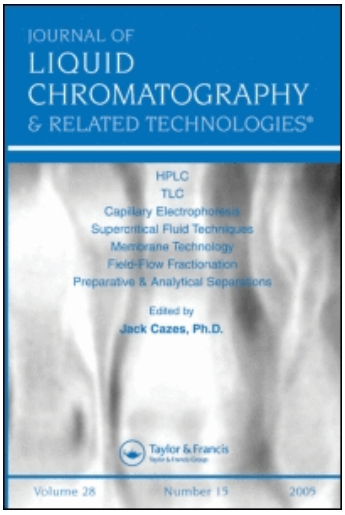

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Rapid HPLC Procedure for the Quantitation of Phytochelatins in Plant Tissue Extracts

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ABSTRACT

A simple HPLC procedure for the identification and quantitation of phytochelatins (PCs) in plant tissue extracts is described. The method, which does not require a derivatization step utilizes only 20 μL of sample volume. Linear quantitative response curve was generated for phytochelatin 3 (PC3) over a concentration range of 1.33 μmol/L–6.66 mmol/L. Linear regression analysis of the standard curve exhibited correlation coefficient of 0.996. Limit of detection (LOD) and limit of quantitation (LOQ) values were 0.1 and 0.5 μmol, respectively. Phytochelatin 3 recovery using this method was relatively high (above 85%). Intra-assay and

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inter-assay precision studies reflected a high level of reliability and reproducibility of the method. The applicability of the method for the quantitation of a wide range of PC concentrations in plant tissue extracts was demonstrated successfully.

Key Words: HPLC; Phytochelatins; Glutathione; Thiols; Heavy metals.

INTRODUCTION

The utilization of plants to remove metal contaminants in soils has been shown to be very effective and non-destructive to the natural environment.[1] One of these metal contaminants is the heavy metal, cadmium (Cd). Cd is an environmental pollutant with established mutagenic, carcinogenic, and teratogenic effects.[2] Although, the mean concentrations of heavy metals in mineral soils worldwide are low, Cd concentration in farmland soils is increasing at a rate of 0.1% each year.[3]

Plants respond to Cd and other metal toxicity by a number of mechanisms, the most important of which, is chelation with phytochelatins (PCs).[4] Phytochelatins are a group of inducible peptides synthesized from glutathione (GSH) and contain the unique sequences of $(\delta\text{-glu-cys})_n$-gly, where n may range from 2 to 11. These peptides bind to and form stable complexes with other heavy metals and place them into vacuoles, thereby neutralizing their toxicity.[5,6]

The presence of Cd in soils may not be stressful to the plants themselves, however, because of their ability to take up Cd, the latter is introduced into the food chain of animals and human beings. In man, Cd is known to accumulate in kidneys where it can cause urea poisoning and, in some cases, irreversible organ damage.[2] Additionally, Cd has been known to have harmful effects on the respiratory system and on bone tissue reproduction.[2]

Because of the potential deleterious effect of heavy metals on human health, PCs, as primary players in detoxification mechanisms in plants, received increased attention from investigators and has been the subject of intensive investigation for the past two decades. This increased interest has lead to the development of several methods for the identification and quantitation of PCs in plant tissue extracts and their metal-binding properties.[5,7] These methods ranged from high performance liquid chromatography (HPLC) to atomic absorption spectrometry (ABS) and x-ray absorption spectroscopy (XRAS).[5–7] Although, all these techniques provide a high degree of specificity and sensitivity, ABS and XRAS procedures are laborious, complicated, and time-consuming. In addition, these instruments are expensive and not readily available in most analytical laboratories. On the other hand, HPLC equipment is

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moderately-priced and is used routinely in research laboratories worldwide, rendering HPLC the most acceptable and widely used chromatographic technique for the separation and quantitation of biological compounds.

To the best of our knowledge, none of the published methods for the quantitation of PCs in plant tissue extracts combine all the desired features for a rapid, reliable, sensitive, and simple assay. In this report, a validated method for the quantitation of PCs is described. This method is simple and reliable. It does not require a liquid–liquid or solid-phase extraction step. Most importantly, it does not require any derivatization step, and it utilizes only 20 μL of sample volume. Additionally, the applicability of the method for quantifying a wide range of concentrations of PCs in plant tissues collected from various environmental regions in Jordan was demonstrated successfully.

EXPERIMENTAL

Materials

High performance liquid chromatography-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). The synthetic phytochelatin polypeptide (PC3) NH_2-(δ-Glu-Cys)$_3$-Gly-COOH (theoretical mean isotopic mass 771.85 Da) was synthesized and purified at the Microchemistry Laboratory at Emory University School of Medicine (Atlanta, GA). Amber autosampler vials of 12×32 mm (crimp-top) with 200 μL limited volume inserts were purchased from Phenomenex (Torrance, CA). Prodigy octadecyl 3 (ODS 3) analytical column (250×4.6 mm, 5 μm) and C18 ODS guard column (4×3 mm) were also purchased from Phenomenex (Torrance, CA). Centrifuge, microfuge tubes, and Nalgene cryovials were purchased from Fisher Scientific (Fair Lawn, NJ). Water used in this work met the specifications for Type II water, according to the guidelines of the National Committee for Clinical Laboratory Standards.[8] Water was filtered through Nanopure System (Barnstead, Dubuque, IA).

Methods

Preparation of Plant Tissue Extracts

Whole plants (4 plant species listed in Table 3) were harvested, immediately and separately weighed, cut into small pieces, placed in plastic bags, and

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transported to the laboratory in liquid nitrogen. In the laboratory, the contents of each bag were ground using a blender. Phytochelatins were extracted by adding 2 mL of 60% perchloric acid per gram fresh weight. Homogenates were vortexed for 1 min, transferred to centrifuge tubes, and centrifuged at 13,000*g* for 5 min. Supernatants were transferred to appropriately labeled Nalgene cryovials and stored at −80°C until use.

Synthesis of Phytochelatin 3

The synthetic PC3 NH_2-(δ-Glu-Cys)$_3$-Gly-COOH (theoretical mean isotopic mass 771.85 Da) was synthesized and purified at the Microchemical facility at Emory University School of Medicine (Atlanta, GA), using tBOC chemistry as described.[9]

Sample Preparation and Construction of the Standard Curve for HPLC Analysis

On the day of analysis, frozen sample extracts were allowed to thaw down to ambient temperature. Once samples reached room temperature, they were placed in ice. Then, 100 μL of each sample were transferred to microfuge tubes and centrifuged at 13,000*g* for 10 min. Supernatants were transferred to autosampler vials for HPLC analysis.

Because PC-deficient plant extract is not available commercially, 0.1% (v/v) TFA (0.1%) was used as the medium to construct the standard curve. A fraction of the purified PC3 was weighed and a standard solution of 50 mmol/L in 0.1% TFA was prepared. For the construction of the standard curve, tubes were spiked with increasing amounts of the prepared synthetic PC3 standard solution (50 mmol/L) to reach final concentrations of 3.33 and 6.66 mmol/L in a final volume of 250 μL each. Volumes were made up to 250 μL with 0.1% TFA. The lowest PC3 concentration point of the standard curve (1.33 μmol/L) calibrator was prepared in a similar fashion, but after serially diluting the PC standard solution 10,000 folds. A volume of 50 μL from each standard was transferred to autosampler vials for HPLC analysis. All spiked tubes used for the construction of the standard curve were treated exactly like actual plant tissue extract samples (with unknown PC concentrations) throughout the analysis. Positive and negative controls were included with every batch run. Autosample vials containing samples and standards were loaded onto a Hewlett Packard 1100 series HPLC system [Hewlett Packard (currently Agilent Technologies), Palo Alto, CA] for analysis.

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Chromatographic Conditions

Samples (20 μL) from the autosample vials were injected into a 250 × 4.6 mm Prodigy ODS (octadecyl 3) column protected by a 4 × 3 mm C18 ODS guard column. The mobile phase consisted of 0.1% TFA (solvent A) and 80% of acetonitrile in 0.1% (v/v) TFA (solvent B). Separation was achieved with a linear gradient of 2% to 100% solvent B at a flow rate of 1.0 mL/min and a column temperature of 30°C. Calibration was conducted daily with the standard curve. Phytochelatins, as well as glutathione were monitored with a Hewlett Packard 1100 Variable Wavelength Detector (Agilent Technologies, Palo Alto, CA) at an absorption wavelength of 214 nm. Data analyses were conducted using HP Chemstation (Agilent Technologies, Palo Alto, CA).

Identification of Native Phytochelatin Peaks

To collect sufficient amounts of native (not synthetic) PCs present in plant extracts for amino acid analysis, peak fractions of multiple runs (5 to 7 runs) with the same retention times were pooled. Amino acid analysis of the pooled fractions was conducted as previously described.[10] Briefly, the pooled fractions were dried and hydrolyzed in 6 N hydrochloric acid for 1 hr at 130°C in evacuated sealed tubes. Amino acid hydrolysates were derivatized with phenylisothiocyanate and separated by reversed-phase HPLC.

RESULTS AND DISCUSSION

A representative HPLC chromatogram of GSH and PCs is shown in Figure 1. The above-described HPLC conditions resulted in a simple and clean chromatogram with well-separated peaks (Fig. 1). The elution profile is in concordance with what has been reported in the literature,[11,12] in which shorter PCs had shorter retention times than longer ones. Retention times with the method described herein are slightly shorter, but generally comparable to what has been reported previously.[11,12] The PC3 peak (peak 4) was identified by two ways: (1) spiking a blank sample (0.1% TFA) with synthetic PC3; and (2) adding synthetic PC3 to a sample of a plant tissue extract already containing PC3 and observing an increase in the corresponding PC3 symmetrical peak. Blank samples not spiked with PC3 did not show any PC3 peak. GSH peak (peak 2) was identified by comparing its retention time to that obtained from a run of a commercially available standard GSH conducted under identical experimental conditions. Other PC peaks, shown in Fig. 1, were identified by

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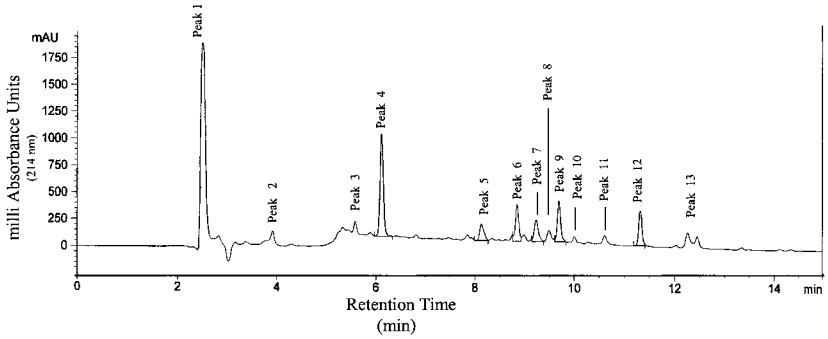

Figure 1. HPLC chromatogram of glutathione and PCs extracted from *Veronical anagalis-aquatica* (whole plant extract). Peak 1 represents the extraction solvent; peaks 2, 3, 4, and 6 correspond to GSH, PC2, PC3, and PC4, respectively. Peaks 5 and 7–13 remain unidentified.

amino acid analysis. From the molar ratio of their amino acid constituents (Glutamic Acid and Cysteine to Glycine), peaks 3 and 6 were identified as phytochelatin 2 (PC2) and phytochelatin 4 (PC4), respectively. Peak 1 represents the extraction solvent. Peaks 5 and 7–13 remain unidentified.

As shown in Table 1, the retention time for synthetic PC3 is 6.11 min. The calibration curve obtained for PC3 was constructed using linear regression analysis. A linear quantitative response curve was achieved over a concentration range of 1.33 μmol/L–6.66 mmol/L. Analysis of the regression line resulted in a correlation coefficient of 0.996 (Table 1). The formula of the line is also shown in Table 1. The limit of detection (LOD) and limit of quantitation (LOQ)

Table 1. Performance parameters for the quantitation of PCs in plant tissue extracts using HPLC.

Performance parameters	Description/value
Retention time of phytochelatin 3 (PC3)	6.11 min
Linearity range	1.33 μmol–6.66 mmol/L
Equation of the line	$RR^a = 0.314 \times AR^b + 7.163 \times 10^{-4}$
Correlation coefficient (r^2)	0.996
Limit of detection	0.1 μmol
Limit of quantitation	0.5 μmol

[a]RR, Response ratio; area of analyte/area of internal standard.
[b]AR, Amount ratio; concentration of analyte/concentration of internal standard.

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for PC3 were determined experimentally ($n = 4$). For LOQ, blank specimens were spiked with a series of decreasing concentrations of PC3. LOD was defined as the concentration corresponding to a signal to noise ratio ≥ 3. Limit of quantitation was defined as the lowest quantitated concentration that was within 10% of the target concentration. As shown in Table 1, LOD and LOQ values obtained for PC3 were 0.1 and 0.5 μmol, respectively.

Table 2 illustrates the percent mean recoveries of PC3 at three concentrations (1.33 μmol/L, 3.33 mmol/L, and 6.66 mmol/L). These three concentration points represent the low, middle, and high portions of the standard curve. As shown in Table 2, percent mean recoveries of PC3 at the high, medium, and low concentrations were 92.6%, 90.4%, and 85.0%, respectively. As expected, the percent recoveries obtained with the three concentrations were relatively high. This is most probably due to the fact that PCs contain δ-peptide bonds between their glutamic acid and cysteine residues, rather than the α-peptide bonds comprising most polypeptides. The presence of these δ-peptide bonds renders PCs resistant to hydrolysis by proteases. It is important to note here, that although plant tissue extracts were treated with perchloric acid to precipitate large proteins, traces of proteases could have been present in our samples, for these enzymes have been known to remain active in tissue extracts even under very acidic conditions.

Intra-assay and inter-assay precision of the analytical procedure, as represented by percent correlation of variance (%CV), is illustrated in Table 2. Precision was determined experimentally ($n = 4$) by spiking blank 0.1% TFA samples with PC3 at concentrations of 1.33 μmol/L, 3.33 mmol/L, and 6.66 mmol/L. At the three concentrations, intra-assay precision (%CV) values ranged from 3.54% to 7.22% (Table 2). Inter-assay precision of the method was determined experimentally in a manner similar to that of intra-assay

Table 2. Percent recovery and precision of the PC assay.

		Intra-assay precision		Inter-assay precision	
PC3 concentration	Percent recovery (%)	Mean concentration[a]	%CV[b]	Mean concentration[a]	%CV[b]
1.33 μmol/L	85.0	1.13 μmol/L	7.22	1.01 μmol/L	9.91
3.33 mmol/L	90.4	3.01 mmol/L	6.98	2.83 mmol/L	8.47
6.66 mmol/L	92.6	6.17 mmol/L	3.54	5.36 mmol/L	7.65

[a] $n = 4$

[b] Coefficients of variations (CV) were calculated as standard deviations expressed as percentage of mean values.

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precision. Spiked blank samples were analyzed on a daily basis for 2 weeks. Inter-assay precision (%CV) ranged from 7.65% to 9.91% (Table 2). The relatively higher %CV values observed with the low PC3 concentrations (7.22 and 9.91 for intra-assay and inter-assay precision, respectively) are because this concentration is closer to the LOQ where greater variation should be expected.

To the best of our knowledge, hitherto, the methodologies described in the literature for the identification and quantitation of PCs did not provide any information on validation. Consequently, there is no reference to which LOQ, precision, and percent recovery values obtained for this method can be compared. The only exception was a report,[11] in which LOD was stated to be 0.1 μmol/g dry weight.

The applicability of this method to determine PC concentrations in whole plant tissue extracts, prepared from plants collected from various regions in Jordan, was demonstrated successfully (Table 3). From an ongoing study that we are pursuing, on the effect of soil heavy metal concentrations on PC content, tissue extracts of four plant species (Table 3) were assayed for these polypeptides. These plants were found to contain varying concentrations of PC3 and PC4 (Table 3).

In a number of published methods,[13–16] PC concentrations were estimated indirectly by subtracting GSH concentration from total acid-soluble thiol concentrations. This indirect way of measuring PC concentrations does not take into consideration the presence of acid-soluble thiols, other than GSH and PCs, hence, leading to overestimation of PC concentrations. The main advantage of the method described herein, is that PC concentrations are measured directly using a standard curve.

Because this method utilizes the absorbance of the peptide bonds occurring between amino acid residues of PC polypeptides, it can be utilized for the identification and quantitation of GSH (Fig. 1). The method is incapable, however, of identifying individual free amino acid thiols that are not part of a polypeptide, such as cysteines or cystines. Preliminary data from

Table 3. Phytochelatin concentrations in whole plant tissue extracts.

Plant species	Most abundant phytochelatin species	Concentration (mmol/g fresh weight)
Veronical anagalis-aquatica	PC3	2.92
Rumex dentatus	PC3	0.66
Rumex vesicarius	PC4	1.80
Inula viscosa	PC3	0.44

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our laboratory suggest that derivatizing the supernatant fractions of the centrifuged plant tissue extracts with the thiol-specific derivatizing agent ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBDF), may increase the sensitivity of the method, hence, rendering it capable of identifying free cysteine (data not shown). The utilization of SBDF for the derivatization and, hence, quantitation of PCs is still in its preliminary stages and under intensive investigation in our laboratory.

CONCLUSION

The method presented here is simple, rapid, and reliable. Because practicality and low instrument cost are central features of most laboratory tests, this method is well within the capabilities of the average analytical laboratory. The assay shows high sensitivity, excellent precision, and high recovery of PCs. More importantly, the method utilizes only 20 μL of sample volume, and PCs are separated from other thiols in a relatively short run time. The present method has been found reliable as a routine assay in our laboratory, and about 20 samples can be processed overnight.

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