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Determination and characterization of cysteine, glutathione and phytochelatins (PC_{2-6}) in *Lolium perenne* L. exposed to Cd stress under ambient and elevated carbon dioxide using HPLC with fluorescence detection

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ABSTRACT

Metal-binding thiols, involved in detoxification mechanisms in plant and other organism under heavy metal stress, are receiving more and more attentions, and various methods have been developed to determine related thiols such as cysteine (Cys), glutathione (GSH) and phytochelatins (PCs). In present study, an HPLC method was established for simultaneous determination of Cys GSH and PC_{2-6} after treatment with disulfide reductant of tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and thiolyte reagent of monobromobimane (mBBr). The separation of thiol derivatives was performed on an Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 30 mm, 1.8 μm) with a linear gradient elution of 0.1% (v/v) trifluoroacetic acid (TFA)–acetonitrile (ACN) at 0.8 mL min^{-1}. The temperature of the column was maintained at 25 °C. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. The thiol derivatives were well separated in 19 min, and the total analysis time was 30 min. The established method was proved selective, specific and reproducible, and could be applicable to determine Cys, GSH and PC_{2-6} and to evaluate their roles in detoxification mechanisms in Cd-treated *Lolium perenne* L. under ambient and elevated carbon dioxide (CO_2). It was found that the total SH contents and proportions of thiols in roots and shoots were dependent on Cd concentration, whereas the total SH contents decreased and the proportions of thiols altered without significance at elevated CO_2 level.

1. Introduction

Phytochelatins (PCs) are cysteine (Cys)-rich peptides in plants and other organisms with general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2–11) and ability of binding metals through SH groups in the Cys parts [1,2]. Synthesis of PCs in plants under heavy metal stress is considered to be crucial to detoxifying mechanisms [3–6], which has been proved right through inhibitor studies [7–9], biochemical studies [10], mutant analyses [11–14] and gene analyses [15–17]. The synthesis was catalyzed by phytochelatin synthase (PCS) using glutathione (GSH) as substrate [14,15,18–21] and metal ion as activator [1,22,23], and related to species, toxic degree of metal ions, and interactions among metals [5,6,24–29]. It was found that PCs production was direct dependent on aqueous free metal-ions [30] and occurred earlier than any other physiological parameter is affected [5,6,24,25]. Therefore, PCs production could be used as biochemical indicators/markers to assess metal toxicity to biota [6,22,23,31], and the assay of PCs would be a better approach than chemical analyses of metals [5,6,24,31].

Besides being involved in PCs synthesis, Cys and GSH are of importance in plants [3]. Biosynthesis of Cys plays a key role in fixing inorganic sulfur from the environment and provides the only metabolic sulfide donor for the generation of many compounds [32]. GSH is involved in defense against reactive oxygen species (ROS), sequestration of heavy metals, detoxification of xenobiotics, regulation of developmental processes such as cell division and flowering, and furthermore a major transport and storage form of reduced sulfur [33–36]. Consequently, it has become a requirement that methods should be established to determine these thiols and to evaluate their roles in plants and other organisms under heavy metal stress.

A range of methods have been developed for determination of thiols, including electrochemical methods such as cathodic stripping voltammetry and polarography [37–43], capillary electrophoresis (CE) equipped with electrochemical detection [44], laser-induced fluorescence detection [45] or photodiode array

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Table 1
Stock and working standards of thiols (μmol L^{-1}).

Thiols	Stock standard (mmol L^{-1})	Working standards (μmol L^{-1})							
		S1	S2	S3	S4	S5	S6	S7	S8
Cys	100.0000	0.2000	0.5000	1.0000	2.0000	4.0000	8.0000	12.0000	16.0000
GSH	10.0000	0.0500	0.1000	0.5000	2.0000	10.0000	20.0000	50.0000	100.0000
PC_2	1.8498	0.0925	0.1850	0.3700	0.7399	1.4798	2.9597	5.9193	11.8387
PC_3	1.2938	0.0323	0.0647	0.1294	0.6469	1.2938	3.8815	7.7630	12.9383
PC_4	0.9950	0.0249	0.0498	0.0995	0.1990	0.3980	0.7960	1.5920	3.1841
PC_5	0.8081	0.0202	0.0404	0.0808	0.1616	0.3233	0.4849	0.6465	0.8081
PC_6	0.6805	0.0170	0.0340	0.0681	0.1361	0.2722	0.4083	0.5444	0.6805

detection [46,47], and HPLC with UV/vis detection [48,49], electrochemical detection [50–52], fluorescence detections [3,6,28,53–67] or inductively coupled plasma-mass spectrometry (ICP-MS) and electrospray-mass spectrometry (ES-MS) [68–80]. These methods were selective and sensitive for determination of thiols in biological samples and their performance characteristics, advantages and disadvantages were summarized in the previous reviews [53,81–83]. The application of MS, especially tandem MS (MS–MS), could provide more information on thiols in organisms, however, HPLC–MS or MS–MS was not available in most laboratories. Therefore, the method using readily available and low-cost instruments should be established to determine thiols in organisms and to evaluate their roles in detoxifying mechanisms under heavy metal stress.

On the basis of comparing existing methods, an HPLC method with fluorescence detection was established for simultaneous determination of Cys, GSH and PC_{2-6}, and applied to assay their concentrations and to evaluate their roles in *Lolium perenne* L. exposed to Cd stress under ambient and elevated CO_2. Furthermore, since GSH and PC_{2-6} have the same unit of [γ-Glu-Cys], there is a hypothesis that the retention time (*RT*) and response (height) would be dependent on the number (*n*) of SH. The objective of present study therefore was also to estimate the role of *n* on *RT* and height.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (TFA, ≥99%), γ-cysteine (Cys, ≥99.5%) and glutathione (GSH, ≥97%) were obtained from Fluka (Milwaukee, WI, USA). Monobromobimane (mBBr, ≥95%) was purchased from Fluka (Buchs, Switzerland). Methanesulfonic acid (MSA, 99.5%), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid sodium salt (HEPES, 99.5%) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma (Louis, MO, USA). Diethylenetriamine-pentaacetic acid (DTPA, >99%) was purchased from Alfa Aesar (Heysham, Lancs, UK). Phytochelatins (PC_{2-6}, >95%) were obtained from AnaSpec (San Jose, CA, USA). HPLC-grade acetonitrile (ACN) was purchased from Tedia (Fairfield, OH, USA). And all other reagents were analytical-reagent grade. Water was purified by a Milli-Q Gradient system (Millipore Corp., Bedford, MA, USA).

2.2. Preparation of standard and reactant solutions

Extraction buffer containing 0.1% TFA and 5 mmol L^{-1} DTPA was prepared in purified water. Standards of Cys, GSH and PC_{2-6} were used for calibration. Stock standard solutions were separately prepared in extraction buffer, divided into several parts and stored in dark at −20 °C. Fresh working solutions were prepared prior to use with extraction buffer. Appropriate portions of each stock were mixed together and further diluted with extraction buffer to create a series of eight working standards (Table 1). HEPES buffer (200 mmol L^{-1}, pH about 9.0) was prepared in 5 mmol L^{-1} DTPA solution; TCEP solution (20 mmol L^{-1}) was made in HEPES buffer and mBBr solution (50 mmol L^{-1}) was made in ACN. The stock standard solutions was divided into several parts and stored in dark at −80 °C and the other solutions were kept in dark at 4 °C.

2.3. Sample preparation

The sample (approximately 0.20 g), previously stored in dark at −80 °C, was ground in liquid N_2 and the thiols were extracted using 1.8 mL of extraction buffer. After the vigorously mixture, the homogenate was centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant or standard solutions (250 μL) was transferred and mixed with 650 μL of HEPES buffer and 25 μL of TCEP solution. This reaction mix was pre-incubated at room temperature (25 °C) for 5 min and the derivatization was then carried out by incubating the mix in dark for 30 min at room temperature (25 °C) after the addition of 20 μL of mBBr solution. The reaction was terminated by the addition of 100 μL of 1 mol L^{-1} MSA. The derivatized samples were filtered with 0.20 μm nylon syringe filters (Millipore Corp., Bedford, MA, USA) for HPLC analyses. The whole protocol was quickly carried out in dark.

2.4. Instrumentation

The separation of thiol derivatives was performed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Hambruecker Landstrasse, Waghaeusel-Wiesental, Germany) consisting of quaternary pump with degasser, thermostat for ALS/FC/Spotter, thermostatted column compartment, diode array detector, fluorescence detector and autosampler fitted with a 100 μL loop. The column was Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 30 mm, 1.8 μm; Agilent Technologies Inc., Princeton, MN, USA). The temperature of the column oven was maintained at 25 °C. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. Data were integrated using ChemStation software (Agilent Technologies Inc., Version B.03.02).

2.5. Chromatographic conditions and peak identifications

Derivatized samples (20, 50, or 100 μL) were run with a linear gradient elution. Solvent A was 0.1% (v/v) TFA in water and solvent B was ACN. The flow rate was 0.8 mL min^{-1}. The gradient profile was described as: 0–20 min, 8–26% B; 20–22 min, 26–100% B; 22–24 min, isocratic 100% B; 24–28 min, 100–8% B; 28–30 min, isocratic 8% B, and total analysis time was 30 min. All solvents were filtered with 0.2 μm nylon filter (Nylaflo; Pall Corp., Ann Arbor, MI, USA) and degassed before use.

Identification of peaks from thiol derivatives was performed through comparing the profiles of blank (extraction buffer), individual standards and standards mix, and the thiol concentrations were calculated using the relationship between thiol concentrations in standard solutions and corresponding peak height.

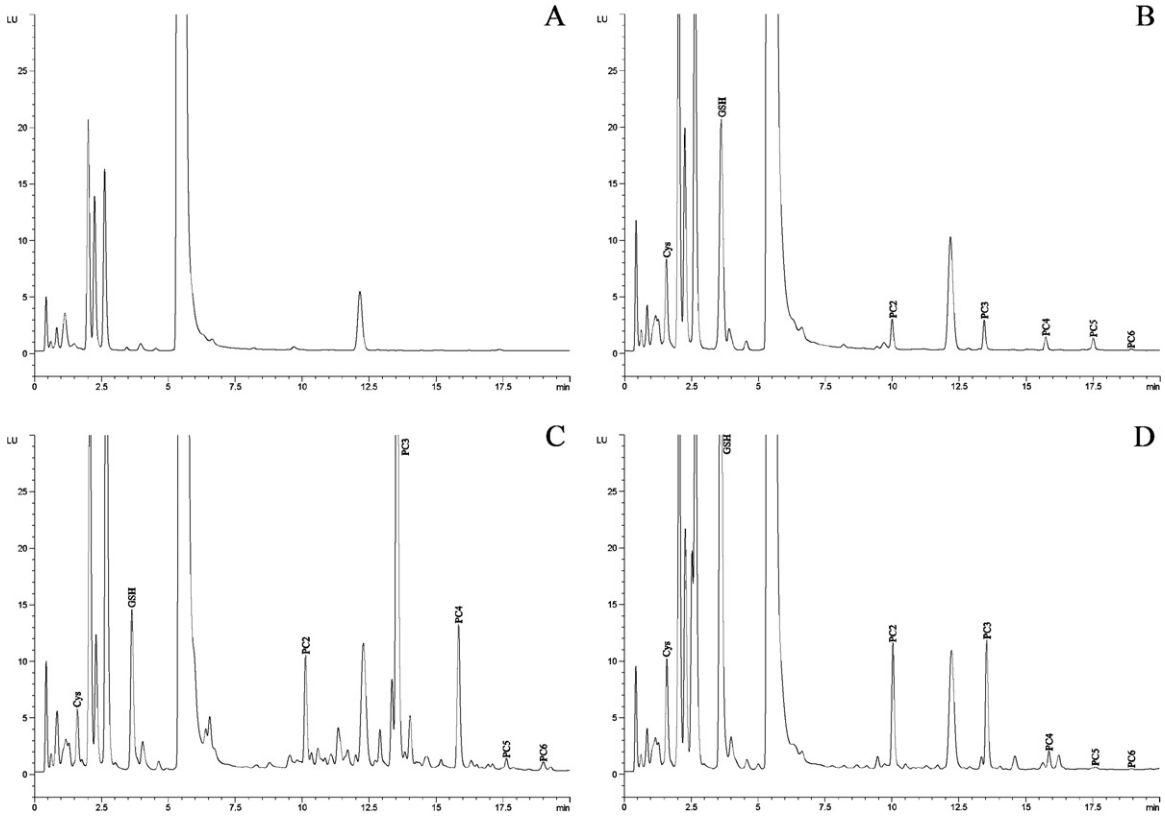

Fig. 1. Separation profiles obtained from of extraction buffer (A), thiol standards mix (S4 in Table 1) (B), and root (C) and shoot (D) extract after exposure for 9 days to Cd stress of 20 μmol L^{-1} at CO_2 concentration of 380 μL L^{-1}.

2.6. Accuracy, precision and recovery

Accuracy, precision and recovery of the method for determining thiols were evaluated with homogenates spiked with the standards at known concentrations. Intra-day precision was performed in four homogenates, and inter-day precision was determined by analyzing four homogenates over 4-day period. Accuracy was evaluated by comparing measured concentration with known concentration of thiol. Recovery (%), precision (RSD, %) and accuracy (bias, %) were calculated according to previous methods [84,85].

2.7. Stability of thiols and their derivatives

Freezing and thawing test is often carried out to evaluate the resistance of compounds to disintegration. Stability of thiols and their derivatives was estimated under different conditions. The accuracy (bias, %) of thiols was calculated to assess the stabilities by comparing measured concentration with the concentration in freshly prepared one.

2.8. Application

Seeds of *L. perenne* were soaked in 1% NaClO for 15 min, washed several times with double-distilled water, and sown in moistened mixture of perlite and vermiculite (1:1). After 14 days growth, healthy and uniform-sized seedlings were randomly selected and transplanted to containers with 10 L of half Hoagland nutrient solution. The Hoagland nutrient solution consisted of 2 mmol L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 0.1 mmol L^{-1} KH_2PO_4, 0.5 mmol L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 mmol L^{-1} KCl, 0.7 mmol L^{-1} K_2SO_4, 10 μmol L^{-1} H_3BO_3, 0.5 μmol L^{-1} $MnSO_4{\cdot}H_2O$, 0.5 μmol L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.2 μmol L^{-1} $CuSO_4{\cdot}5H_2O$, 0.01 μmol L^{-1} $(NH_4)_6Mo_7O_{24}$ and 100 μmol L^{-1} Fe-EDTA [86]. Solution pH was adjusted to about 6.5 by 0.1 mol L^{-1} NaOH or 0.1 mol L^{-1} HCl.

After growth of the seedlings in half Hoagland nutrient solution for 7 days, the containers were filled with full Hoagland nutrient solution, and the culture medium was renewed every 7 days and continuously aerated with an aquarium pump. After incubation for 21 days, the seedlings were sorted randomly into two sets, and exposed to Cd levels of 0, 20 and 80 μmol L^{-1} in each set, respectively. Solution Cd^{2+} was supplied with $2CdCl_2{\cdot}5H_2O$. The experiment was conducted with four replicates. During the experimental period, the two sets of *L. perenne* in container were grown in two controlled growth chambers with identical temperature, light and moisture. The two growth chambers had almost the same growth conditions with an exception of CO_2 concentration. Day/night time was 16/8 h, temperature was 25 °C, light during daytime was 105 μmol m^{-1} s^{-1}, and relative humidity was 60%. CO_2 concentration in one chamber was maintained at 760 μL L^{-1}, and in the other one at 380 μL L^{-1}.

After exposure to Cd stress for 9 days, the seedlings were harvested, washed with double-distilled water, and separated manually into roots and shoots. Roots and shoots (approximately 0.20 g) were keep in liquid N_2 and stored in dark at −80 °C till analyses.

2.9. Statistical analysis

PASW Statistics 18 (SPSS Inc., USA, version 18.0.0) and OriginPro (OriginLab Corp., USA, v8.0724) were used for statistical analyses and figure drawing, respectively. Results were expressed as mean ± SD. A *P*-value of <0.05 was considered to be statistically significant.

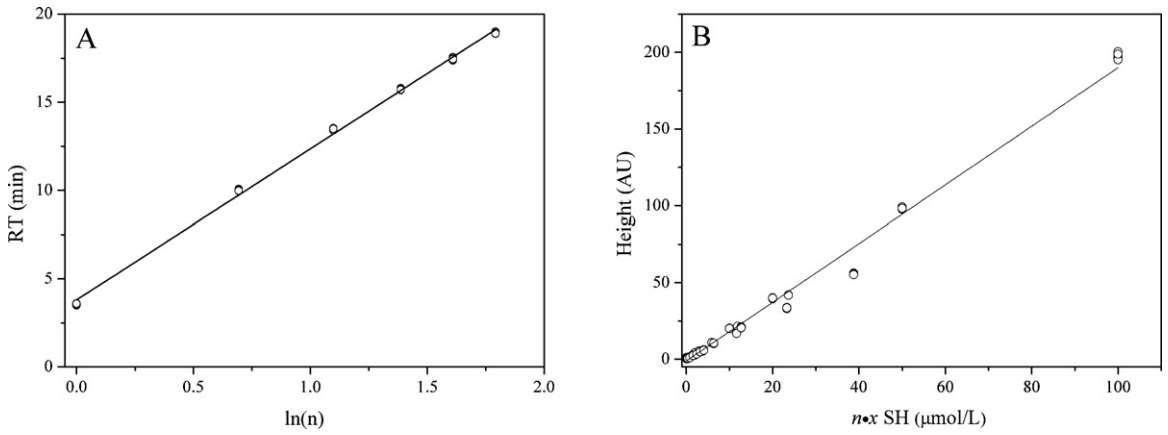

Fig. 2. The relationship between number of [SH] (n) and retention time (RT) (A), and the relationship of n, concentration (x) and height (y) (B).

3. Results and discussion

Since thiols are susceptible to oxidation, several measures should be taken for determination including alkylation, full conversion of disulfide to free SH and appropriate preparation protocol [6,41,45,53,64]. HPLC methods were previously developed to determine thiols through post-column derivatization with Ellman's reagent [5,5′-dithio-bis(2-nitrobenzoic acid), DTNB] [18,78] and pre-column derivatization with mBBr [3,53,56,87,88]. In present study, the latter was used, since it is very sensitive and the deriva-

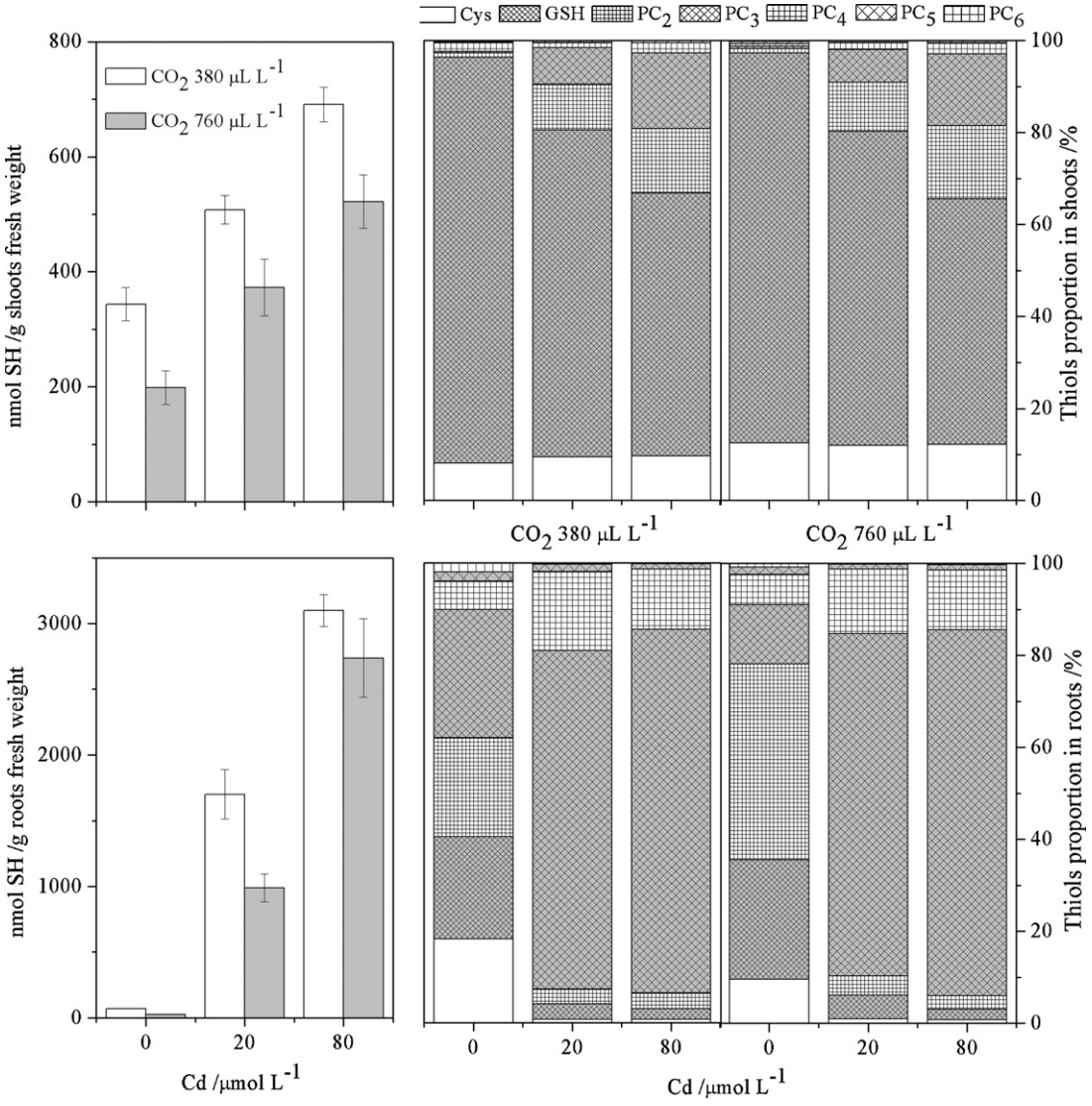

Fig. 3. Thiols in roots and shoots of *Lolium perenne* L. exposed to Cd stress at CO_2 concentrations of 380 and 760 μL L^{-1}, respectively.

Table 2
Retention time and calibration curve of thiols.

Thiols	*RT*[a] (min)	Regression equation[b]	r^2
Cys	1.56 ± 0.01 (0.55)	$y = 2.1137x + 0.8726$	0.9997
GSH	3.55 ± 0.03 (0.82)	$y = 1.9714x + 0.3979$	0.9999
PC_2	9.99 ± 0.03 (0.26)	$y = 3.5313x + 0.2146$	0.9999
PC_3	13.43 ± 0.03 (0.21)	$y = 4.3009x - 0.0102$	0.9999
PC_4	15.72 ± 0.03 (0.17)	$y = 6.5272x - 0.1168$	0.9995
PC_5	17.47 ± 0.04 (0.24)	$y = 7.3479x - 0.0328$	0.9974
PC_6	18.91 ± 0.03 (0.15)	$y = 1.3568x - 0.0251$	0.9942

[a] Retention time was expressed as mean ± SD (RSD, %) of 48 duplicates.
[b] Standard curves were run with 8 points, each point had 6 duplicates. *x* and *y* in the equations are concentrations and corresponding heights, respectively.

tives are relatively stable [3,53,56,65]. The derivatives of thiols were found to be stable under tested conditions [3,29,53,57,65], but sensitive to repeat freezing and thawing, and light [65]. And the latter was previously used to determine 2, 3-dimercaptosuccinic acid (DMSA) in mice blood and tissues [89]. In order to convert the disulfide to free SH, TCEP was used as disulfide reductant due to its advantages [53,83]. For the sample preparation, the tissue was ground in liquid N_2, and 0.1% TFA in 5 mmol L^{-1} DTPA was used as extraction buffer by comparing the previous report [61,90–92].

3.1. Method validation

3.1.1. Selectivity and specificity

The separation of thiol derivatives was performed under different chromatographic conditions with different gradient profiles in previously published reports [3,53]. In present study, the solvent composition and the gradient profile were optimized for simultaneous determination of Cys, GSH and PC_{2-6}, and the optimal chromatographic conditions were described above. Several typical HPLC traces obtained from the analyses of thiols are shown in Fig. 1. Fig. 1A represents the trace obtained from the derivatization of extraction buffer (blank) and Fig. 1B stands for a standard solution (standards mix). Fig. 1C and D shows traces obtained from the derivatization of root and shoot extract, respectively. The interference peaks from derivative reagent and extracts had no affect on identification and quantification of thiols studied. Though the method was not better than that reported by Lima et al. [93] in terms of selectivity and resolution quality, the present method was time-saving, and selective and specific enough to determine thiols in samples collected.

3.1.2. Linearity

The commercial PC standards could be obtained from a limited number of biochemistry laboratories and are usually expensive, whereas GSH standard could be obtained at different purity levels from a number of companies [29]. GSH calibration was used to quantify PCs concentrations with an assumption that the fluorescence response is directly proportional to the number of SH groups [6,56]. In our laboratory, Cys, GSH and PC_{2-6} standards were obtained commercially, and thiols were quantified using the external standard method with eight-point calibration curves (Table 1). The retention times of thiol derivatives demonstrated good reproducibility and the r^2 value was higher than 0.99 for all thiols studied (Table 2). The linear range of detection varied with thiols, showing a similar trend as previous report [3].

Taken the number (n) of [γ-Glu-Cys] into account, the retention time (RT) of GSH and PC_{2-6} were expressed as $RT = 8.5507 \ln(n) + 3.8034$ ($r^2 = 0.9984$, $P < 0.001$, $n = 1, 2, 3, 4, 5, 6$) and shown in Fig. 2A, and height (y) of GSH and PC_{2-6} expressed as $y = 1.9151nx - 1.3916$ ($r^2 = 0.9876$, $P < 0.001$, $n = 1, 2, 3, 4, 5, 6$) and shown in Fig. 2B. The coefficient of each independent variable was significant with $P < 0.001$. The two equations could be used to esti-

Table 3
Precision (RSD, %), accuracy (bias, %) and recovery of thiols for samples analyses.

	Intra-day precision ($n = 4$)				Inter-day precision ($n = 16$)				Recovery (%)
	Homogenate[a]	Spiking[a]	Homogenate + spiking[a]	Accuracy	Homogenate[a]	Spiking[a]	Homogenate + spiking[a]	Accuracy	
Root									
Cys	0.7918 ± 0.0387 (4.89)	4.0154 ± 0.0186 (0.46)	4.9465 ± 0.0325 (0.66)	2.90	0.6013 ± 0.0231 (3.83)	4.0278 ± 0.0512 (1.27)	4.8431 ± 0.1125 (2.32)	4.62	98.04
GSH	0.6346 ± 0.0345 (5.44)	10.1857 ± 0.2071 (2.03)	12.9293 ± 0.2299 (1.78)	19.49	0.7754 ± 0.0312 (4.02)	9.8110 ± 0.2970 (3.03)	13.0494 ± 0.4160 (3.19)	23.26	117.91
PC_2	0.3365 ± 0.0118 (3.50)	1.4481 ± 0.0086 (0.60)	1.8039 ± 0.0159 (0.88)	1.08	0.3439 ± 0.0194 (5.63)	1.4347 ± 0.0172 (1.20)	1.7781 ± 0.0993 (5.58)	−0.02	99.67
PC_3	7.7338 ± 0.2224 (2.88)	1.2832 ± 0.0042 (0.33)	9.3205 ± 0.0970 (1.04)	3.37	7.7832 ± 0.1742 (2.24)	1.2809 ± 0.0163 (1.27)	9.1176 ± 0.3655 (4.01)	0.59	103.38
PC_4	0.3099 ± 0.0089 (2.89)	0.3972 ± 0.0035 (0.89)	0.6777 ± 0.0066 (0.98)	−4.16	0.3071 ± 0.0125 (4.09)	0.3981 ± 0.0028 (0.71)	0.6901 ± 0.0163 (2.36)	−2.13	95.94
PC_5	0.4356 ± 0.0123 (2.83)	0.3386 ± 0.0068 (2.02)	0.7752 ± 0.0167 (2.16)	0.12	0.4101 ± 0.0108 (2.64)	0.3411 ± 0.0062 (1.83)	0.7281 ± 0.0158 (2.16)	−3.07	101.13
PC_6	0.3576 ± 0.0031 (0.86)	0.2405 ± 0.0019 (0.79)	0.5925 ± 0.0138 (2.33)	−0.95	0.3750 ± 0.0204 (5.44)	0.2432 ± 0.0037 (1.54)	0.5912 ± 0.0143 (2.42)	−4.36	102.46
Shoot									
Cys	0.5985 ± 0.0280 (4.68)	3.9816 ± 0.0230 (0.58)	4.7398 ± 0.0457 (0.96)	3.49	0.6036 ± 0.0328 (5.44)	3.9985 ± 0.0256 (0.64)	4.8605 ± 0.1496 (3.08)	5.61	98.36
GSH	2.1339 ± 0.0532 (2.49)	10.0158 ± 0.2753 (2.75)	15.4019 ± 0.1832 (1.19)	26.77	2.0874 ± 0.0608 (2.91)	10.2213 ± 0.3159 (3.09)	15.4444 ± 0.1683 (1.09)	25.48	125.93
PC_2	0.1288 ± 0.0054 (4.21)	1.4418 ± 0.0038 (0.27)	1.5650 ± 0.0103 (0.66)	−0.36	0.1161 ± 0.0078 (6.73)	1.4450 ± 0.0068 (0.47)	1.4722 ± 0.0645 (4.38)	−5.69	95.42
PC_3	0.1927 ± 0.0064 (3.35)	1.2755 ± 0.0066 (0.52)	1.4367 ± 0.0272 (1.90)	−2.14	0.2176 ± 0.0113 (5.18)	1.2793 ± 0.0064 (0.50)	1.4379 ± 0.0551 (3.83)	−3.94	96.79
PC_4	0.0828 ± 0.0012 (1.41)	0.3990 ± 0.0024 (0.59)	0.4596 ± 0.0053 (1.14)	−4.60	0.0706 ± 0.0029 (4.07)	0.3983 ± 0.0051 (1.27)	0.4452 ± 0.0092 (2.08)	−5.06	94.48
PC_5	0.0286 ± 0.0005 (1.64)	0.3435 ± 0.0058(1.70)	0.3392 ± 0.0036 (1.06)	−8.86	0.0494 ± 0.0028 (5.59)	0.2685 ± 0.0079 (2.95)	0.2874 ± 0.0090 (3.12)	−9.61	90.87
PC_6	0.0328 ± 0.0007 (1.99)	0.2458 ± 0.0036(1.47)	0.2424 ± 0.0011 (0.47)	−12.99	0.0374 ± 0.0016 (4.33)	0.2995 ± 0.0089 (2.98)	0.2985 ± 0.0094 (3.13)	−11.37	88.11

[a] Thiol amounts per column; expressed as mean ± SD (RSD, %) μmol L^{-1}.

Table 4
Stability of thiol standards and their derivatives after freezing test, and freezing–thawing test in dark at −80 °C (n = 4).

	Thiol standards				Derivatives			
	Freezing one week[a]	Freezing one month[a]	Freeze–thaw once[a]	Freeze–thaw twice[a]	Freezing one week[a]	Freezing one month[a]	Freeze–thaw once[a]	Freeze–thaw twice[a]
S3								
Cys	−1.79	2.86	−2.50	−7.99	−2.74	−3.29	−3.33	−54.28
GSH	−0.44	1.21	−1.78	−4.04	−1.16	−2.71	−1.96	−60.68
PC_2	−0.17	−1.37	−2.33	−9.69	−2.15	−5.05	−1.76	−0.35
PC_3	−2.25	−5.12	−7.68	−13.14	−3.98	−4.34	−4.66	−4.24
PC_4	−2.17	−2.10	−8.23	−15.48	−4.36	−4.19	−4.78	−5.12
PC_5	−1.63	−4.87	−2.95	−13.07	−1.91	−2.27	−1.44	−1.59
PC_6	−1.46	−3.66	−4.52	−16.58	−3.22	−1.76	0.63	0.52
S7								
Cys	−0.27	1.68	−4.92	−7.09	−2.18	−2.89	−2.41	−13.22
GSH	−4.53	−7.77	−5.69	−11.85	−0.60	−3.43	−4.67	−31.23
PC_2	−4.06	−2.19	−3.96	−5.96	0.60	−3.56	−0.67	−2.58
PC_3	−1.27	−4.55	−5.54	−15.62	0.05	−1.89	−2.88	−3.98
PC_4	−0.89	−6.37	3.14	−10.99	−0.45	−2.50	−5.89	−5.13
PC_5	−1.63	3.43	−3.85	−12.76	−2.08	−1.26	−4.76	−7.22
PC_6	2.00	1.23	−4.46	−16.78	−1.22	−0.52	−3.96	−3.44

[a] Accuracy (bias, %) = (measured concentration/freshly prepared concentration × 100) − 100.

mate the retention time and concentrations of PC_{2-6}, when only GSH standard is available.

3.1.3. Recovery, precision and accuracy

Recovery was checked for the studied thiols by addition of known amounts of the thiols working standards to extracts (Table 3). The recoveries were acceptable with an exception that the recoveries of GSH in root and shoot extract were respectively 117.91% and 125.93%, and the reasons are unknown, which should be explored in further studies. Intra- and inter-day precision were also summarized in Table 3. The intra- and inter-day precision (RSD, %) are all less than 10% for all thiols. The accuracies (bias, %) of GSH were more than 15% in root and shoot samples, and the others within ±15%. The results demonstrated that the method was reproducible with acceptable precision and accuracy. The results obtained were partly similar with those reported [3,43]. The difference might be due to different protocols of sample preparation.

3.1.4. Stability

Stability of thiols and their derivatives were estimated in present study. The stability of thiol standards in extraction buffer and their derivatives in dark at 4 °C was firstly evaluated. The results demonstrated that thiol standards and their derivatives were all stable in 7 days without significant loss but with a trend of reducing over time. The thiol standards and their derivatives were all stable in dark at −80 °C for 1 month without loss, and the accuracies of all thiol standards were acceptable after freeze–thaw twice but significantly affected, the derivatives except Cys and GSH were found stable after freeze–thaw twice. The results provided the information that the derivatives of Cys and GSH were more sensitive to freezing and thawing than derivatives of PC_{2-6}. The data were presented in Table 4.

The stability of thiols in root and shoot extracts was furthermore estimated (Table 5). When the root and shoot extracts were stored in dark at 20 °C and at 4 °C, the longer time the more loss of thiols. The results also showed that thiols in root and shoot extracts were stable when stored in dark at −20 °C and at −80 °C. The stability of thiols in extracts was sensitive to freeze–thaw. The differences of stability of thiols in dark at 4 °C may be due to presence of more compounds in root and shoot extract than those in extraction buffer.

The results obtained were partly in agreement with those previously reported [3,29,53,57,65] and provided useful information for analyses of thiols. Firstly, plants samples should be quickly prepared and analyzed, otherwise should be quickly frozen with liquid nitrogen and stored in dark at −20 or −80 °C, and they are better to avoid freeze–thaw before analyses to protect thiols from oxidation and disintegration. Secondly, samples are ground in liquid N_2

Table 5
Stability of thiols in extracts of samples (n = 4).

	At 20 °C		At 4 °C		At −20 °C		At −80 °C		Freeze–thaw	
	2 days[a]	10 days[a]	2 days[a]	10 days[a]	2 days[a]	10 days[a]	2 days[a]	10 days[a]	Once[a]	Twice[a]
Root										
Cys	−7.55	−32.08	−6.06	−37.69	2.05	−3.56	−5.39	−3.59	−2.08	−6.85
GSH	−6.02	−34.64	−3.80	−28.34	−4.91	−1.66	−1.57	−0.98	−1.88	−6.01
PC_2	−35.77	−40.35	−29.84	−33.73	0.94	−5.60	0.58	−1.47	−2.94	−10.46
PC_3	−9.35	−48.88	−6.98	−32.48	−2.16	−3.33	−4.46	−7.96	−8.40	−16.01
PC_4	−14.50	−51.30	−0.64	−35.83	−4.33	−2.94	−4.42	−3.76	−6.86	−17.11
PC_5	−29.28	−53.53	−9.82	−39.44	−2.01	−6.05	−1.25	−0.14	−2.96	−15.36
PC_6	−32.89	−62.20	−13.97	−43.86	−4.36	−3.95	−0.87	0.72	−3.97	−18.66
Shoot										
Cys	−9.90	−61.52	0.34	−22.57	−4.00	−6.29	−1.89	−4.26	−3.79	−8.54
GSH	−2.46	−39.27	−1.46	−24.99	−2.34	−6.39	−0.42	−1.40	−5.54	−7.86
PC_2	−10.99	−47.87	−6.16	−34.75	−3.60	−6.51	−3.08	−2.88	−5.57	−9.63
PC_3	−8.04	−34.86	−4.51	−25.89	−2.27	−3.43	−4.03	−7.39	−8.25	−18.15
PC_4	−23.11	−74.95	−3.82	−75.84	−4.07	−5.48	−4.21	−6.77	−6.17	−15.16
PC_5	−15.85	−49.00	−6.10	−53.19	−5.42	−6.50	−3.82	−6.09	−7.83	−19.75
PC_6	−38.00	−53.81	−8.00	−20.75	−7.06	−8.27	−5.12	−7.32	−6.46	−16.42

[a] Expressed as accuracy (bias, %) = (measured concentration after treatment/freshly prepared concentration × 100) − 100.

Table 6
Correlation matrix of CO_2, Cd and thiols, and the effects of CO_2 and Cd on thiols in roots and shoots.

		Correlation matrix																ANOVA (Sig.)		
		Factor		Roots							Shoots							CO_2	Cd	$CO_2 \times$ Cd
		CO_2	Cd	Cys	GSH	PC_2	PC_3	PC_4	PC_5	PC_6	TSH	Cys	GSH	PC_2	PC_3	PC_4	PC_5	PC_6	TSH			
Factor	CO_2	1.000																				
	Cd	0.000	1.000																			
Roots	Cys	−0.317	0.855**	1.000																**	**	**
	GSH	−0.165	0.818**	0.738**	1.000															**	**	ns
	PC_2	−0.206	0.931**	0.833**	0.917**	1.000														**	**	*
	PC_3	−0.138	0.957**	0.820**	0.918**	0.974**	1.000													**	**	*
	PC_4	−0.213	0.891**	0.775**	0.922**	0.935**	0.964**	1.000												**	**	**
	PC_5	−0.264	0.863**	0.785**	0.903**	0.917**	0.947**	0.986**	1.000											**	**	**
	PC_6	−0.263	0.906**	0.852**	0.892**	0.924**	0.960**	0.979**	0.978**	1.000										**	**	**
	TSH	−0.155	0.950**	0.820**	0.926**	0.975**	0.999**	0.974**	0.961**	0.969**	1.000									**	**	**
Shoots	Cys	−0.107	0.926**	0.801**	0.926**	0.942**	0.967**	0.941**	0.921**	0.928**	0.968**	1.000								ns	*	ns
	GSH	−0.739**	0.460*	0.633**	0.677**	0.663**	0.593**	0.630**	0.636**	0.644**	0.607**	0.539**	1.000							**	**	ns
	PC_2	−0.118	0.939**	0.795**	0.909**	0.943**	0.976**	0.962**	0.930**	0.944**	0.978**	0.960**	0.568**	1.000						*	**	ns
	PC_3	−0.183	0.951**	0.841**	0.862**	0.966**	0.971**	0.924**	0.907**	0.916**	0.967**	0.935**	0.578**	0.951**	1.000					**	**	**
	PC_4	−0.324	0.904**	0.929**	0.774**	0.895**	0.885**	0.809**	0.784**	0.850**	0.876**	0.855**	0.668**	0.862**	0.919**	1.000				**	**	*
	PC_5	−0.179	0.919**	0.746**	0.814**	0.922**	0.930**	0.888**	0.853**	0.879**	0.926**	0.889**	0.613**	0.917**	0.912**	0.876**	1.000		*	**	ns
	PC_6	−0.690**	0.228	0.411*	0.131	0.319	0.219	0.198	0.195	0.253	0.218	0.199	0.523**	0.196	0.311	0.475*	0.310	1.000		**	**
	TSH	−0.470	0.824**	0.837**	0.899**	0.933**	0.909**	0.907**	0.895**	0.908**	0.916**	0.871**	0.865**	0.895**	0.901**	0.899**	0.882**	0.427*	1.000	**	**	ns

ns, not significant.
* Significant at the 0.05 level.
** Significant at the 0.01 level.

without repeat freeze–thaw during the protocol, and the extracts should be immediately derivatized with mBBr or stored in dark at −20 or −80 °C without repeat freeze–thaw. Thirdly, the derivatives of thiols should be analyzed using HPLC as soon as possible, otherwise stored in dark at −80 °C without freeze–thaw till analyses. Fourthly, the procedure of sample preparation is better to be quick and carried out in dark.

3.2. Application

It was reported that elevated CO_2 ameliorated Cd toxicity in *Lolium mutiforum* and *L. perenne* under Cd stress through increasing photosynthesis and enhancing antioxidant capacity, and the syntheses of PCs played key roles in detoxification mechanism [94], however, more information is in need to support and explain the views. In present study, after exposure to Cd stress for 9 days at CO_2 concentrations of 380 and 760 μL L^{-1}, the contents and proportion of thiols changed, and the data obtained are summarized in Fig. 3 and Table 6. The results demonstrated that the effects of CO_2 and Cd stress on thiols were respectively negative and positive, and there were interactive effects (Table 6). The total SH contents increased with the Cd concentration increasing regardless of CO_2 concentration, and decreased at elevated CO_2 comparing to those at ambient CO_2, being in agreement with the results obtained by Jia et al. [94]. The decrease of total SH contents may be due to the reduction of Cd contents in plant with an explanation of the dilution phenomenon induced by elevated CO_2 [94].

It was noted that the total SH contents in roots were more than in shoots, and thiols in roots were mainly PC_3 and PC_4, while GSH, PC_2 and PC_3 in shoots (Fig. 3) indicating that different thiols preformed detoxification in roots and shoots. Regardless of CO_2 level, the proportion of PCs, especially PC_3 and PC_4 in root, and PC_2 and PC_3 in shoot, showed a tendency of increase, meanwhile the proportions of Cys in root and GSH in root and shoot decreased, and Cys in shoot altered without significance at both CO_2 levels. Exposed to the same Cd concentration, no significant differences were observed in proportions of thiols between at elevated CO_2 and at ambient CO_2. It was also noted that PCs production occurred at Cd concentration of 0 μmol L^{-1}, whereas PCs were barely detected in the shoots of the control plants, and minor amounts of PCs were found in the roots of these plants [94], being due to the presence of other metal ions except Cd with more mobility and activity in solution than in soil.

Different thiols in roots or shoots were highly correlated, as well as thiols in roots and shoots (Table 6). The high correlation between different thiols in roots or shoots was due to the fact that thiols with high molecular weight are synthesized from thiols with low molecular weight as a substrate [14,15,18–21]. And the high correlation between thiols in roots and shoots may be due to another fact that PCs have the ability to undergo long-distance mutual transport between roots and shoots [95,96].

4. Conclusions

The method established was valid and applicable for simultaneous determination of Cys, GSH and PC_{2-6} to evaluate their roles in detoxification mechanisms in plants under heavy metal stress. Moreover, the analytical method provides an alternative way to investigate biogeochemical importance *in vivo*, and thiols especially PCs could be potentially used as biomarkers to assess metal toxicity to organisms.

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