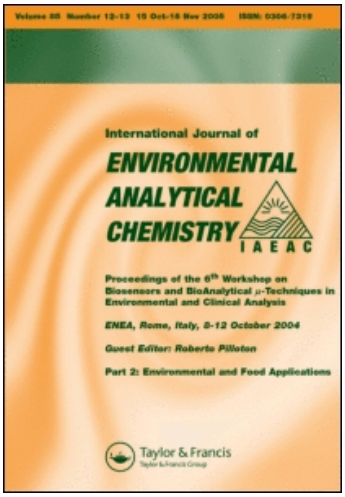

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Roxane England[a]; Kevin J. Wilkinson[a]
[a] Department of Chemistry, University of Montreal, Montreal, QC, H3C 3J7, Canada

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Determination of phytochelatins in algal samples using LC-MS

Roxane England and Kevin J. Wilkinson*

Department of Chemistry, University of Montreal, PO Box 6128, Downtown Station, Montreal, QC, H3C 3J7, Canada

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The objective of this study was to develop a tool to assess the bioavailability of Cd in freshwaters. An analytical method was developed to measure the induction of phytochelatins (PC) by *Chlamydomonas reinhardtii* using online HPLC-ESI-MS/MS. PC determinations were validated and detection limits were determined. It was shown that the presence of dithiothreitol (DTT) was necessary in order to maintain the PC in their reduced form. Sample purification was shown to be extremely important. The stability of PC was evaluated for several temperature storages (−20°C, 1°C and room temperature): PC degraded with time under all examined temperatures, including freezing.

Keywords: LC-MS; phytochelatin; biomarker; toxicity; bioavailability

1. Introduction

The bioavailability of trace metals is very much influenced by their chemical speciation in the natural environment, the presence of trace metal competitors and hardness metals (Ca, Mg) and physical parameters such as temperature and ionic strength [1]. It is thus difficult to perform simple *chemical* measurements that are useful for monitoring subtle perturbations of trace metal bioavailability or risk in the natural environment. Biomarkers can be one tool to evaluate organism health in the presence of contaminants such as trace metals.

Phytochelatins (PC) are intracellular polypeptides having the general structure $(\gamma\text{Glu-Cys})_n\text{-Gly}$ ($n = 2$ to 11) [2,3] that are produced by a number of aquatic organisms following their exposure to toxic trace metals such as Cd, Cu, Zn, Hg and Pb [2,4–6]. Indeed, several studies on marine and freshwater alga have demonstrated that intracellular PC concentrations increase with increasing concentrations of metals [4,7–11], including Cd [2,10,12,13]. For example, Ahner *et al.* [10] have shown that several species of phytoplankton from Cd contaminated ($\geq$30 nM) marine systems had elevated PC concentrations that were in line with concentrations found in the laboratory under similar conditions [7]. Such studies suggest that PC concentrations could be used as a biomarker for trace concentrations of metals.

Several analytical techniques have been employed to quantify PC. For example, reverse phase liquid chromatography (RP-HPLC) [14,15] has been employed for the past twenty years in concert with absorbance or fluorescence detection, following deritivisation [15]. Voltamperometric detection [14–17] appears to be as much as 1000x more sensitive than

*Corresponding author. Email: kj.wilkinson@umontreal.ca

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absorbance [16]. More recently, PC identification has been accomplished by electrospray ionisation mass spectrometry either alone (ESI-MS) or in tandem (ESI-MS/MS) [14,17–19]. By coupling RP-HPLC and mass spectometric detection, instrument detection limits (IDL) on the order of the femtomole have been attained for the determination of oxidised and reduced thiols in animal fluids [17].

The overall goal of this study was to develop an analytical method to evaluate Cd bioavailability in natural waters, more precisely by quantifying phytochelatin production by a unicellular alga, *Chlamydomonas reinhardtii*. In a first step, the chromatographic separation (RP-HPLC) was optimised using a mixture of phytochelatin standards (PC2, PC3 and PC4) in water. Following electrospray ionisation, the PC were analysed by a triple quadrupole MS in selected reaction monitoring (SRM) mode in order to determine calibration curves and detection limits. Finally, phytochelatins were determined in algae that had been exposed to Cd in the laboratory.

2. Experimental

2.1 *Materials*

All polymerware employed for the experiments was first soaked for 24 hours in 0.1 M HNO_3 (Sigma-Aldrich), rinsed 6 times with Milli-Q water ($R > 18\,M\Omega\,cm$, $TOC < 2\,\mu g\,C\,L^{-1}$) and then dried in a laminar flow hood.

2.2 *Maintenance and exposure of the algae*

Wild type WT-2137 *C. reinhardtii* was obtained from the Chlamy Center of Duke University (Durham, NC). A small quantity of algae was sampled from the agar plate and inoculated in a sterilised TAP (Tris-Acetate-Phosphate) growth medium [20] under a 12:12 h light : dark regime of 50 μE of fluorescent white light and rotary shaking, (100 rpm) at 20°C (Multitron, Infors HT). Algae were grown until their mid exponential growth ($1.5–3.0 \times 10^6$ cell mL^{-1}) as determined by particle counter measurements (Multisizer 3, Beckman Coulter). Cell surface areas were also determined with the particle counter. The cells were diluted to 10^5 cell mL^{-1} in a renewed growth medium and once again incubated until mid-exponential growth, prior to the centrifugation and washing of the cell pellet.

An aliquot of the algal cell concentrate was pipetted into a TAP exposure solution containing no trace metals (TAP-TM) except Cd ($\leq 10^{-6}$ M) in order to obtain a final algal surface area of 0.8–1.2 $cm^2\,mL^{-1}$ (corresponding to $(2–5) \times 10^5$ cells mL^{-1}). Citrate (0.01 M) was added to the solutions to buffer Cd^{2+} concentrations in order to avoid Cd depletion that would limit Cd bioavailability. Sixty mL of algal solution was sampled following exposures of 1, 2, 4, 6 and 8 hours. Cd bioaccumulation was stopped by adding 5 mL of EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate, Sigma-Aldrich, final concentration 0.001 M) to the algae prior to their centrifugation for 4 minutes at $215 \times g$ [21].

2.3 *Extraction of phytochelatins*

The algal pellet was washed with a TAP-TM solution, transferred to an Eppendorf tube where it was re-centrifuged for 5 minutes at $10390 \times g$ (4°C, AccuSpin Micro R, Fisher Scientific) and then frozen (−80°C) until extraction. One (1.0) mL of 25 mM DTT

(4°C, Sigma-Aldrich) was added to the thawed pellet (no DDT was employed in preliminary experiments). The sample was vortexed for 1 minute, sonicated (40 kHz, Branson5510) for 2 minutes then again centrifuged for 20 minutes at $10390\times g$ (4°C). Following removal of the supernatant, the pellet was extracted an additional 2 times. The combined supernatants were centrifuged for 4 minutes at $16320\times g$ (Multifuge 1SR, Heraeus) then lyophilised (Freezemobile 35 LE, VirTis) and frozen (−80°C) until analysis.

2.4 *Validation of the HPLC-ESI-MS/MS technique*

PC2, PC3 and PC4 were quantified by HPLC-ESI-MS/MS (ThermoFinnigan Surveyor HPLC system, Thermo Fisher Scientific TSQ Quantum Ultra AM quadrupole). Validation consisted of a linearity determination from a standard addition of 5 PC standards (Anaspec, 1–1000 $\mu g\,L^{-1}$) in water or in algae that were not exposed to Cd. Instrument and method detection limits were determined from the signal obtained from a blank solution or a solution containing 300 $\mu g\,L^{-1}$ of PC. The chromatographic parameters used for the detection of the PC are given in Table 1.

3. Results and discussion

3.1 *Validation*

For the PC standards in water (without added DTT), good linearity was obtained as a function of concentration for PC2 (Figure 1; $R^2 = 0.985$). For PC3 ($R^2 = 0.851$) and PC4 ($R^2 = 0.541$), an increase in measured peak areas was observed with concentration, however, the correlation was very poor (Figure 1). For similar (mass) concentrations of the three PC standards, peak areas for PC4 were significantly lower than those observed for PC2 and PC3. It was hypothesised that the smaller peak areas obtained for PC4 and the poor correlation obtained for PC3 and PC4 may have been due to the formation of

Table 1. Conditions used for the separation and detection of phytochelatins.

HPLC			MS		
Column	Eclipse XDB-C18		Ionisation	ESI	
Flow rate	500 $\mu L\,min^{-1}$		Analyser	Triple quadrupole	
Injected volume	3 μL		Mode	SRM	Positive
Mobile phase	A: H_2O, 0.1% formic acid			Precursor ion (m/z)	Product ion (m/z)
	B: Acetonitrile, 0.1% formic acid			540.200	336.100
Gradient	Minutes	Solvent A			411.200
	0.00–10.00	95.0%		772.200	336.100
	10.00–11.00	50.0%			465.100
	11.00–13.00	10.0%		1004.300	540.100
	13.00–13.10	10.0%			697.100
	13.10–18.00	95.0%	Mode	Full scan (m/z) 100.000–1100.000	

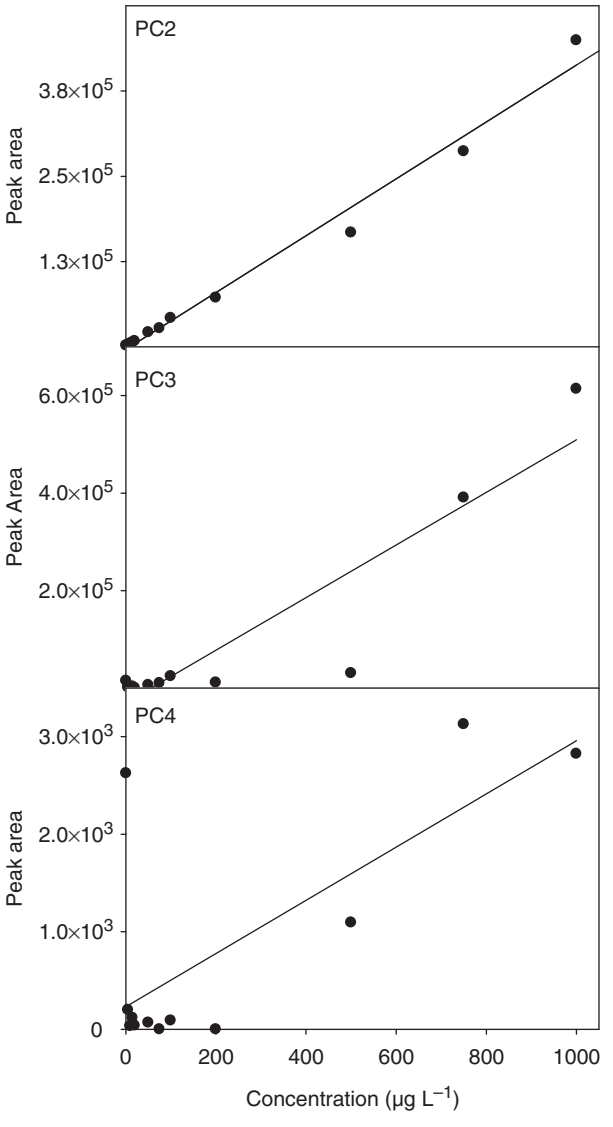

Figure 1. Peak areas determined from HPLC-ESI-MS/MS analysis of three phytochelatin standards (PC2, PC3, PC4) prepared in water without addition of DTT.

intramolecular disulphur bridges. Fortunately, it is possible to maintain the reduced –SH group by adding reducing agents to the PC such as DTT (DL-dithiothreithol) or TCEP (tris(2-carboxyethyl)phosphine) [14]. Given its lower cost and lower corrosiveness, DTT was employed in this study. Indeed, the presence of 5 mM of DTT resulted in a significant increase in the peak areas for PC3 and PC4 and a better linearity at the low concentrations that were examined (0–200 μg L^{-1}) (see Figures 1 and 2). Although the addition of both 25 and 50 mM DDT consistently improved linearity and signal, no consistent advantage was found to adding the higher concentration. When PC standards were re-run in the presence

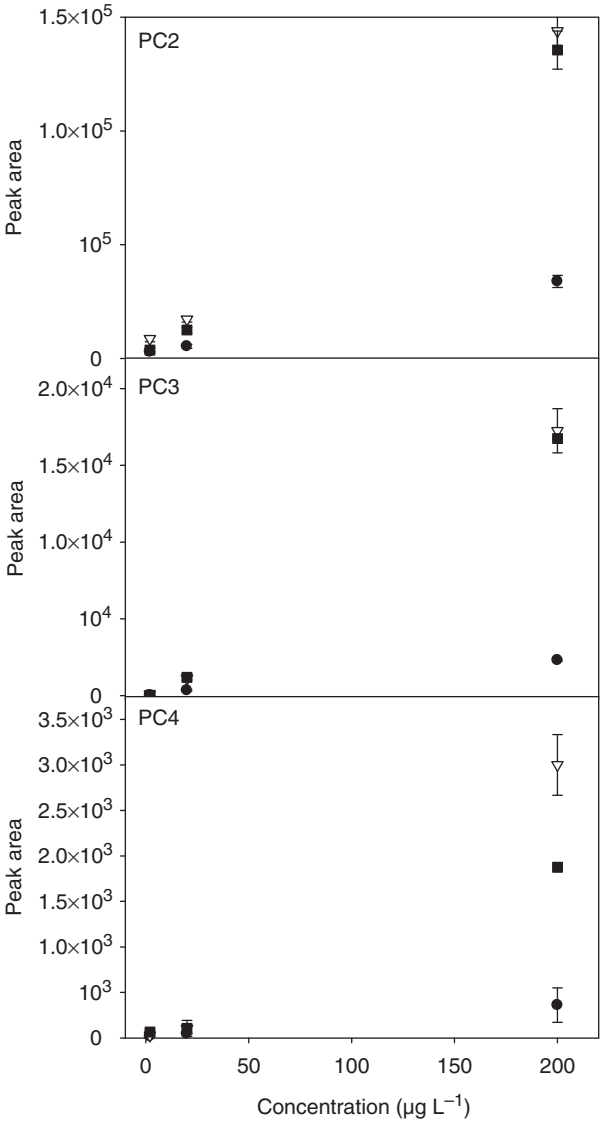

Figure 2. Effect of the reducing agent on surface areas for different concentrations of the phytochelatins: 0 mM DTT (black circle), 25 mM DTT (white triangle) and 50 mM DTT (black square).

of 25 mM of DTT (Figure 3), both determination coefficients (PC2: 0.998; PC3: 0.997; PC4: 0.986) and slopes were improved (PC2: 2290; PC3: 289; PC4: 34) as compared to results obtained in its absence. Instrument detection limits (IDL) and quantification limits (IQL) determined from those analyses are given in Table 2.

Elution profiles (Figures 4 and 5) resembled those of El-Zohri *et al.* [14] and Simmons *et al.* [22], except that elution times were substantially shorter for the PC. When PC standards were spiked into algal homogenates, a suppression of the MS signal was

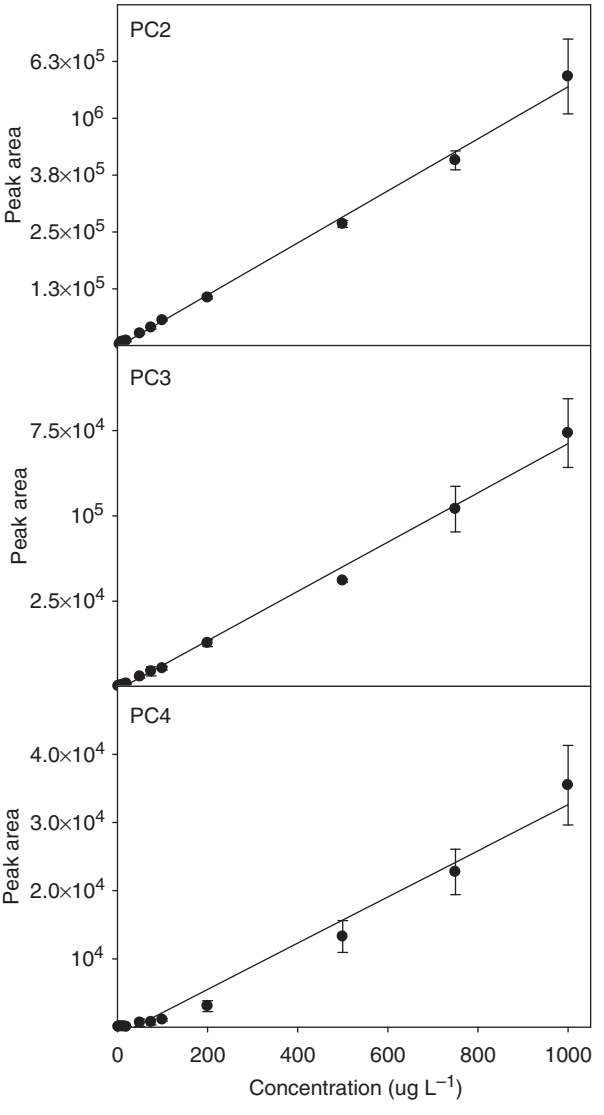

Figure 3. Linearity domain for the three phytochelatins determined from HPLC-ESI-MS/MS analysis of PC standards in a reducing homogenate (25 mM DTT).

Table 2. Instrumental detection limit and instrumental quantification limit for phytochelatin analysis following HPLC-ESI-MS/MS determination.

Phytochelatin	Instrumental detection limit	Instrumental quantification limit
PC2	12 μg L^{-1}	27 μg L^{-1}
PC3	31 μg L^{-1}	69 μg L^{-1}
PC4	85 μg L^{-1}	196 μg L^{-1}

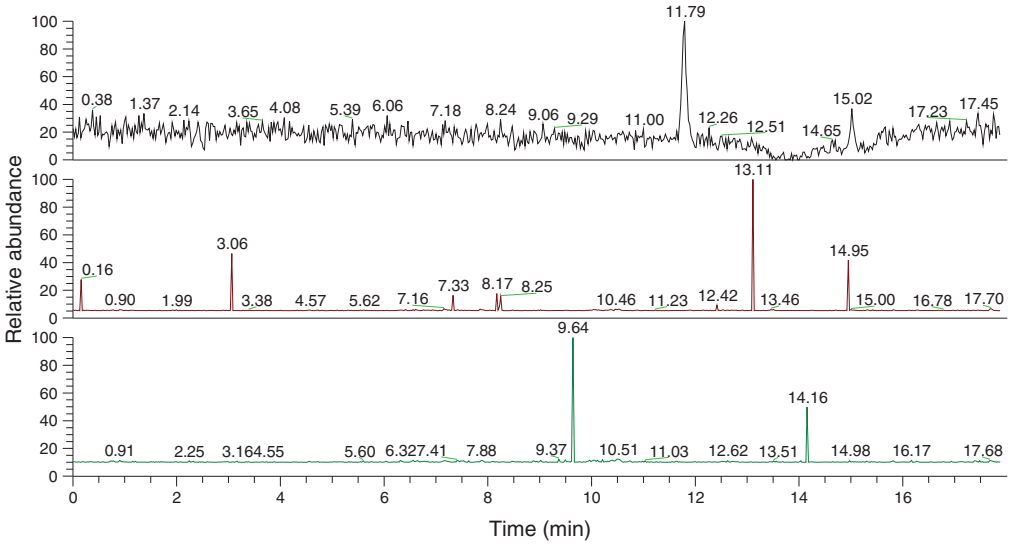

Figure 4. HPLC-ESI-MS/MS analysis of the algal homogenate (matrix).

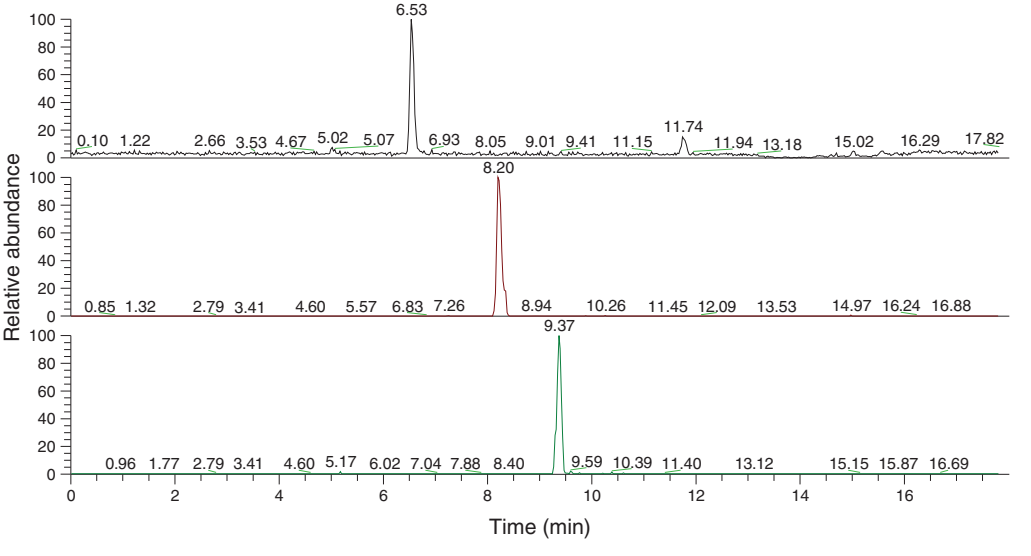

Figure 5. HPLC-ESI-MS/MS analysis of 500 μg L^{-1} of the phytochelatin standards spiked into the algal homogenates (matrix). Top chromatogram corresponds to PC2, middle chromatogram to PC3 and final chromatogram to PC4.

observed for each of the PC (see Figures 4 and 5) and the regression of signal intensity as a function of concentration was less linear (Figure 6). Although calibration curves could still be employed for quantitative determinations, clearly, more optimisation is still warranted prior to systematic use of the method. Several attempts were made to purify the PC using Cd bound IMAC (NTA Superflow, Qiagen) columns (data not shown), SPE (Oasis MAX, Waters) columns (data not shown) and simply centrifugation (16320×*g*). While the signal

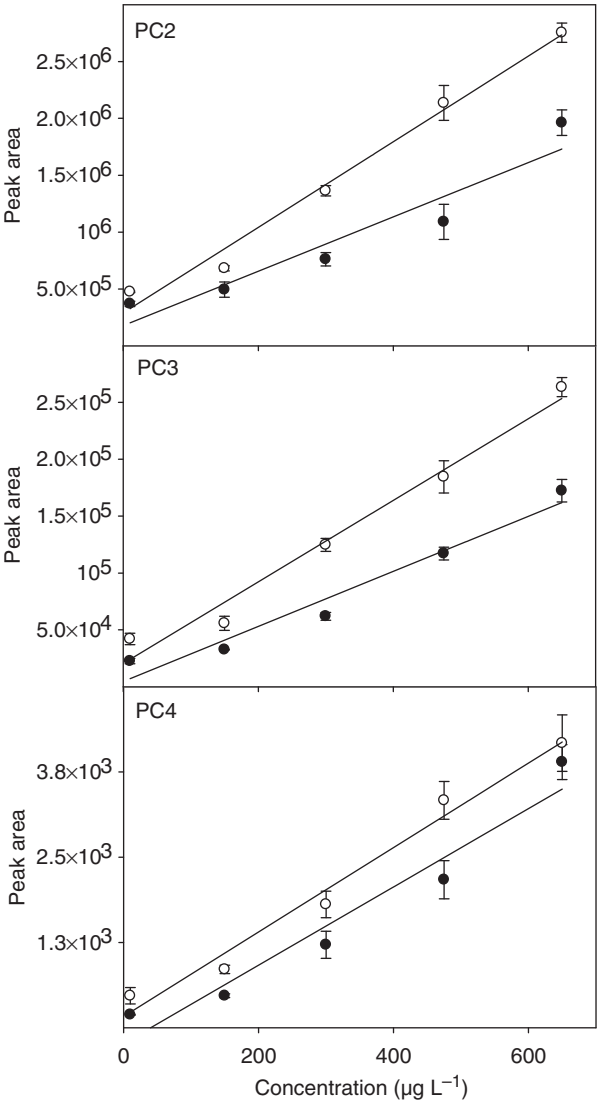

Figure 6. Linearity of the HPLC-ESI-MS/MS analysis of phytochelatin standards in 25 mM DTT with (black circle) or without (white circle) algal matrix.

reduction was still large, the most reproducible improvements of PC signal quality were obtained by the removal of algal cell debris by centrifugation.

As mentioned above, calibration slopes consistently decreased when in the presence of algal matrix. Nonetheless, a note of caution is required. Experiments were repeated in triplicate on three separate days. It was observed that peak areas determined on the first day of experiments were significantly higher that those obtained on subsequent days (Table 3). The source of this observed decrease in slope was thus evaluated by evaluating the stability of the PC standards with time for a number of different sample preservation conditions.

Table 3. Slopes and the determination coefficients for phytochelatin standards homogenate in 25 mM DTT with or without algal matrix.

		PC2		PC3		PC4	
Analyte matrix		Without	With	Without	With	Without	With
Day 1	Slope	4167	2388	407	242	27	23
	R^2	0.997	0.907	0.997	0.958	0.988	0.945
Day 2	Slope	2439	1180	222	132	15	11
	R^2	0.995	0.738	0.998	0.864	0.961	0.942
Day 3	Slope	2394	1156	196	117	13	13
	R^2	0.995	0.923	0.991	0.979	0.994	0.955

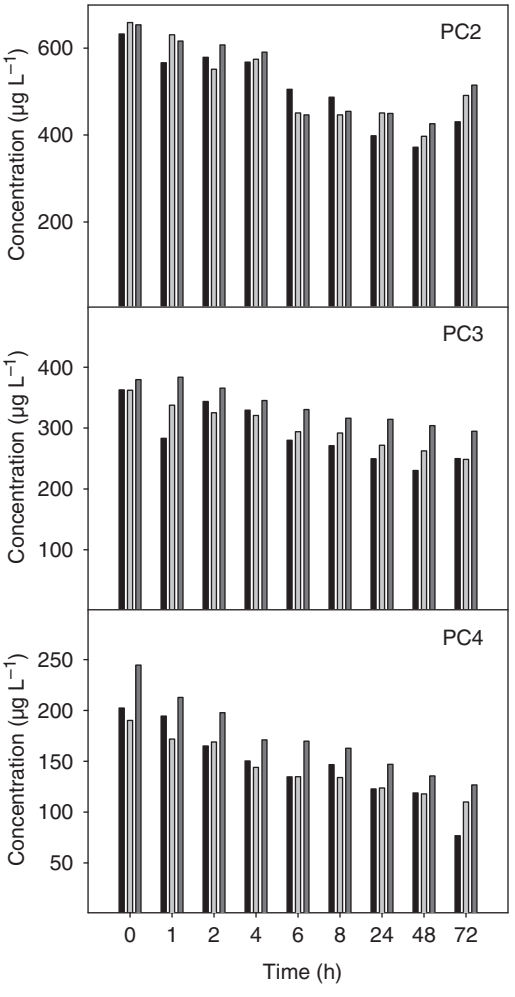

Figure 7. Stability of the phytochelatins according to their storage condition, −20°C (black), 1°C (light grey) and room temperature (dark grey).

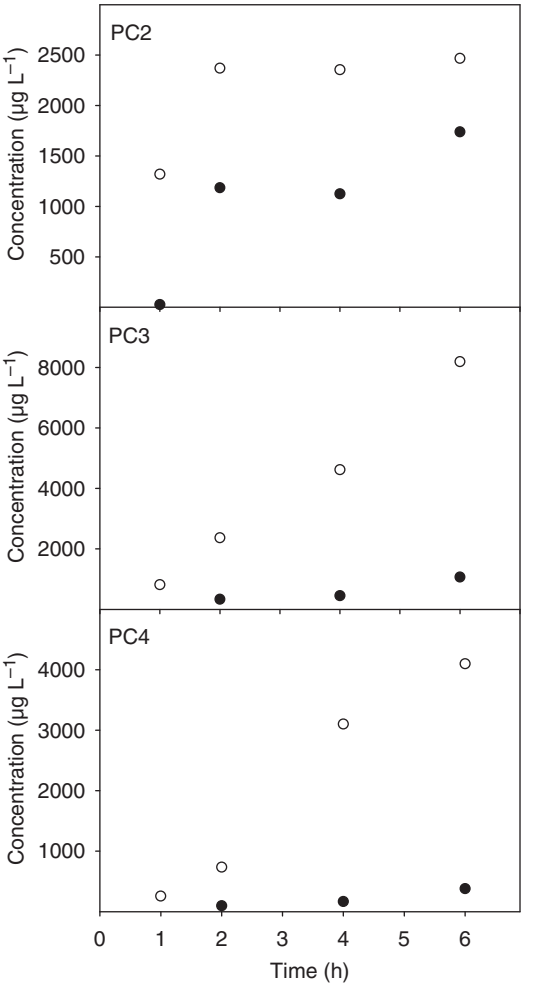

Figure 8. Analysis of phytochelatins induced by *C. reinhardtii* that were exposed to 10^{-7} M Cd (black circle, 10^{-8} M Cd^{2+}) and 10^{-6} M Cd (white circle, 10^{-7} M Cd^{2+}).

PC were prepared then run following storage at 3 different temperatures (−20°C, 1°C and room temperature). For PC2 and PC3, storage under all three conditions had a small, but significant effect on measured peak areas (Figure 7). Furthermore, for PC4, a very large decrease in surface area was observed. It was surprising to observe that peak areas decreased even when the sample was stored at −20°C. Indeed, the decrease in signal appeared to be less important at room temperature than for the samples at the controlled (colder) temperatures. This suggested that the PC degradation may have been, at least partially caused by a physical (e.g. due to ice formation) rather than chemical or biological degradation. The presence of small amounts of oxygen or transition metals could also have contributed to the decreased signal due to oxidation, however, it should be noted that these experiments were performed in the absence of the algal matrix and in the presence of 25 mM DTT. In the presence of algae, intracellular enzymes would surely be expected to

contribute to an additional biochemical degradation of the phytochelatins. Recent studies have demonstrated the critical importance of reducing phytochelatin degradation. For example, Simmons *et al*. [22] greatly reduced PC degradation by preparing their samples in an oxygen free environment (Ar). Similarly, phytochelatins were stable for more than 4–6 weeks at 4°C once derived with mBBr for fluorescence analysis [23]. At the very least, Figure 7 demonstrates that great care and multiple calibrations are required for LC-MS analysis of the PC.

3.2 *PC analysis in* C. reinhardtii *following exposure to Cd*

PC concentrations were evaluated following the exposure of *C. reinhardtii* to Cd. PC were generally observed for each of the Cd exposure concentrations (1×10^{-7} M and 1×10^{-6} M) and at all exposure times (1, 2, 4 and 6 hours) with the exception of PC3 and PC4, which were not observed at 1 hour (Figure 8). Globally, induction was superior following exposure to 10^{-6} M Cd (10^{-7} M Cd^{2+}) as compared to 10^{-7} M Cd (10^{-8} M Cd^{2+}). In the case of PC2, peak areas appeared to stabilise for both Cd concentrations while they appeared to increase over time for PC3 and PC4. This saturation of the PC2 concentrations was expected, as the smaller PC2 peptides are used to synthesise the larger peptides (PC3, PC4). Furthermore, as expected, PC concentrations increased more quickly following induction at 10^{-7} M Cd^{2+} with respect to 10^{-8} M Cd^{2+}. Both Cd concentrations generally resulted in PC concentrations that were well above the detection limits of the HPLC-ESI-MS/MS.

4. Conclusion

In summary, it was shown that the PC form disulphur bridges in aqueous media, but for analytical purposes the bridges can be dissolved using 25 mM DTT. In DTT, the PC could be quantified by HPLC-ESI-MS/MS for concentrations ranging from $1\,\mu g\,L^{-1}$ to $1.0\,mg\,L^{-1}$. While signal suppression was observed in the presence of the algal matrix, the calibration curve remained fairly linear. Further studies should focus on the sample clean-up step in order to completely reduce signal suppression. Additionally, analysts should pay special attention to standard and sample conditions since all PC solutions were shown to degrade with time whether at −20°C, 1°C or room temperature. It is thus likely to be important to analyse natural PC samples as quickly as possible in order to minimise degradation or to work under inert conditions, especially in the presence of the algae. Finally, the developed analytical technique was successful to measure the induction of PC by *C. reinhardtii* exposed to 10^{-7} and 10^{-6} M Cd under laboratory conditions. With some modifications of the analytical method, including better PC clean-up, the technique will surely become a useful biomarker for Cd bioavailability in natural waters.

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References

[1] V.I. Slaveykova and K.J. Wilkinson, Environ. Chem. **2**, 9 (2005).
[2] E. Grill, E.L. Winnacker, and M.H. Zenk, Science **230**, 674 (1985).
[3] G. Howe and S. Merchant, Plant Physiol. **98**, 127 (1992).
[4] B.A. Ahner, S. Kong, and F.M.M. Morel, Limnol. Oceanogr. **40**, 649 (1995).
[5] C. Cobbett and P. Goldsbrough, Ann. Rev. Plant Biol. **53**, 159 (2002).
[6] W.E. Rauser, Ann. Rev. Biochem. **59**, 61 (1990).
[7] B.A. Ahner, N.M. Price, and F.M.M. Morel, Proc. Nat. Acad. Sci. U.S.A. **91**, 8433 (1994).
[8] S.B. Le Faucheur, R. Behra, and L. Sigg, Environ. Sci. Technol. **39**, 8099 (2005).
[9] S.B. Le Faucheur, R. Behra, and L. Sigg, Environ, Toxicol. Chem. **24**, 1731 (2005).
[10] B.A. Ahner and F.M.M. Morel, Limnol. Oceanogr. **40**, 658 (1995).
[11] S.B. Le Faucheur, R. Behra, and L. Sigg, Aquat. Toxicol. **80**, 355 (2006).
[12] E. Grill, E.L. Winnacker, and M.H. Zenk, Proc. Nat. Acad. Sci. U.S.A. **84**, 439 (1987).
[13] K. Knauer, B. Ahner, H.B. Xue, and L. Sigg, Environ. Toxicol. Chem. **17**, (12), 2444 (1998).
[14] M.H.A. El-Zohri, R. Cabala, and H. Frank, Anal. Biooanal. Chem. **382**, 1871 (2005).
[15] M. Perez-Rama, E.T. Vaamonde, and J.A. Alonso, Electrophoresis **26**, 610 (2005).
[16] B. Yosypchuk, I. Sestakova, and L. Novotny, Talanta **59**, 1253 (2003).
[17] S.K. Kawakami, M. Gledhill, and E.P. Achterberg, Trac-Trends Anal. Chem. **25**, 133 (2006).
[18] V. Vacchina, H. Chassaigne, M. Oven, M.H. Zenk, and R. Lobinski, Analyst **124**, 1425 (1999).
[19] W.H. Zhang and Y. Cai, Anal. Chem. **75**, 7030 (2003).
[20] E. Harris, *The Chlamydomonas Sourcebook* (Academic Press, London, 1989), p. 2000.
[21] C.S. Hassler, V.I. Slaveykova, and K.J. Wilkinson, Limnol. Oceanogr. Meth. **2**, 237 (2004).
[22] D.B.D. Simmons, A.R. Hayward, T.C. Hutchinson, and R.J.N. Emery, Anal. Bioanal. *Chem.* **39**, 809 (2009).
[23] R. Minocha, P. Thangavel, O.P. Dhankher, and S. Long, J. Chromatogr. A. **1207**, 72, (2008).