Characterisation and determination of phytochelatins in plant extracts by electrospray tandem mass spectrometry

The Analyst

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A method based on pneumatically assisted electrospray ionisation tandem mass spectrometry (ESI MS-MS) was developed for the identification, sequencing and determination of phytochelatin (PC) peptides in plant tissue and plant cell cytosols. The ionization and fragmentation conditions were optimized using a series of $(GluCys)_2Gly$ (PC_2), $(GluCys)_3Gly$ (PC_3), and $(GluCys)_4Gly$ (PC_4) standards prepared from glutathione by enzymatically (γ-glutamylcysteine dipeptyl transpeptidase) assisted biosynthesis in the presence of Cd^{2+}. Phytochelatins were found to ionize readily to produce a characteristic mono-protonated ion. The collision-induced dissociation (CID) of this ion followed by mass spectrometry (MS-MS mode) allowed the determination of the amino acid sequence of each of the PCs. Calibration curves were linear up to a concentration of 2 μg ml^{-1} in the MS and MS-MS modes with the detection limits at the low ng ml^{-1} level. The method was applied to the determination of phytochelatin peptides biosynthesized by a number of plant cell cultures exposed to the Cd stress. The results agreed with those obtained by an independent procedure based on reversed-phase HPLC with post-column derivatization of the –SH groups with 5,5′-dithiobis-2-nitrobenzoic acid and spectrophotometric detection.

Introduction

Speciation of heavy metals in plants has been attracting considerable interest as a way to understand the internal mechanisms allowing the living organisms to grow in an environment contaminated by heavy metals.[1] Most frequently, as a response to the metal stress, plants biosynthesize a ligand, such as a phenolic compound, organic acid, or oligo- or polypeptide that is able to complex the excess of the toxic element into a compound innocuous to the organism.[1–4]

Particular attention has been paid to phytochelatins (PCs) which are a class of peptides composed only of three amino acids: cysteine (Cys), glutamic acid (Glu) and glycine (Gly) and in which glutamic acid is linked to cysteine through a γ-peptide linkage. Their general formula is $(GluCys)_nGly$ where n is between 2 and 11.[2–4] They are synthesized from glutathione (GSH) in the presence of some heavy metals during a reaction catalysed by the enzyme γ-glutamylcysteine dipeptyl transpeptidase (PC-synthase).[5] PCs can detoxify these metals by forming a metal–PC complex in which the metal is bound to the thiol group of the cysteine unit.[2–5] The general structure of phytochelatins is conservative in a wide variety of plants but some modifications may occur on the C-terminal amino acid. For example, instead of glycine, β-alanine was found in some plants (*Fabacea*),[6,7] serine[8] and glutamic acid[9] were reported in rice and maize, respectively, whereas des-Gly phytochelatins $(GluCys)_n$ were found in yeast.[10] These modified PCs are named iso-PCs.

Even though some hyphenated techniques, such as size-exclusion chromatography (SEC) with inductively coupled plasma mass spectrometric (ICP-MS) detection offer an attractive way to monitor the induction of PCs and binding of heavy metals to these ligands, the poor resolution of this separation technique and the lack of the molecular specificity of the detector allow neither the differentiation between the different PCs and iso-PCs complexes nor the differentiation between PCs and other ligands.[11,12] The key to the understanding of the heavy metal detoxification mechanisms in plants is the unambiguous identification, characterization and quantification of the bio-induced ligands.

The classical approach to the analysis of PCs is reversed phase HPLC with post-column derivatization of the sulfhydryl groups with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) and spectrophotometric detection at 410 nm.[13–16] The detection is not specific to PCs; any compound containing a sulfhydryl group is able to produce a signal. The signal identification needs therefore to be based on matching the retention times of the analyte compounds with the corresponding standards. The latter are usually unavailable. Even if they were available, ambiguities with the identification using this approach may occur, especially in the case of iso-PCs which have a similar structure and retention times to the corresponding PCs. The use of an analytical technique able to detect compounds specifically, for example mass spectrometry, is therefore required.

Positive ion fast atom bombardment tandem mass spectrometry (FAB MS-MS) was proposed as an elegant and species specific method for fingerprinting of PC peptides.[17,18] This technique lacks the sensitivity and is difficult to use for quantitative analysis. These drawbacks can be overcome by using electrospray tandem mass spectrometry (ESI MS-MS) that is becoming a well-established tool for peptide identification and sequencing in biological samples.[19,20] ESI MS-MS using a triple quadrupole configuration has also an emerging potential as a sensitive species-selective quantification technique. Recently, it was proposed for quantitative species-selective analysis of cobalamin analogues at ng levels.[21]

The objective of this work was to develop a simple sensitive method for the identification, sequencing and quantitative determination of Cd-induced phytochelatins in plants and plant cell cultures by ESI MS-MS following a custom-designed sample preparation procedure.

Experimental

Apparatus

All experiments were performed using a Perkin-Elmer SCIEX (Thornhill, ON, Canada) API 300 pneumatically-assisted electrospray (ion-spray) triple-quadrupole mass spectrometer. A Model 1100 HPLC pump (Hewlett-Packard, Wilmington, NC, USA) was used as the sample delivery system for the purification of the plant and plant cell extracts by reversed-phase chromatography. A Hitachi (Tokyo, Japan) Model CS 120 GX refrigerated ultracentrifuge was used for the separation of the supernatant after leaching of Cd species from plant tissues and cell cultures. The solvents were degassed by means of an ultrasonic bath.

Materials

A reversed-phase Vydac (Hesperia, CA) C_8 150 mm × 4.6 mm id column was used for the purification of the plant tissues and plant cell extract. Acetonitrile and methanol (Sigma-Aldrich, Saint-Quentin Fallaviec, France) were of HPLC grade. Trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from Sigma-Aldrich. Water purified using a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout.

Cell cultures of plants: *Silene cucubalus*, *Agrostis tenuis*, and *Rauvolfia serpentina* were investigated. They were grown for four days in a 300 μM Cd^{2+} solution.

Phytochelatin standards

The synthesis of phytochelatins was carried out according to Grill *et al.*[2] In brief, 2000–10 000 pkat of PC-synthase[5] were incubated at 25 °C with 1 mM GSH and 0.8 mM $Cd(NO_3)_2$ in 1.2 l of buffer solution (pH 8.0). An amount of 0.02% of NaN_3 was added to retard the bacterial growth.

Proteins were precipitated from the resulting solution by the addition of $(NH_4)_2SO_4$ to reach a concentration of 85% followed by centrifugation at 8000*g* for 30 min. Phytochelatins were precipitated from the supernatant by the addition of 20 ml of 1 M $Cd(NO_3)_2$ solution. The precipitated mixture of phytochelatins was centrifuged and washed twice with water. The washed precipitate was stable for months when stored at −20 °C.

The precipitated phytochelatins were dissolved in 3.5 M HCl and separated by semi-preparative HPLC using elution with a concentration gradient of acetonitrile in water.[2] The fractions with the individual phytochelatin (PC_2, PC_3, PC_4) peaks were heart-cut. The acetonitrile was rotavaporated and the fractions were lyophilized. The heart-cut fractions were repurified in the same way to produce compounds used as standards below.

Analytical procedures

Sample preparation. Cells were vacuum filtered and washed. Plant roots were cut from the rest of the plant and washed (in both cases only with water). From here on, both cells and plants were treated the same way. They were frozen in liquid nitrogen to break the cell wall, ground with a pestle and mortar and extracted with water or with 10 mM TRIS-HCl buffer (pH = 8). They were centrifuged (30 min, 10 000*g*, 4 °C), filtered and lyophilized.

A sample of 30 mg of the freeze-dried material was dissolved in 500 μl of water containing 0.1% of TFA (pH = 2.3). The solution was filtered and injected on the reverse-phase purification column. An aliquot of 100 μl was eluted with 0.1% TFA in water for 5 min followed by a linear increase of acetonitrile concentration in the eluent up to 50% during 30 min. The flow rate was set at 0.75 ml min^{-1}. The eluate containing acetonitrile was collected and incubated for 20 min at 25 °C with 5 mM DTT. The acetonitrile was removed by rotavaporation and the aqueous residue was freeze-dried. The dried residue was dissolved in 200 μl of 0.06 M acetic acid in 30% methanol, diluted if necessary to fit the linearity range of the calibration curve, and analysed by ESI MS.

Instrumental ESI MS-MS conditions. In the MS mode, Q1 was swept over a given mass range and Q3 was operated in rf-only mode. The orifice potential was 40 V, the ion spray voltage was 4100 V and the ion multiplier potential was 2400 V. The total spectra of PCs were acquired in the range 50–1100 Da using a 10 ms dwell time and a 0.5 Da step size during 10 scans.

In the MS-MS mode, the product ion scan mode was chosen for data acquisition. The mass of Q1 was fixed and Q3 was swept over a given mass range to determine the ions which result from the fragmentation of the precursor ion. The collision gas was nitrogen and the collision energies were 24 eV for GSH, 26 eV for PC_2, 34 eV for PC_3 and 49 eV for PC_4.

For the calibration curves in direct introduction, the step size was 0.05 and the dwell time was 5 ms. The mass ranges selected for the calibration curves in the MS mode were 290–320 u for GSH, 530–550 u for PC_2, 760–790 u for PC_3 and 990–1020 u for PC_4. In the MS-MS mode the mass ranges selected were: 305–311 u, 536–543 u, 769–775 u and 1001–1007 u for GSH, PC_2, PC_3 and PC_4, respectively. The biggest *y*-fragment in the MS-MS mode was monitored in the ranges: 176–182, 408–414, 640–646, and 872–878 for GSH, PC_2, PC_3 and PC_4, respectively.

Results and discussion

Preliminary experiments were aimed at maximizing the signal/background ratio obtained in the MS and MS-MS modes, by direct introduction of the sample solution. Methanol and acetonitrile were investigated as organic solvents whereas acetic acid and HCl were used for acidification. The most intense signals were obtained for a mixture of methanol with water 30 + 70 v/v). The maximum signal was obtained at a concentration of 0.06 M acetic acid.

Characterization of PCs standards in the MS mode

Fig. 1 shows mass spectra obtained for the individual GSH, PC_2, PC_3 and PC_4 standards. These spectra are principally constituted by the protonated molecule ion $[M + H]^+$ which allows the identification of each PC according to its mass. There are also intense but unidentified signals present at 159 and 288 u and some other minor background signals. In the case of PCs, even if the dissociation of the protonated molecule ion is weak, the formation of certain fragments cannot be prevented, especially that of the heaviest *y*-fragment of the protonated molecule ion. This is, for example, the case of the m/z = 411 u peak in the PC_2 spectrum or of the m/z = 643 u peak in the PC_3 spectrum.

Characterization of PCs standards in the MS-MS mode

In the MS-MS mode, the protonated molecule ion is fragmented by collision induced dissociation (CID) with inert gas molecules. Peptides fragment primarily at the amine bonds to produce a ladder of sequence ions.[20] The charge can be retained on the amino terminus (type *b*-ion) or on the carboxy terminus (type *y*-ion). Thus a complete series made of ions from both

types allows the determination of the amino acid sequence by subtraction of the masses of adjacent sequence ions.

Fig. 2 shows the mass spectra produced by the fragmentation of the molecular peaks observed in Fig. 2 for each of the compounds investigated. Most of the expected *b*- and *y*-type fragments can be identified. As observed elsewhere in FAB MS the γ-GluCys linkage is hardly broken.[18] The peaks corresponding to the loss of a γ-GluCys group are less intense. This is for example the case for the peak at 362 u in the PC_2 spectrum.

In the MS-MS mode, two ions are characteristic of a PC: the protonated molecule ion and the *y*-type fragment of the biggest mass. For lower masses, the MS-MS spectrum of PC_n overlaps

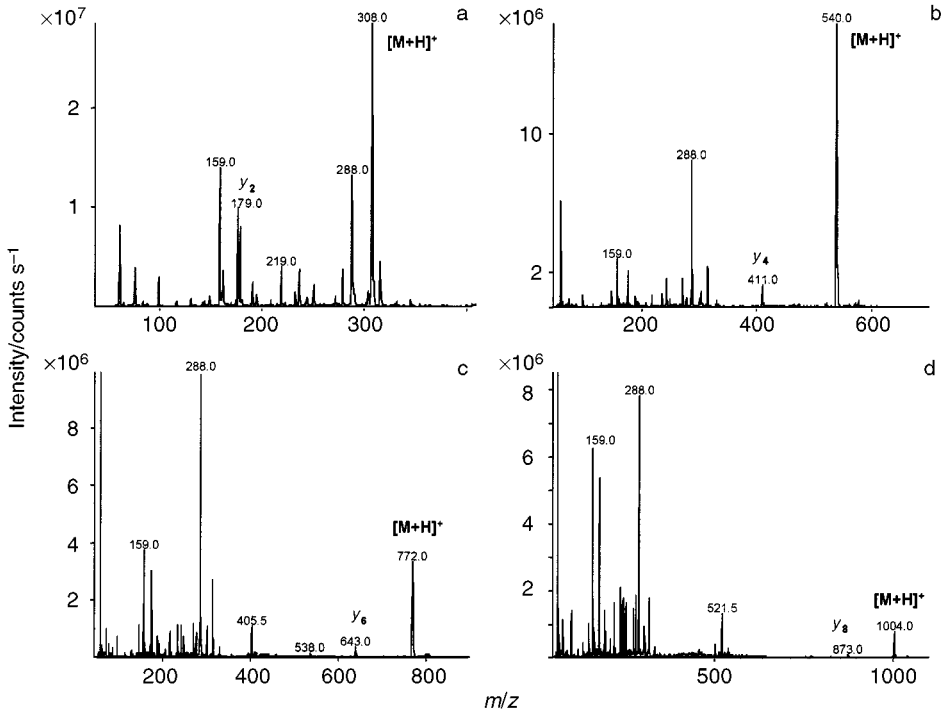

Fig. 1 Electrospray mass spectra of GSH and purified PCs standards subtracted from the blank. (a) GSH; (b) PC_2; (c) PC_3; (d) PC_4. *Ca.* 1 μg ml^{-1} of each PC.

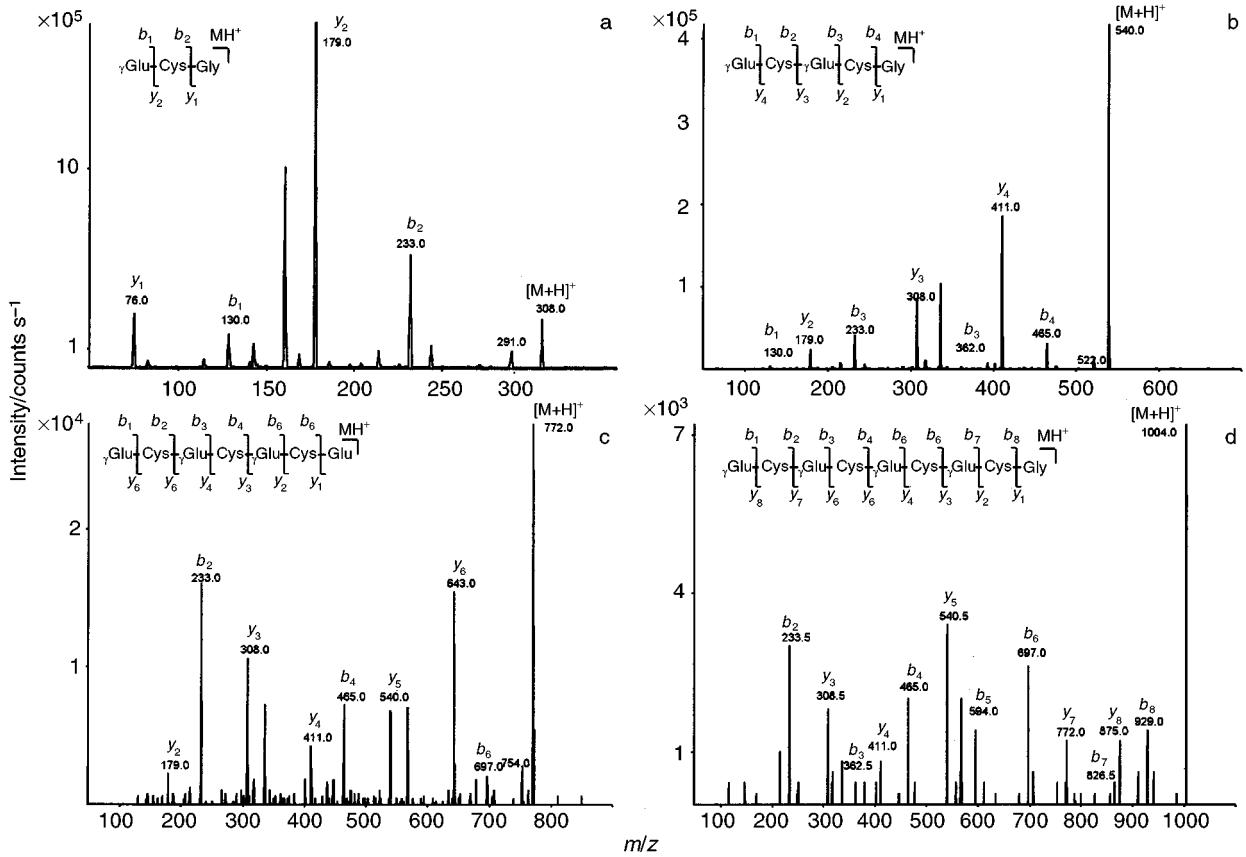

Fig. 2 Electrospray tandem mass spectra of GSH and purified PCs standards. (a) GSH, collision energy = 24 eV; (b) PC_2, collision energy = 26 eV; (c) PC_3, collision energy = 34 eV; (d) PC_4, collision energy = 49 eV. *Ca.* 1 μg ml^{-1} of each PC.

with the PC_{n-1} one. Nevertheless the MS-MS mode offers a possibility to detect a PC unambiguously, even if its molecular peak overlaps with one or two more compounds in the ESI MS spectrum.

Precision, linearity and detection limits

ESI MS offers an until now unexplored opportunity of the quantitative species-selective determination of the individual phytochelatins in plant extracts. Calibration curves were established for the molecular MS mode and the tandem (MS-MS) mode. Precision, determined by five fold injection at the 1 μg ml^{-1} level, was in the range 3–5%. Calibration curves were plotted for the characteristic ion of each of the compounds determined. In the MS mode it was the protonated molecule ion $[M + H]^+$, whereas in the MS-MS mode the use of the protonated molecule ion and the *y*-type fragment having the biggest mass was investigated for the purpose of quantification.

The linearity of the instrumental response as a function of PC concentration was investigated in the range 0–10 μg ml^{-1}. It was found that, both in the MS and the MS-MS mode, the signal intensity is a linear function of the PCs concentration up to 2 μg ml^{-1}. Table 1 summarizes the sensitivities (slopes), correlation coefficients and detection limits (DL) obtained in each case. In the case of interferences with compounds of the same mass, PCs can be identified and quantified using the largest *y*-type fragment.

The detection limits were calculated as three times the standard deviation of the blank measured for a given ion. The detection limits in the MS and the MS-MS modes are similar. They deteriorate rapidly with the increasing molecular mass of the PC-species investigated.

Analysis of the biosynthesis post-reaction mixture

The method developed was applied to the analysis of PCs mixture resulting from the reaction of glutathione with the enzyme phytochelatin synthase. The mass spectrum obtained is shown in Fig. 3 The intense peaks at 288 and 159 u are still present but the molecular peak of GSH at 308 u, PC_2 at 540 u, PC_3 at 772 u and PC_4 at 1004 u can be identified unambiguously. The CID fragmentation of all those molecular peaks leads to the characteristic peaks of PCs as in Fig. 2 (data not shown). This allows the identification of the PCs present in the mixture without the need for HPLC.

The different analytical modes: ESI MS, ESI MS-MS using the molecular peak and ESI MS-MS using the biggest *y*-type fragment were compared for the determination of phytochelatins in the crude post-reaction mixture. The results are shown in Table 2. It is evident that glutathione which is the substrate for the enzyme-assisted PC synthesis is almost totally consumed. PC_2, PC_3 and PC_4 are synthesized in proportions 10 : 5 : 3. The analytical result is practically independent of the instrumental operating mode used for quantification.

Determination of phytochelatins in plant extracts

The method developed was further applied to the characterization and determination of PCs in extracts of three different plant cell cultures (*Silene cucubalus*, *Agrostis tenuis*, and *Rauvolfia serpentina*) known to biosynthesize phytochelatins when exposed to Cd^{2+}. The procedures investigated to extract the Cd complexes included extraction with water and with 10 mM TRIS-HCl buffer (pH 8.0). Table 3 shows that the extraction procedure has hardly any effect on the recovery of PC from a sample. Water was therefore used for extraction of metal–peptide complexes in order not to introduce additional salt to the extract that would be preconcentrated by freeze-drying.

Even when water is used as extractant, the extracts contain a considerable concentration of salt and cannot be analysed directly by ESI MS. The expected concentrations in the plant cell cultures analysed are much lower than in the case of the post-reaction mixture referred to above so the reduction of the salt concentration by dilution cannot be applied. The use of a size-exclusion desalting column as used in the protein analytical biochemistry fails because the PC-species are much smaller

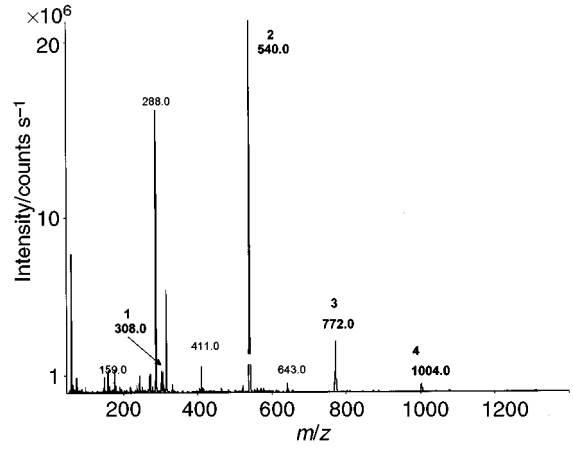

Fig. 3 Electrospray mass spectrum obtained for the post-reaction mixture in the enzymatically mediated biosynthesis of PC from glutathione. Peak identification: 1, GSH; 2, PC_2; 3, PC_3; 4, PC_4.

Table 2 Comparison of the results of the quantitative determination of glutathione and phytochelatins in the post-rection mixture of enzymatically assisted biosynthesis using the different data acquisition modes

	Concentration in the analysed solution/μg l^{-1}		
Compound	Protonated molecule ion in the MS mode	Protonated molecule ion in the MS-MS mode	Heaviest *y*-type fragment in the MS-MS mode
GSH	35 ± 2	37 ± 2	29 ± 2
PC_2	1056 ± 53	1104 ± 56	1193 ± 60
PC_3	519 ± 26	462 ± 24	607 ± 31
PC_4	354 ± 18	392 ± 20	411 ± 21

Table 1 Figures of merit of ESI MS and ESI MS-MS for the quantitative determination of glutathione and phytochelatins using different data acquisition modes

	Protonated molecule ion in the MS mode			Protonated molecule ion in the MS-MS mode			Heaviest *y*-type fragment in the MS-MS mode		
Compound	Slope, counts s^{-1}/μg l^{-1}	r^2	DL/μg l^{-1}	Slope, counts s^{-1}/μg l^{-1}	r^2	DL/μg l^{-1}	Slope, counts s^{-1}/μg l^{-1}	r^2	DL/μg l^{-1}
GSH	8602	0.9985	1	118.9	0.9918	17	727.4	0.9982	0.5
PC_2	9471	0.9982	9	209.2	0.9964	9	91.0	0.9952	6
PC_3	585.7	0.9985	7	17.3	0.9967	38	6.1	0.9837	43
PC_4	144.1	0.9962	28	2.5	0.9979	42	1.0	0.9960	40

than proteins and may co-elute with the salt when diluted from a desalting column. Therefore, reversed-phase chromatography was applied (see Fig. 4). Apophytochelatins (non-metallated PCs) are known to be retained on a C_8 support from aqueous and slightly (up to 5%) organic media containing 0.1% TFA[5] under which conditions the salts are eluted. PCs can be recovered from the column by increasing the concentration of an organic modifier (acetonitrile) in the mobile phase.

Such a procedure eliminates salts but introduces TFA that is known to suppress electrospray ionization, and leads to a considerable dilution of the PCs solution. Freeze-drying was attempted to remove both TFA and the solvent. It turned out, however, that PC present before freeze-drying underwent oxidation and no original non-oxidized PC forms could be recovered. The use of β-mercaptoethanol, commonly used as an anti-oxidant in analytical chemistry of thiol compounds[22] offered only a limited improvement. The best results were obtained with dithiothreitol (DTT) that allowed the recovery of the original PCs and did not influence the ESI MS signal.

The matrix effect of the solution obtained after dissolving the freeze-dried eluate was investigated by spiking a PC standard and examining its signal. It turned out that the slopes of the PC standard calibration curve and a standard addition curve obtained by spiking the sample solution with the PC standards were identical. This indicates the possibility of using external calibration for the determination of PCs in extract purified by reversed-phase chromatography. The results obtained, expressed as the concentration of PC in dry mass of the cell culture extract, are shown in Table 3.

Table 3 Determination of phytochelatins in plant cell cultures. Comparison of ESI MS with HPLC with post-column derivatization. The values correspond to the amount of each phytochelatin (in μg) in 1 mg of the powder obtained after purification by reversed-phase HPLC and lyophilization

	Extracted with water		Extracted with TRIS buffer	
	ESI MS analysis	Reference method	ESI MS analysis	Reference method
Silene cucubalus—				
PC_2	3.11 ± 0.16	3.6	3.48 ± 0.17	2.8
PC_3	10.9 ± 0.5	10.5	6.21 ± 0.31	9.3
PC_4	0.68 ± 0.03	3.6	0.69 ± 0.03	3.1
Agrostis tenuis—				
PC_2	1.14 ± 0.16	0.9	0.55 ± 0.03	0.98
PC_3	2.69 ± 0.14	2.3	3.3 ± 0.17	2.7
PC_4	—	0.16	0.16 ± 0.019	0.22
Rauvolfia serpentina—				
PC_2	0.28 ± 0.02	0.3	0.56 ± 0.03	0.3
PC_3	3.3 ± 0.17	2.7	3.4 ± 0.17	2.7
PC_4	0.66 ± 0.03	1.3	0.65 ± 0.03	1.3

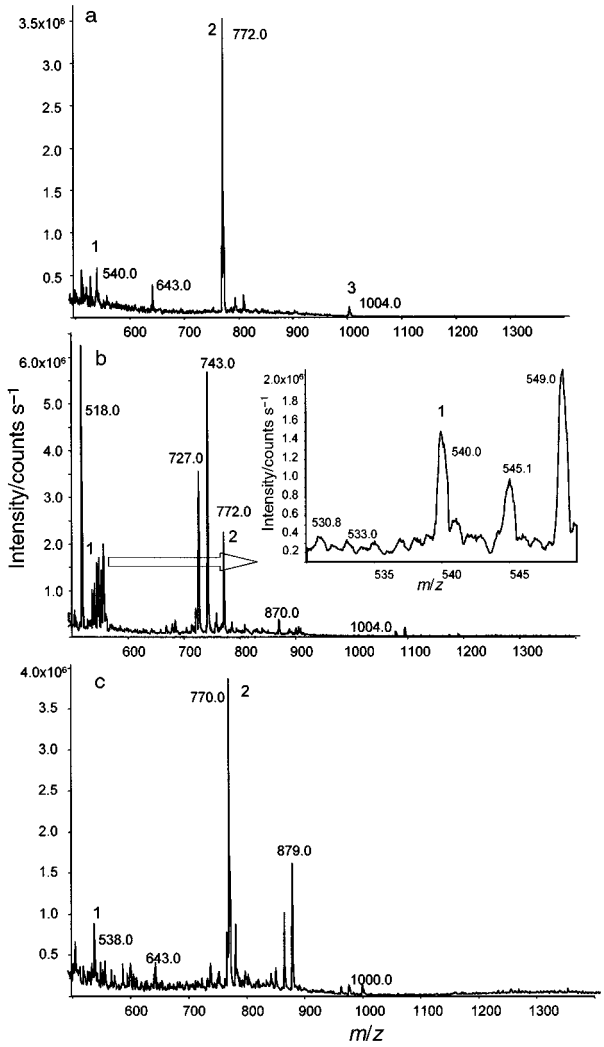

Fig. 4 Electrospray mass spectra obtained for plant culture extracts after their purification by reversed-phase HPLC and lyophilization. (a) *Silene cucubalus*; (b) *Agrostis tenuis*; (c) *Rauvolfia serpentina*. Peak identification: 1, PC_2; 2, PC_3; 3, PC_4. The signals in Fig. 4(c) correspond to the oxidized forms of the corresponding phytochelatins.

Validation of the determination of phytochelatins in plant extracts

Since reference materials with certified phytochelatin concentrations in plant samples are not available, the only way to validate the method developed was to compare the results obtained with those obtained by an independent analytical method. Table 3 shows results of the determination of phytochelatins in the analysed samples obtained by reversed-phase HPLC with post column derivatization of the sulfhydryl groups of the individual peptides with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid), and spectrophotometric detection at 410 nm. The measurements were realised in a different laboratory by a different operator. As can be seen from Table 3 the agreement can be considered satisfactory, irrespective of whether water or buffer solution were used for the extraction of phytochelatins.

Conclusion

Pneumatically assisted electrospray mass spectrometry allows not only the identification of phytochelatins but also their quantitative determination with detection limits in the low ng ml^{-1} range. Tandem mass spectrometry enables the on-line determination of the amino acid sequence of a PC and thus unambiguous identification of the compound determined even in a complex mixture. A purification step using a reversed-phase column is necessary prior to ESI MS-MS to separate the bulk of phytochelatins from the matrix salts that would suppress the ionization. The on-line coupling with RP HPLC suffers from definitely poorer detection limits because of the need for TFA in the mobile phase that affects negatively the electrospray ionization.

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