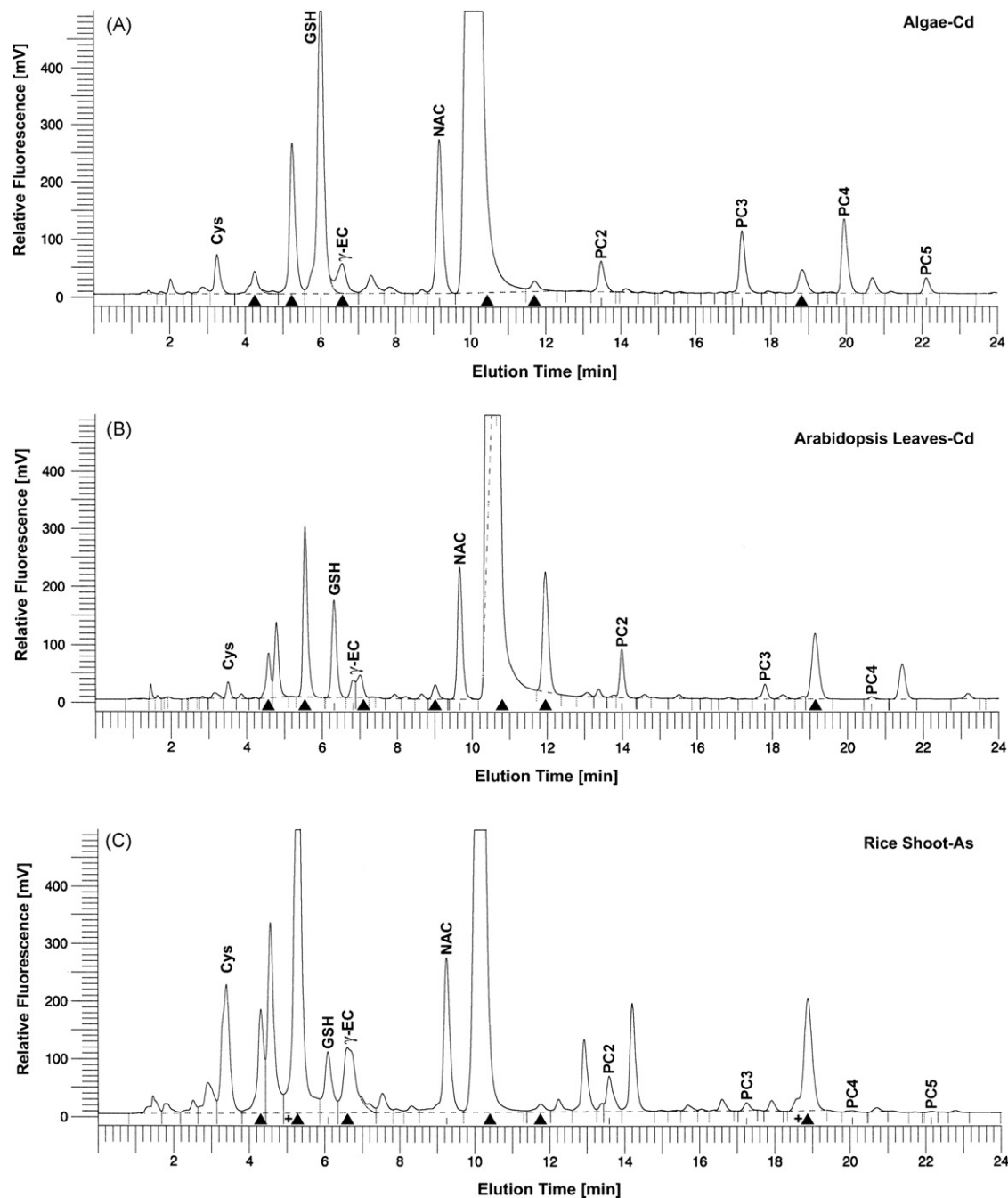


Fig. 2. Separation profiles of mBBR derivatized thiols in (A) marine green algal cultures (*Dunaliella tertiolecta*) treated with cadmium chloride (100 μM), (B) *Arabidopsis* (*Arabidopsis thaliana*) leaves treated with cadmium chloride (250 μM) and (C) Rice (*Oryza sativa*) shoots treated with sodium arsenate (150 μM). Other details same as described under Fig. 1 legend.

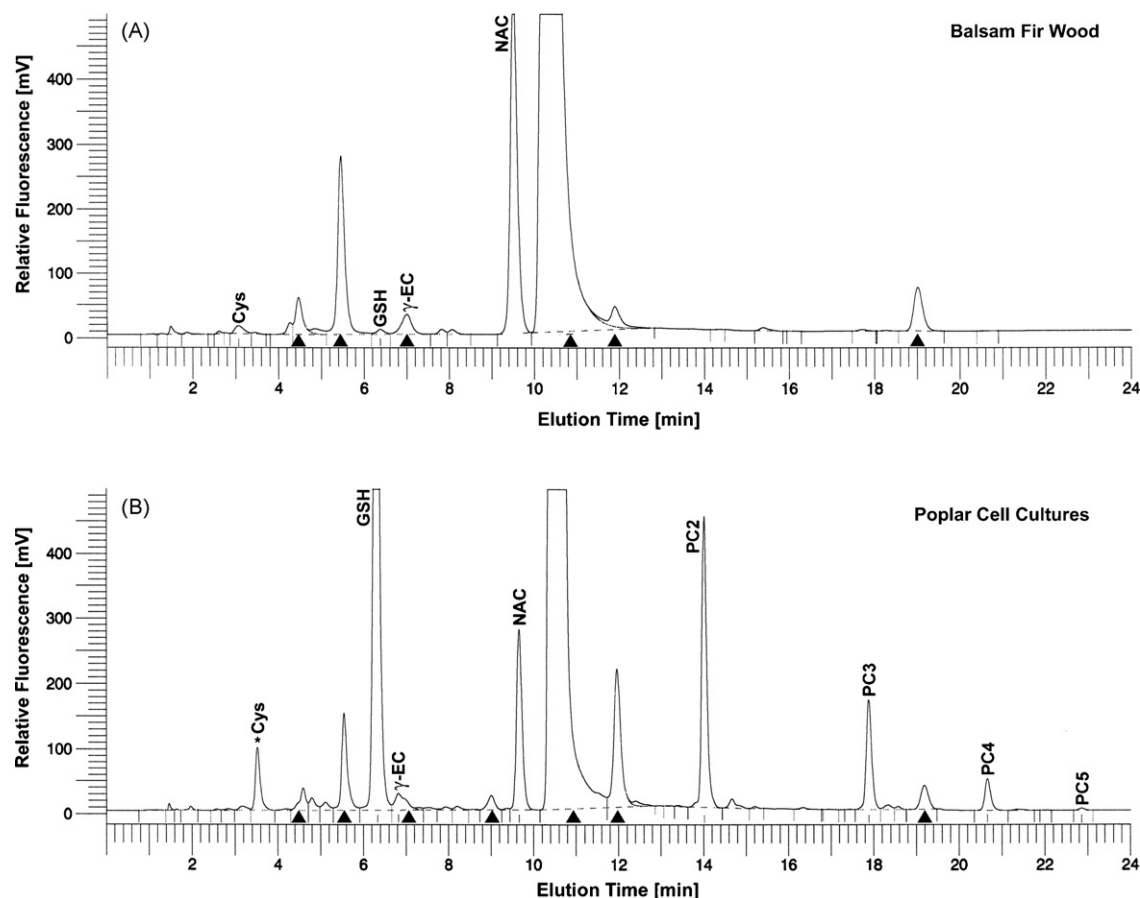


Fig. 3. Separation profile of mBBR derivatized thiols in (A) wood of balsam fir (*Abies balsamea*) and (B) hybrid poplar cell cultures (*Populus nigra* × *maximowiczii*). Other details same as described under Fig. 1 legend.

4, –20 or –70 °C [37]. However, mBBR derivatives of PCs in phytoplanktons were found to be stable for only 4–6 weeks at 4 °C [11,15]; 30% degradation was found for mBBR derived GSH in phytoplankton samples after 15 days of storage at 4 °C [15].

Sneller et al. [12] reported that the efficiency of derivatization of thiols with mBBR is temperature dependent. The optimum conditions they reported were 30 min reaction time at 45 °C with 10 µL of 25 mM mBBR. We validated their reaction conditions in our laboratory by incubating a known concentration of standards mix for either 15 or 30 min at 45 °C after adding 10 µL of either 25 or 50 mM mBBR. Previously our laboratory [23] reported that the optimum conditions for the mBBR derivatization of monothiols and PC₂ were a 30 min incubation with 10 µL of 50 mM mBBR at 45 °C. Reaction conditions remained the same when higher PCs were added to the standards mix in the present study. Tang et al. [13] suggested that the amount of mBBR added should be at least 200 times more than the total thiol concentration in samples to obtain adequate intensity and complete recovery. However, an increase in mBBR concentrations from 50–100 mM resulted in the appearance of a peak that often co-eluted with γ-EC. Similar to Sneller et al. [12], the present study also showed that the efficiency of derivatization using mBBR decreased as the chain length of PCs increased, when equal concentrations of all PCs were used in the reaction. At concentrations higher than 50 pmol for PC₃ and PC₄ and 25 pmol for PC₅ (for a 10 µL injection) derivatization becomes non-linear. The concentration of PCs was kept lower compared to the smaller size thiols (Cys, GSH and γ-EC) to ensure the completion of reaction in the standards. Our samples were always within the limits of PCs concentrations used for the standards.

Cysteine has been used to react with excess mBBR in some studies [38,39]. However, in our study, no significant differences were observed when blanks and standards were derivatized with or without 20 µL of 100 mM Cys (data not presented). Instead, Cys was used as one of the standards to evaluate its concentrations in samples.

A broad reagent peak was observed at approximately 10.5 min in samples derivatized with mBBR. Kawakami et al. [15] identified this compound as tetramethylbimane (Me₄B) and reported that Me₄B is part of the synthesis of mBBR. Whereas the area of the Me₄B peak was similar for standards and cell culture samples, it was approximately three times lower in foliage samples from some tree species such as willow clones (Fig. 4) and sugar maple (data not shown). We hypothesize that this may be due to the binding of thiols, thiol derivatives and/or mBBR with other unknown compounds within the foliar sample extract.

3.3. Separation of thiol compounds

3.3.1. Peak identification

An extremely sensitive technique is required for the detection of thiol compounds that are often present at low levels in biological samples. In the present method, the quantitation limit was 0.1 pmol per 10 µL injection for all thiols except for PC₅ in which case it was ≥0.5 pmol. This method is more sensitive in terms of detection and quantitation limits as compared to most other methods published earlier ([24,11–14,40] and table of detection limits from other published work therein, [41–43]). Dupont and Ahner [44] described a method with similar sensitivity as the method described here

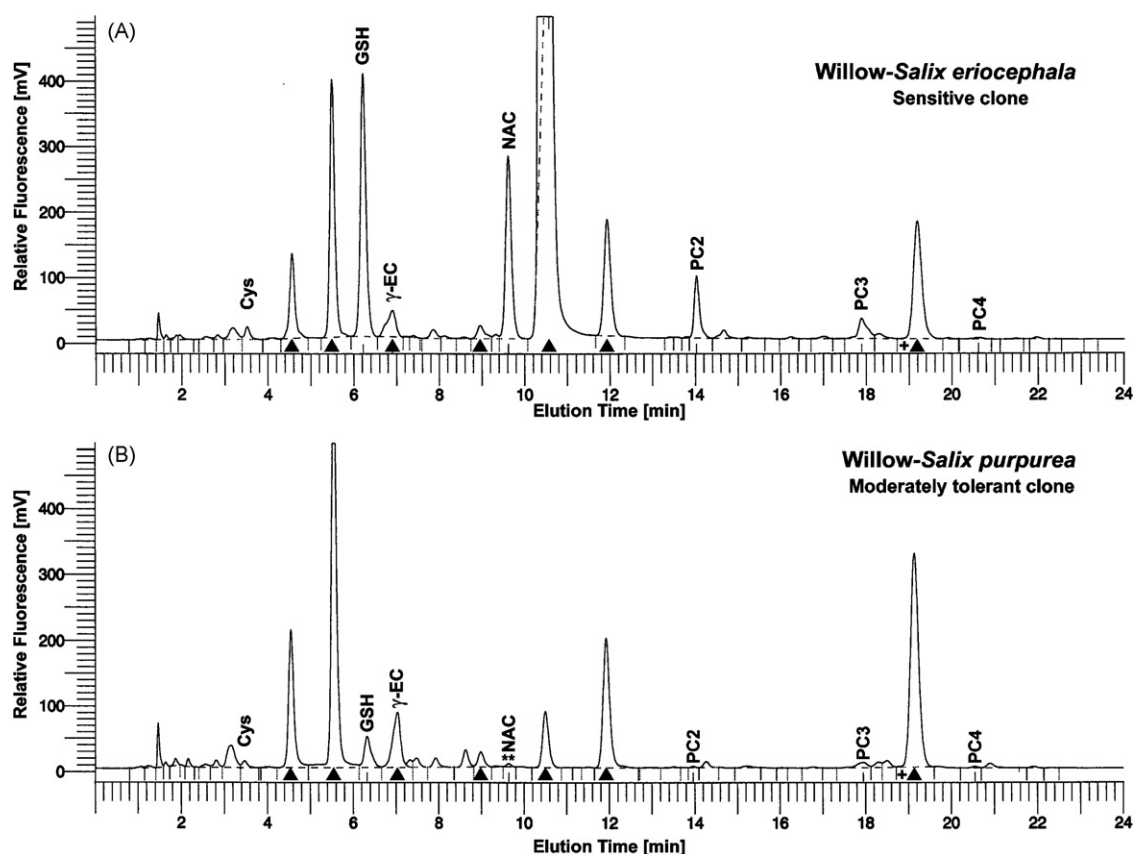


Fig. 4. Separation profiles of mBBR derivatized thiols in sodium arsenate (250 µM) treated shrub willow clones (A) *Salix eriocephala* (arsenate sensitive) and (B) *Salix purpurea* (arsenate tolerant). Other details same as described under Fig. 1 legend.

though their method had a much longer total run time. Wei and Ahner [45] described a method which was more sensitive. However, they used a small bore column and the total run time for that method was much longer than the method presented here. Sample spiking was used to identify the known peaks that co-eluted with other unknown peaks; for example, γ -EC in most samples analyzed and GSH and PC₂ in sugar maple foliage.

3.3.2. Calibration curves

Thiols were quantified using an internal standard (NAC) method using a six or seven-point calibration curve (Table 2). The standards mixture contained monothiols (Cys, GSH and γ -EC) and polythiols

(PC₂ to PC₅). The r^2 value for standard curves was 0.99 or higher for all thiol compounds tested except for PC₅ (0.98) (Table 2). Fig. 1 shows the representative chromatographic profile of a reaction blank (A) and standards mixture of mono and polythiols (B). The known monothiols along with the added internal standard eluted within 9.64 min; polythiols eluted between 13.68 and 22.86 min for the standards mixture. The retention times were reproducible between runs (Table 3). The total run time (34.8 min) is significantly shorter than most other published HPLC methods for non-protein thiol analyses using pre-column derivatization with mBBR. A small peak of known area that originated from reaction ingredients often merged with γ -EC in the standards (Fig. 1A and B). In addition,

Table 4

Percent recovery of standards added to various tissue samples. Four different concentrations of standards were run separately to develop a standard curve against which all the recoveries were calculated. Each sample was derivatized five times; spiked either with buffer or each of the four concentrations of standards before derivatization. Recovery data for each species is based on 4 replicates (mean $\pm$ S.E.)

Name	Cys	GSH	γ -EC	PC ₂	PC ₃	PC ₄	PC ₅
Adjusted amounts							
Average standard amount ($n=2$), pmol μL^{-1}							
S2 (1.0 monothiols and 0.2 PCs)	0.98	0.97	1.14	0.20	0.20	0.20	0.22
S3 (1.5 monothiols and 0.3 PCs)	1.52	1.54	1.54	0.30	0.27	0.30	0.30
S4 (2.0 monothiols and 0.4 PCs)	2.03	2.02	2.00	0.41	0.41	0.41	0.39
S5 (2.5 monothiols and 0.5 PCs)	2.49	2.48	2.44	0.50	0.49	0.50	0.52
Percent recovery ($n=4$, spiked with S2–S4)							
Red spruce cell culture	99.7	114.6	69.8	120.3	108.4	91.2	139.9
Algae	93.9	53.3	97.7	94.9	108.0	115.3	182.5
Arabidopsis	139.5	18.3	19.3	108.7	112.9	101.5	151.4
Crambe shoot	97.1	104.6	83.5	110.5	112.6	106.1	114.9
Red spruce current year needles	78.4	99.0	102.5	71.1	24.8	15.5	0.0
Balsam fir wood plugs	95.2	106.8	84.0	101.1	110.9	107.0	130.6
Sugar maple							

Most of the added thiols bound to unknown compounds in leaf extract

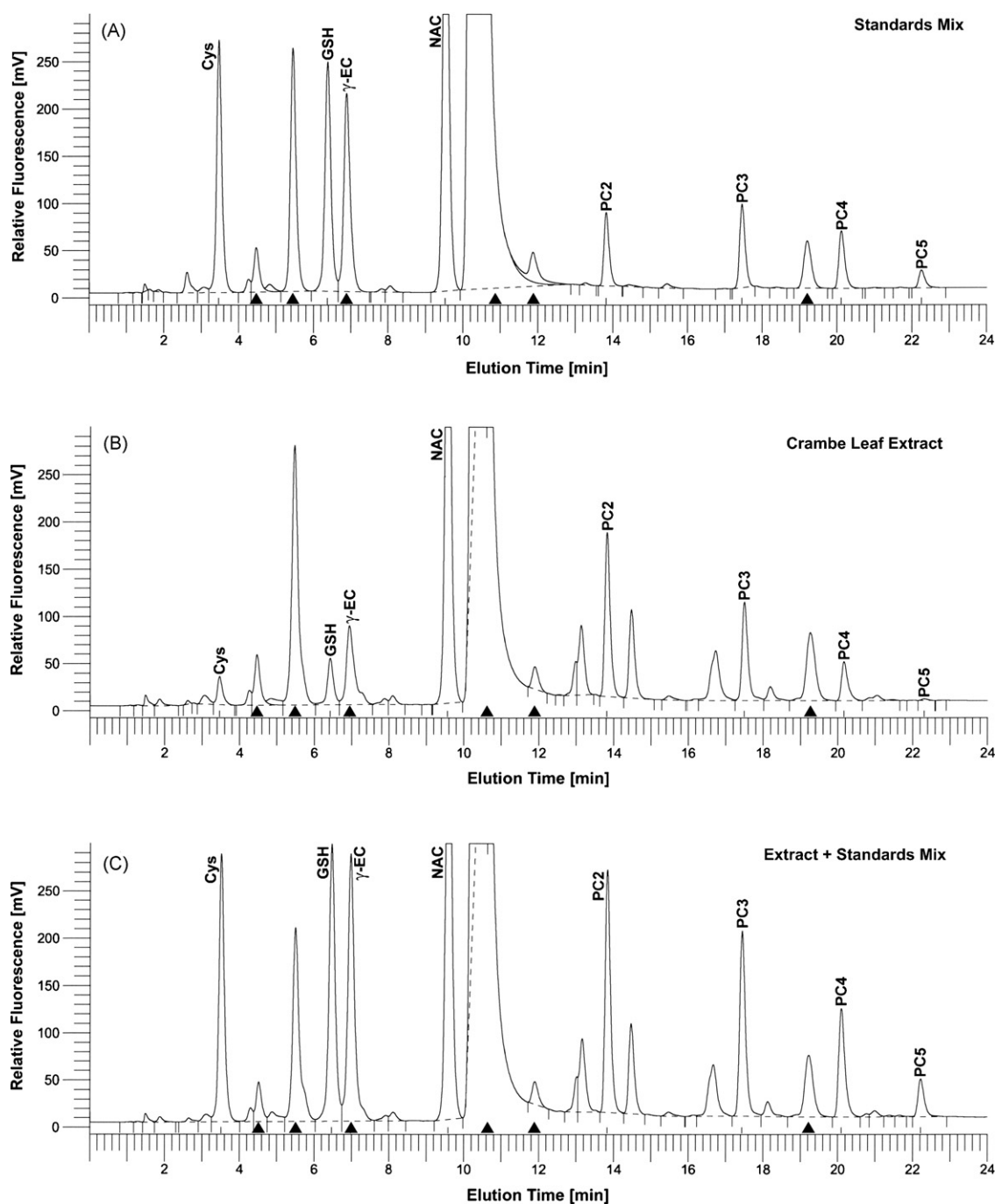


Fig. 5. Recovery of thiol standards from shoot extract of crambe (*Crambe abyssinica*) treated with sodium arsenate (250 μM) for 24 h. Extracts were derivatized with or without the addition of thiol standards. Separation profiles of mBBR derivatized thiols in (A) standards mix (25 pmol of Cys, GSH, γ -EC and NAC and 5 pmol of PC₂–PC₅), (B) tissue extract and (C) tissue extract spiked with thiol standards mix. Other details same as described under Fig. 1 legend.

two blank peaks were observed before GSH and one before PC₄ but these peaks did not interfere with the quantification of known thiols (Fig. 1A and B). However, the blank peak that co-eluted with γ -EC did not interfere with the quantification of γ -EC because the linear regression equation used for the standard curve had a non-zero intercept which equaled the area of this blank peak. The linear range of detection varied with thiols (Table 2). In general, the larger number of thiol groups in a phytochelatin the lower its range of detection under these reaction conditions. This method was tested on several cell cultures and plant tissue types. A wide variety of chromatographic profiles from various tissues are presented below.

Irrespective of species, γ -EC co-eluted with an unknown and/or blank peak in all foliar samples.

3.3.3. Plant tissues analyzed

Fig. 2 shows the HPLC profiles of three different species. In all three profiles presented in Fig. 2, γ -EC co-eluted with an unknown thiol peak along with the blank reagent peak. In the marine green alga, *D. tertiolecta* exposed to 100 μM of Cd for 24 h, PC₄ was the predominant phytochelatin (Fig. 2A). The present study corroborates the studies of Hirata et al. [46] and Tsuji et al. [47] for the same species at 400 μM of Cd expo-

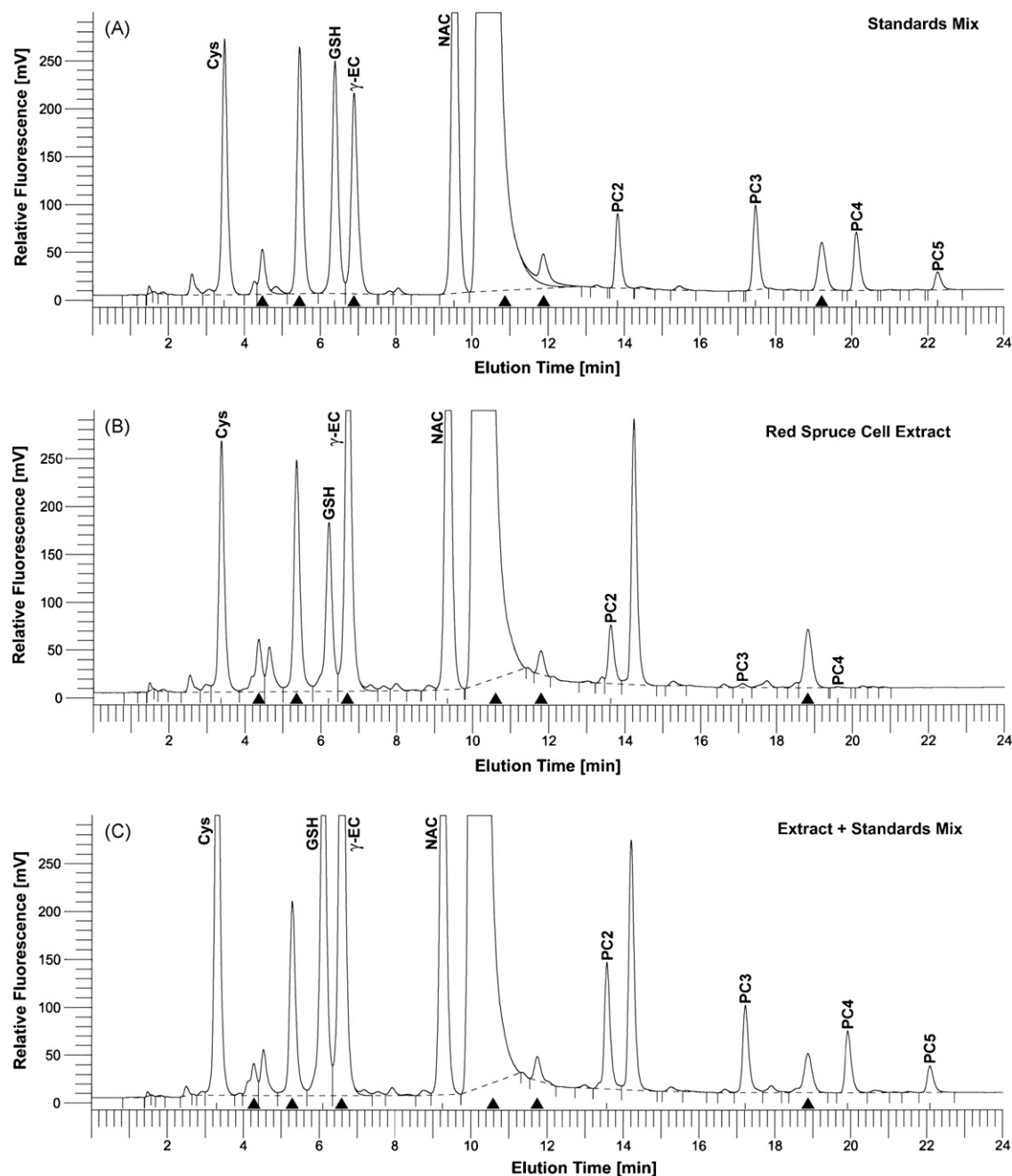


Fig. 6. Recovery of thiol standards from extract of red spruce (*Picea rubens*) cells grown in suspension cultures. Extracts were derivatized with or without the addition of thiol standards. Separation profiles of mBBR derivatized thiols in (A) standards mix (25 pmol of Cys, GSH, γ -EC and NAC and 5 pmol of PC₂–PC₅), (B) cell extract and (C) cell extract spiked with thiol standards mix. Other details same as described under Fig. 1 legend.

sure for 24 h. In the case of *Arabidopsis*, Cys, GSH, PC₂ and PC₃ were induced by Cd addition (Fig. 2B). Shoots of arsenate-treated rice (Fig. 2C) showed a clear separation of GSH and PC₃. However, based on peak shape, Cys co-eluted with an unknown thiol peak.

There were no polythiols and only very low levels of monothiols present in experimentally untreated wood plugs of balsam fir (Fig. 3A). These sample extracts had no unknown thiol peaks. Untreated control (GUS-transformed) cell cultures of poplar showed high levels of GSH, and PC₂ along with small size peaks of PC₃ and PC₄. Similar to most other species, poplar also had a small peak originating from the reaction ingredients that merged with γ -EC (Fig. 3B).

Fig. 4 shows the separation profiles of mBBR derivatized foliar extracts of two different shrub willow clones exposed to 250 μ M sodium arsenate: (A) *S. eriocephala* (clone ID 00x-026-082), a clone sensitive to arsenate and (B) *S. purpurea* (clone ID 99113-012), a clone moderately tolerant to arsenate. The extract of the tolerant clone contained compounds that interfered with the derivatization with mBBR as evidenced by the presence of very small peaks of the internal standard NAC and Me₄B of Fig. 4B in comparison with Fig. 4A. It can be hypothesized that the leaf extract of the tolerant clone contained negatively charged compounds such as organic acids or amino acids. If similar compounds are produced in roots and excreted into the growth medium they may bind with arsenic, thus preventing its entry into the cells. Under the derivati-

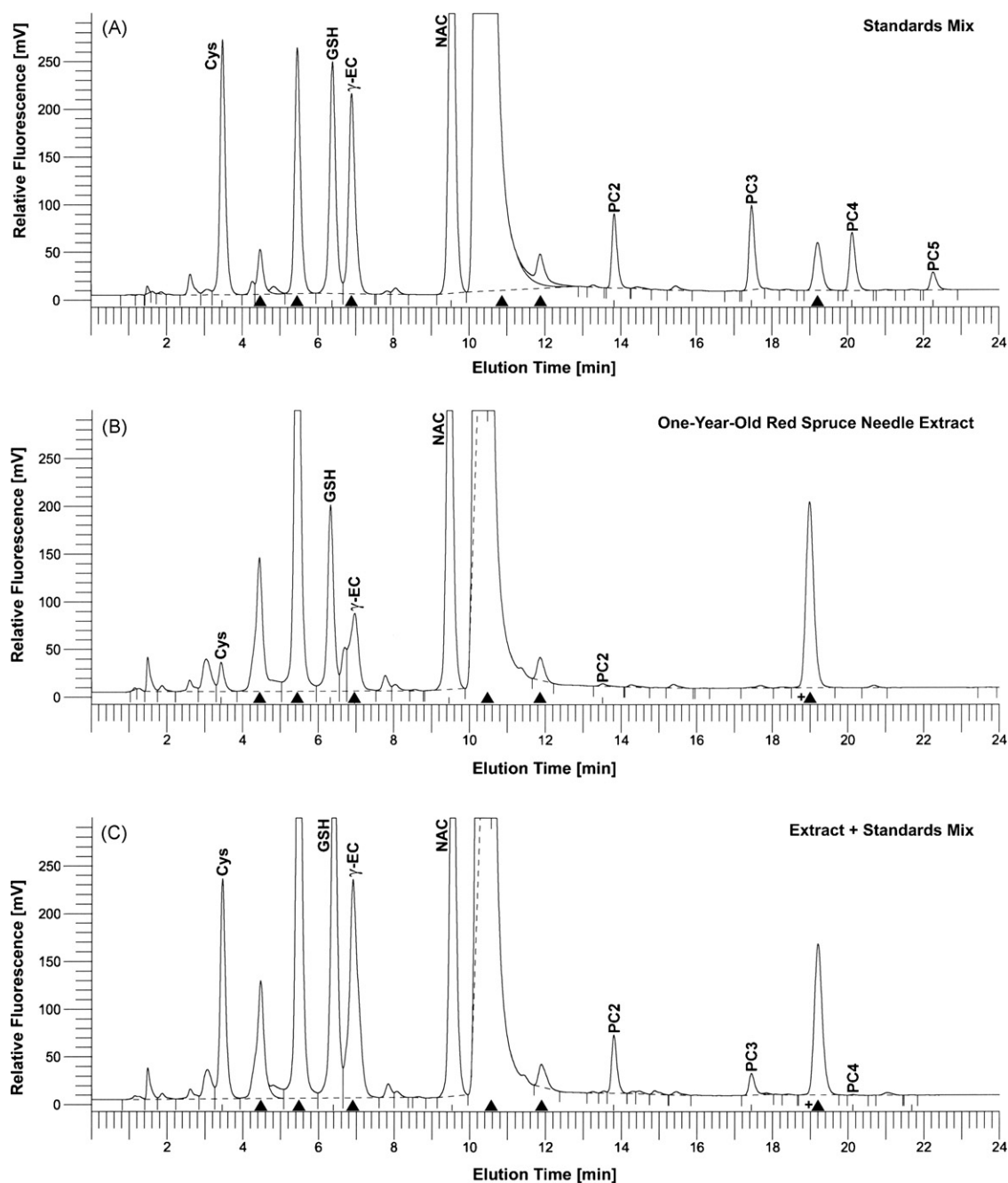


Fig. 7. Recovery of thiol standards from extract of 1-year-old red spruce (*Picea rubens*) needles collected from Hubbard Brook Experimental Forest, NH, USA. Extracts were derivatized with or without the addition of thiol standards. Separation profiles of mBBBr derivatized thiols in (A) standards mix (25 pmol of Cys, GSH, γ -EC and NAC and 5 pmol of PC₂–PC₅), (B) needle extract and (C) needle extract spiked with thiol standards mix. Other details same as described under Fig. 1 legend.

zation conditions, the negatively charged compounds in the sample extract were able to bind with leaf thiols (produced in response to arsenate treatment), and the added internal standard (NAC) during the 10 min pre-incubation, thus making them unavailable for derivatization with mBBBr. This would explain the presence of lower levels of free thiols in the tolerant clone compared to the sensitive clone, if in fact they have an additional defense mechanism that would prevent heavy metal from entering the cells. In support of this explanation, Purdy and Smart [29] showed that the more tolerant clone accumulated 2.5–4 times less arsenate as compared to the sensitive clone under these conditions.

3.3.4. Recovery of known quantities of added thiols

There are compounds present in many plant extracts that interfere with the derivatization process by binding with thiols during pre-incubation. Experiments were conducted to evaluate the extent of this type of interference with the derivatization process that could produce misleading results. This was accomplished by determining the recovery of known amounts of standards added to sample extracts of 4 different species. Each extract was derivatized alone or with each of 4 concentrations of a mix of seven standards (Table 4). Four different concentrations of a mix of seven standards were also derivatized in order to calculate the percent recovery of standards in each

case. Leaves of crambe (a member of *brassica* family) resulted in complete recovery of all thiol standards including NAC. The only exception was an 80% recovery of γ -EC (Fig. 5 and Table 4). Spiking of red spruce cell extracts with thiol standards resulted in complete recovery of all thiols including NAC, except for γ -EC and PC₄ where the recovery was 70 and 90%, respectively (Fig. 6 and Table 4). Interestingly, the spiking of extracts of 1-year-old needles of red spruce trees resulted in different levels of recovery. Whereas GSH, γ -EC and NAC yielded 100% recovery among monothiols, Cys recovery was only 80%; the recovery of polythiols decreased from 70% (PC₂) to 0% (PC₅) as the chain length increased (Fig. 7 and Table 4). Unlike the cell suspension culture and herbaceous plant samples, the extracts of foliage of some forest trees (e.g., sugar maple, red spruce) have many unknown thiol and/or other compounds such as tannins, phenolics and flavonoids that interfere with the separation of thiols by potentially disrupting the derivatization reaction. Sugar maple foliar extracts contained a yellow particulate even after filtering the derivatized samples with a 0.45 μ m nylon syringe filter. All standards added to sugar maple extract were completely bound by unknown compounds in the extract and could not be quantified in this case (Table 4). There is no explanation available for more than 100% recovery of PC₅ in all cases except for Crambe.

3.3.5. Internal standard

The addition of an internal standard to samples does not compensate for the losses during the derivatization protocol due to oxidation of thiols or other chemical reactions since the rates of these reactions can be different for different thiols [37]. However, the addition of an internal standard could help to improve the analytical results by compensating for losses during sample processing. Additionally, the area of internal standards of a sample may be indicative of the extent of binding of thiols with unknown compounds in the sample extracts before the derivatization reactions occur. This was observed in the foliage of sugar maple analyzed for the present study where NAC (internal standard) peak areas were minimal. As analyzed in our laboratory, eastern hemlock, Norway spruce, red spruce (Fig. 7), white pine, red pine, pitch pine, American beech, black birch and black cherry extracts did not contain compounds that bind with NAC (data not shown). The degree of binding of thiols was different for different species (Fig. 7 and Table 4) and different clones within a species (e.g., willow; Fig. 4). Extracts of cell cultures did not contain compounds that bind with NAC. A similar situation was also encountered with sugar maple leaves where binding of amino acids, polyamines, and the internal standard (heptane diamine) to unknown compounds occurred before derivatization with dansyl chloride [48].

4. Conclusions

The HPLC protocol described in Thangavel et al. [23] for analyses of red spruce cell cultures was improved further for broad applicability to other plant cultures and tissues including conifer and hardwood trees. This protocol was also extended to quantify thiols higher than PC₂. The solvent composition and solvent profile were modified for greater reproducibility of retention times for the analyses of mBBR tagged mono and polythiols. The total run time of 34.8 min is significantly shorter than other published HPLC methods. This method showed improvement in the reproducibility of retention times and can be used with a variety of tissues and species for the separation of most thiols with the exception of γ -EC due to the interference from another unknown peak in the extract of some of these samples. However, standards and several cell cultures could be analyzed for γ -EC without a problem.

The combination of sample filtration and frequent column cleaning steps has enabled us to achieve better separation for many thiols from these tissues and substantially increased column life. The use of a shorter length column has few disadvantages; possibly the separation problems observed with γ -EC. However, using a shorter column had significant economic benefits including savings in personnel time, solvent usage and organic waste generation. These advantages far outweigh the problems with the γ -EC separation and add a positive feature in terms of environmental safety and awareness.

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