

## Characterization and quantification of thiol-peptides in *Arabidopsis thaliana* using combined dilution and high sensitivity HPLC-ESI-MS-MS

Erika Bellini<sup>a,d</sup>, Marco Borsò<sup>b</sup>, Camilla Betti<sup>c</sup>, Laura Bruno<sup>d</sup>, Andrea Andreucci<sup>a</sup>,  
Monica Ruffini Castiglione<sup>a</sup>, Alessandro Saba<sup>b,\*\*</sup>, Luigi Sanità di Toppi<sup>a,\*</sup>

<sup>a</sup> Dipartimento di Biologia, Università di Pisa, Italy

<sup>b</sup> Dipartimento di Patologia Chirurgica, Medica, Molecolare e Dell'Area Critica, Università di Pisa, Italy

<sup>c</sup> Dipartimento di Medicina, Università di Perugia, Italy

<sup>d</sup> Dipartimento di Biologia, Università di Roma "Tor Vergata", Italy

### ARTICLE INFO

#### Keywords:

*Arabidopsis thaliana* (Brassicaceae)  
Cadmium  
Glutathione  
Heavy metal  
HPLC-ESI-MS-MS  
Isotope-labelled internal standard  
Mass spectrometry  
Phytochelatin  
Thiol-peptide

### ABSTRACT

Although thiol-peptide compounds, such as reduced glutathione (GSH),  $\gamma$ -glutamylcysteine ( $\gamma$ -EC), and phytochelatins, play fundamental roles in plants, their analytical determination and characterization is still somewhat problematic, mainly due to their high polarity and oxidation propensity. Thus, in this work a reliable and sensitive HPLC-ESI-MS-MS method was developed, in order to simultaneously assay, within 14-min instrumental runs,  $\gamma$ -EC, GSH, and phytochelatins up to phytochelatin 4. This analytical method was validated in shoot and root extracts of the model plant *Arabidopsis thaliana* (Brassicaceae) and guaranteed accurate quantification by using specific isotope labelled-internal standards for both GSH and phytochelatins, as well as standards for external calibration. Good linearities in the method performance were observed ( $R > 0.99$ ), with a dynamic range over three orders of magnitude in thiol-peptide concentrations. In MRM mode, the detection sensitivity of the thiol-peptides was equal to approximately 16, 6, 7, 13, 10 fmol for  $\gamma$ -EC, GSH, phytochelatin 2, phytochelatin 3, and phytochelatin 4, respectively (20  $\mu$ l injection each). The reproducibility of the method was confirmed by high intra- and inter-day accuracy and precision values. The recovery rates were estimated approximately in the range of 73.8–91.0% and the matrix effect evaluation revealed that all analytes exhibited ionization suppression. The use of stable isotope-labelled analogs of the thiol-peptides as internal standards was particularly worthy of note: it offered the considerable advantage of overcoming the consequences of matrix effect and thiol-peptide loss through sample preparation, by normalizing the analyte signal during the quantification process. Thus, by validating the method's sensitivity, accuracy, precision, reproducibility, stability, recovery, and matrix effect, data reliability and robustness were ensured.

### 1. Introduction

Glutathione (GSH), a thiol-tripeptide consisting of  $\gamma$ -glutamate, cysteine and glycine, plays a key role in all organisms: i) in quenching reactive oxygen species (ROS) (Zenk, 1996); ii) in maintaining the redox status of enzymatic and non-enzymatic proteins (Orwar et al., 1995); and iii) in regulating the catabolism of certain xenobiotics (Schröder and Collins, 2002). Phytochelatins (PCn) are thiol-oligopeptides directly derived from GSH, and have the general structure ( $\gamma$ -glutamate-cysteine)<sub>n</sub>-glycine, with  $n$  usually ranging from 2 to 5 (Grill et al., 1985). PCn exhibit a high affinity for various metal(loid)s, including cadmium (Cd), arsenic (As), lead (Pb), mercury (Hg), zinc (Zn)

and copper (Cu), mainly due to the thiol groups present in their cysteine residues (Gupta et al., 2013). Thus, PCn can easily chelate the above metal(loid)s and, as a result, prevent them from circulating inside the cytosol by compartmentalizing them in the vacuole. For this reason, PCn are considered the main system of metal(loid) detoxification in the plant cell (Sanità di Toppi and Gabbrielli, 1999; Cobbett and Goldsbrough, 2002). Interestingly, more recently, PCn have been also proposed to play additional functions in the co-regulation of the physiological need of iron [Fe(II)/(III)], even in early plants as bryophytes and charophytes (Degola et al., 2014; Fontanini et al., 2018).

In general, both GSH and PCn are highly sensitive to spontaneous oxidation, which sometimes contributes to provide inaccurate

\* Corresponding author. Dipartimento di Biologia, Università di Pisa – via Luca Ghini 13, I-56126, Pisa. Italy.

\*\* Corresponding author. Dipartimento di Patologia Chirurgica, Medica, Molecolare e dell'Area Critica, Università di Pisa – via Roma 67, I-56126, Pisa. Italy.

E-mail addresses: [alessandro.saba@med.unipi.it](mailto:alessandro.saba@med.unipi.it) (A. Saba), [luigi.sanita@unipi.it](mailto:luigi.sanita@unipi.it) (L. Sanità di Toppi).

analytical results as far as their quantification and characterization are concerned (Simmons et al., 2009). Moreover, GSH,  $\gamma$ -glutamylcysteine ( $\gamma$ -EC), PCn and their derivatives are amphoteric, highly polar and lacking in strong chromophores. This peculiarity makes challenging to quantify them by traditional analytical methods (Mou et al., 2016), such as reverse-phase high performance liquid chromatography (r.p.-HPLC) followed by post-column derivatization with 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (Grill et al., 1991), or preceded by pre-column derivatization with monobromobimane (Sneller et al., 1999; Minocha et al., 2008). Thiol-peptide derivatization is advantageous because it improves the stabilization of these compounds, but such technique is time-consuming and not particularly specific for PCn and related peptides, as the derivatizing reagents can react with any thiol group. As a result, the method selectivity might be often not satisfactory for HPLC analysis (Bräutigam et al., 2010; Cao et al., 2015). Moreover, some derivatization techniques may erroneously overestimate thiol-peptides, due to the possible detection of other, non-thiolic, molecules. For example, Berlich et al. (2002) demonstrated that a positive post-column Ellman's reaction could also be conferred by coumarins, detected under the basic pH value of the Ellman test.

Indeed, a previous study developed an IL-LC-ESI-MS-MS method using a derivatization strategy to detect PCn in plant samples without losing selectivity and sensibility (Liu et al., 2015). Anyway, when not strictly necessary, derivatization should be avoided as it is time-consuming, makes the sample preparation more complicated and could affect the assay accuracy. Moreover, results are dependent on the derivatizing agent. For these reasons, analysis without derivatization appears to be preferable, in order to develop a fast and useful method. Accordingly, HPLC-ESI-MS-MS potentially allows specific quantification of several analytes, with less chance of false positive results. However, assays using MS are still challenging due to some fluctuation in this technique's response. This is due to the significant variability of the concentrations and to the varying ionization efficiencies in matrices from different samples, even under the same HPLC conditions (Huang et al., 2014). Furthermore, using ESI as an ionization technique, a matrix-dependent signal suppression could be present and differ from sample to sample (Wood and Feldmann, 2012). To reduce these potential negative effects, the use of stable isotope-labelled internal standards (which coelute with the analytes) is advised, since this improves quantification accuracy by considering a truly matrix-matched effect and an ion suppression effect. It is worth noting that external standard methods developed for PCn quantification are not an optimal choice (El-Zohri et al., 2005; Bräutigam et al., 2010; Cao et al., 2015), as they do not consider the loss of analyte through the extraction procedure and the above-mentioned matrix effect. Moreover, only a few attempts have been developed using isotope internal standards. Concerning this, Simmons et al. (2009) outlined a method using glycine- $^{13}\text{C}_2$ -GSH as IS, whereas Liu et al. (2015) developed a stable isotope-labelling strategy for the quantitative profiling of PCn, by using  $\omega$ -bromoacetylquinolinium bromide (BQB) and BQB- $d^7$  as isotope-labelling reagents.

In this regard, given the important biological roles of thiol-peptides in plants, it is crucial to develop a sensitive and accurate analytical method that allows the simultaneous detection and quantification of these compounds. Thus, the aim of this work was to provide an efficient, accurate and versatile HPLC-ESI-MS-MS method for detecting GSH,  $\gamma$ -EC, and PCn in plants, with particular reference to shoots and roots of *Arabidopsis thaliana* (Brassicaceae). In particular, we have developed a method based on a simple and fast extraction procedure with a suitable reducing agent that mitigates PCn oxidation; this method also relies on the use of specific isotope-labelled compounds as internal standards, both for GSH and PCn, in order to improve the quantification accuracy.

## 2. Results and discussion

### 2.1. The adopted procedure provides high reproducibility and yield in the extraction of thiol-peptides from plant material

The quantification of analytes in plant extracts can be usually challenging due to the quite high biological and instrumental variability. Traditionally, mortar and pestle are used to homogenize the plant material; nevertheless, the method reliability and reproducibility is in this case usually poor and highly dependent on the manual skills of the operator(s). In order to overcome such problem, in this study the plant material (shoots and roots from axenic cultures of *Arabidopsis thaliana*, Columbia ecotype) was homogenized using a mixer mill, which significantly improved the reproducibility and yield of the extraction (Fig. S1).

Another common criticism is PCn sensitivity to oxidation reaction, which frequently involves both inter- and intra-molecular disulfide bond formation during either sample preparation or storage. Studies in literature confirm that inter- and intra-molecular oxidations of PC<sub>2</sub> produce ions at  $m/z$  1077 and 770 Da, respectively, while inter-molecular oxidation of PC<sub>3</sub> gives rise to an ion at  $m/z$  1543 Da (El-Zohri et al., 2005; Cao et al., 2015). To address this concern, in our study, Tris (2-carboxyethyl) phosphine (TCEP), an efficient reducing agent which is reliable at a low pH, stable in air and not able to reduce metals, was added to the extraction buffer in order to improve the procedure, as demonstrated in previously studies (Gupton-Campolongo et al., 2013; Kawakami et al., 2006; Liu et al., 2015; Simmons et al., 2009). TCEP is suitable for this purpose because, unlike the dithiothreitol (DTT), its chemical structure does not contain any thiol group and cannot compete with the analytes of interest.

Another remarkable point of the method here described is the use of the stable isotope-labelled PC<sub>2</sub> as internal standard, according to the standard isotope-dilution assay techniques (Chace et al., 1993). This compound has physical-chemical properties similar to those of the endogenous PCn and, in addition, makes it ideal for monitoring PCn throughout the sample processing.

### 2.2. The established HPLC method assures fair separation amongst thiol-peptides

A baseline separation is not always necessary when highly specific SRM-based mass spectrometry is used. However, in this work, great attention has been paid to the retention-times of GSH and PCn, in order to prevent sensitivity loss associated with ionic suppression phenomena, mainly induced by the huge amounts of electrolytes present in the plant sample, which elute with the HPLC solvent front. In particular, GSH was a matter of concern because, according to its hydrophilicity, it eluted very close to the solvent front. However, the HPLC method set up assured a very good separation among the thiol-peptides (Fig. 1), as well as guaranteed a moderate matrix effect for all of them, as demonstrated by the tests carried out during the validation procedure.

Previous studies described a loss of separation efficiency for GSH and PCn when using HPLC columns packed with standard silica-based reverse stationary phases, due to the adsorption of matrix components (El-Zohri et al., 2005). Our studies confirmed such behavior; consequently, HPLC column was periodically washed counterflow with acetonitrile and water in order to remove adsorbed material and preserve the overall performances.

### 2.3. The adoption of isotope-labelled internal standards compensates matrix effect and improves method validation

The analytical method was submitted to internal validation, and the results are reported below. The use of stable isotope-labelled internal standards, which coelute with the analytes, is recognized as the most

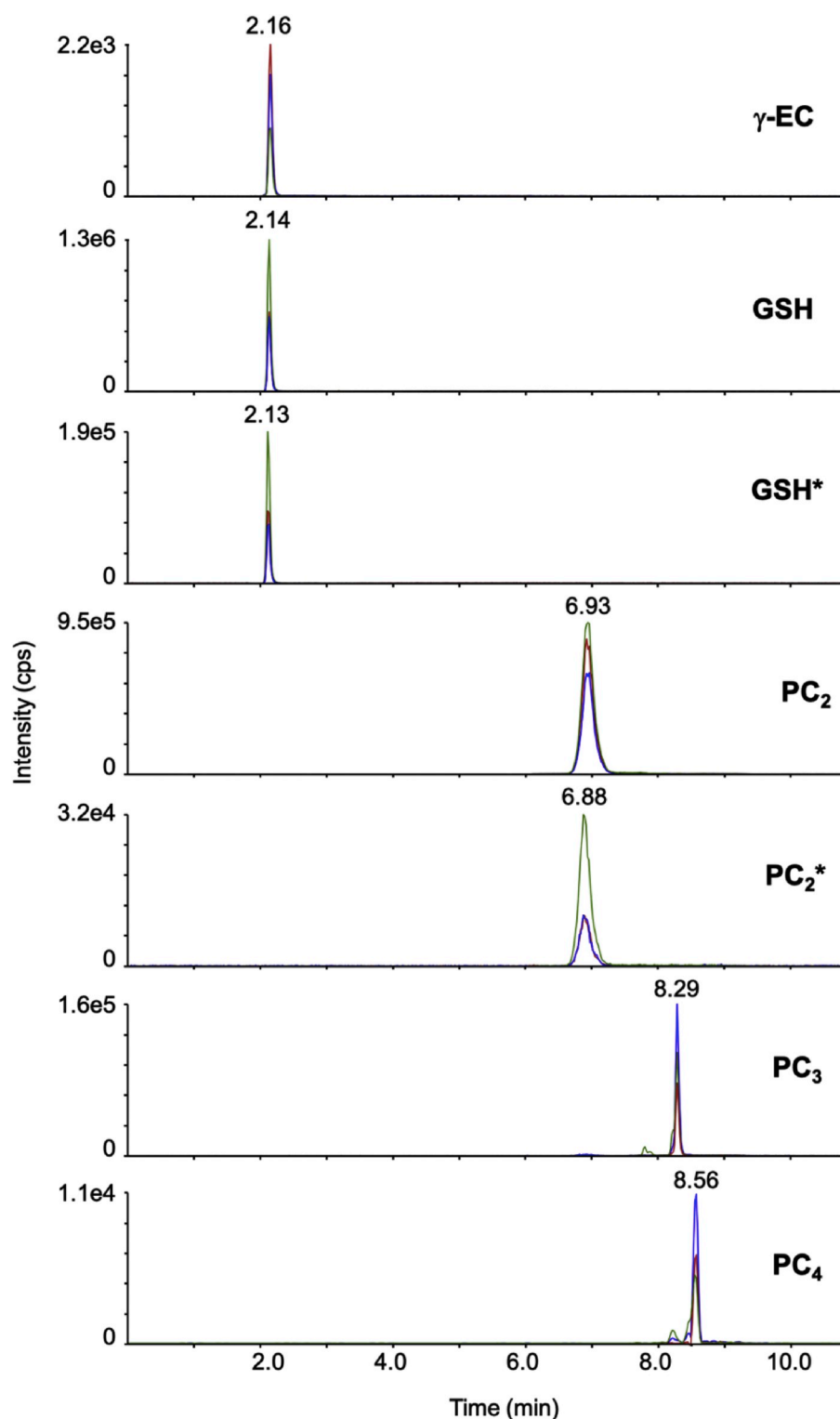


Fig. 1. SRM chromatograms of a representative shoot extract of *A. thaliana* in the time-range of 0–10 min.  $\gamma$ -EC and GSH were diluted 1:100 and analyzed by the second HPLC-ESI-MS-MS method described in point 4.5, whereas for the PC<sub>2-4</sub> the third method was used (point 4.5). Internal standards are marked by an asterisk (\*).

efficient way for compensating the matrix effect, thus improving quantification accuracy. According to the experimental evidences,  $^{13}\text{C}_2, ^{15}\text{N}$ -GSH should be considered as a satisfactory internal standard also for  $\gamma$ -EC, and  $^{13}\text{C}_2, ^{15}\text{N}$ -PC<sub>2</sub> for PC<sub>3</sub> and PC<sub>4</sub>. Experimental results reported in Table 1 confirmed that chromatographic retention-times were stable and that calibration curves (general equation,  $y = mx + n$ ), obtained from linear regression with a weighting of  $1/x$ , had a good

linearity in the tested range. In fact, their correlation coefficients (R) were always higher than 0.999, with reproducible slopes (m) and low intercepts (n). The instrumental limits of detection (LODs) and the limits of quantification (LOQs) were evaluated in standard solutions, due to the impossibility of having a real matrix completely devoid of thiol-peptides. However, LODs and LOQs matched with the method requirements, being much lower with respect to the effective

**Table 1**

Some of the method performances: retention-time and calibration curve equation parameters, namely equation slope (m), equation intercept (n), and correlation coefficient (R). All values are expressed as means  $\pm$  s.d..

Calibration curve				
Analyte	Retention-time (min)	Slope (m)	Intercept (n)	Correlation (R)
$\gamma$ -EC	2.15 $\pm$ 0.006	0.0039 $\pm$ 0.00013	0.0025 $\pm$ 0.00033	0.9993
GSH	2.14 $\pm$ 0.005	0.0076 $\pm$ 0.00007	0.0066 $\pm$ 0.00013	0.9996
PC <sub>2</sub>	6.87 $\pm$ 0.044	0.0385 $\pm$ 0.00041	0.0075 $\pm$ 0.00091	0.9994
PC <sub>3</sub>	8.29 $\pm$ 0.005	0.0134 $\pm$ 0.00041	0.0019 $\pm$ 0.00036	0.9998
PC <sub>4</sub>	8.56 $\pm$ 0.008	0.0049 $\pm$ 0.00005	0.0016 $\pm$ 0.00002	0.9992

**Table 2**

LODs and LOQs of the assayed thiol-peptide compounds.

Analyte	LOD		LOQ	
	(ng ml <sup>-1</sup> )	(nM)	(ng ml <sup>-1</sup> )	(nM)
$\gamma$ -EC	0.2	0.65	0.5	1.62
GSH	0.1	0.79	0.2	1.99
PC <sub>2</sub>	0.2	0.37	0.8	1.48
PC <sub>3</sub>	0.5	0.65	1.0	1.29
PC <sub>4</sub>	0.5	0.65	1.0	0.99

concentrations of the analytes present in the samples, as LODs ( $S/N \cong 3$ ) were in the range 0.2–0.5 ng ml<sup>-1</sup>, i.e. 0.37–0.79 nM, and LOQs ( $S/N \cong 10$ ) in the range of 0.5–1.0 ng ml<sup>-1</sup>, i.e. 0.99–1.99 nM (Table 2). Indeed, the method employed in this work was more sensitive than other methods based on HPLC-ESI-MS-MS, which exhibited a LOD range of 5.8–16.8 nM (Bräutigam et al., 2010), or HILIC-ESI-MS-MS, which presented a LOD range of 3–15 nM for the same analytes (Cao et al., 2015).

Accuracy is an important validation parameter and represents how the assayed concentration is close to the nominal concentration of the analyte. It was evaluated by adding known amounts of thiol-peptide standards to homogenates (shoots and roots of *A. thaliana*) at three concentration levels. Another relevant parameter is precision, which describes the closeness of repeated individual measurements of thiol-peptides, and it is expressed as relative standard deviation (r.s.d.%). The assays of control solutions (CSs) provided very high intra- and inter-day accuracy and precision, as shown in Tables 3 and 4, thus

**Table 3**

Intra-day accuracy and precision (expressed as relative standard deviation, r.s.d.%) either in shoot or in root extracts of *A. thaliana*. Spiked analytes and mean assayed concentrations are indicated as "Spiked C" and "Assayed C", respectively.

Analyte	Spiked C (ng g <sup>-1</sup> fr. wt.)	Shoot			Root		
		Assayed C (ng g <sup>-1</sup> fr. wt.)	r.s.d.(%)	Accuracy(%)	Assayed C (ng g <sup>-1</sup> fr. wt.)	r.s.d.(%)	Accuracy(%)
$\gamma$ -EC	Endogenous	38.1			32.7		
	15	53.9	4.00	104.9	47.9	5.71	101.4
	150	212.6	2.80	116.3	196.5	2.30	109.2
	600	657.6	2.50	103.3	625.2	3.47	98.7
GSH	Endogenous	117.9			73.5		
	15	132.8	9.80	99.0	89.0	6.87	103.0
	150	279.3	1.31	107.6	231.9	4.02	105.6
	600	708.0	5.03	98.3	636.5	2.00	93.8
PC <sub>2</sub>	Endogenous	98.1			51.9		
	15	113.1	4.00	100.0	66.3	2.16	96.0
	150	251.1	2.98	102.0	197.7	3.40	97.2
	600	622.1	7.90	87.3	579.3	1.80	87.9
PC <sub>3</sub>	Endogenous	85.8			44.7		
	15	101.7	1.70	106.0	59.7	2.15	100.0
	150	227.4	2.40	94.4	180.9	2.77	90.8
	600	634.8	2.54	91.5	609.3	3.52	94.1
PC <sub>4</sub>	Endogenous	25.5			17.1		
	15	40.1	4.03	97.2	31.8	4.10	98.0
	150	185.1	2.06	106.4	147.5	1.78	86.9
	600	589.9	1.93	94.1	545.1	2.36	88.0

confirming the achievement of good results for all analyte concentrations.

In order to estimate the efficacy of the analytical method to obtain the thiol-peptides throughout the entire preparation process, recovery was evaluated by comparing the peak area of internal standards spiked before and after extraction. The recovery rates in shoot extracts were estimated at 91.02% and 83.90% for glycine-<sup>13</sup>C<sub>2</sub>,<sup>15</sup>N-labelled GSH and glycine-<sup>13</sup>C<sub>2</sub>,<sup>15</sup>N-labelled PC<sub>2</sub>, respectively, while in root extracts the recovery rates were 73.78% and 88.96% for glycine-<sup>13</sup>C<sub>2</sub>,<sup>15</sup>N-labelled GSH and glycine-<sup>13</sup>C<sub>2</sub>,<sup>15</sup>N-labelled PC<sub>2</sub>. Thus, the recovery of the spiked internal standards allowed a better set up of the entire process.

It is well known that ESI-MS is significantly affected by the so-called matrix effect, according to which the matrix in the sample has an influence on the signal response of the analytes, inducing either its suppression or enhancement. In particular, the sample composition could be responsible for poor analytical accuracy, linearity, reproducibility and robustness. To the best of our knowledge, this study is the first to evaluate the matrix effect on PCn analysis. This was assessed by comparing the peak area of each thiol-peptide spiked into plant extracts and of the neat solutions, according to the procedure described in point 4.7. Interestingly, all thiol-peptides exhibited ionization suppression which, if expressed as (100 – matrix effect%), resulted in the range of 3.3–35.9% and 6.9–29.8% for shoot and root extracts, respectively (Table 5). It is worth noting that the use of stable isotope-labelled analogs of the analytes as internal standards usually offers the considerable advantage of overcoming the consequences of matrix effect by normalizing the analyte signal during the quantification process.

**Table 4**

Inter-day accuracy and precision (expressed as relative standard deviation, r.s.d.%) either in shoot or in root extracts of *A. thaliana*. Spiked analytes and mean assayed concentrations are indicated as "Spiked C" and "Assayed C", respectively.

Analyte	Spiked C (ng g <sup>-1</sup> fr.wt)	Shoot			Root		
		Assayed C (ng g <sup>-1</sup> fr.wt)	r.s.d.(%)	Accuracy(%)	Assayed C (ng g <sup>-1</sup> fr.wt)	r.s.d.(%)	Accuracy(%)
γ-EC	Endogenous	38.1			32.7		
	15	52.7	4.00	97.2	46.8	5.71	93.8
	150	203.2	2.80	110.0	180.6	2.30	98.6
	600	615.0	2.50	96.2	572.1	3.47	89.9
GSH	Endogenous	117.9			73.5		
	15	131.9	9.80	93.3	87.7	6.87	94.7
	150	282.7	1.31	109.8	228.4	4.02	103.3
	600	681.4	5.03	93.9	624.1	2.00	91.8
PC <sub>2</sub>	Endogenous	98.1			51.9		
	15	112.6	4.00	96.6	66.0	2.16	93.8
	150	243.9	2.98	97.2	184.5	3.40	88.4
	600	659.1	7.90	93.5	580.8	1.80	88.2
PC <sub>3</sub>	Endogenous	85.8			44.7		
	15	100.1	1.70	95.0	57.9	2.15	88.0
	150	225.7	2.40	93.3	168.6	2.77	82.6
	600	609.6	2.54	87.3	554.7	3.52	85.0
PC <sub>4</sub>	Endogenous	25.5			17.1		
	15	39.2	4.03	91.3	30.5	4.10	89.6
	150	168.6	2.06	95.4	153.0	1.78	90.6
	600	562.5	1.93	89.5	557.4	2.36	90.1

**Table 5**

Matrix effect (ME%) and relative precision (expressed as relative standard deviation, r.s.d.%) in shoot and root extracts of *A. thaliana*. Concentration of the spiked analytes is indicated as "Spiked C". ME values > 100% indicate ionization enhancement; ME values < 100% indicate ionization suppression.

Analyte	Spiked C (ng g <sup>-1</sup> fr.wt.)	Shoot		Root	
		ME(%)	r.s.d.(%)	ME(%)	r.s.d.(%)
γ-EC	15	76.4	3.80	75.3	2.76
	150	90.2	3.76	89.7	1.85
	600	95.9	2.20	73.3	1.29
GSH	15	85.6	9.53	72.2	3.64
	150	92.4	9.58	89.7	3.40
	600	96.3	5.05	70.5	2.81
PC <sub>2</sub>	15	87.0	10.01	83.3	3.92
	150	93.4	9.76	85.1	6.10
	600	86.7	8.79	73.6	5.24
PC <sub>3</sub>	15	92.5	2.80	88.1	1.99
	150	85.7	6.02	88.0	5.80
	600	64.1	4.30	73.4	6.03
PC <sub>4</sub>	15	95.3	5.21	90.8	6.42
	150	96.7	3.26	93.1	2.52
	600	72.9	3.71	70.2	2.30

### 3. Concluding remarks

This study set up and validated an efficient and versatile HPLC-ESI-MS-MS method to simultaneously assay GSH, γ-EC, and PC<sub>2-4</sub> in shoots and roots of the model plant *A. thaliana*. One of the strengths of the developed procedure is the very broad linear range of the method, which made possible the quantification of thiol-peptides in the concentration-range expected in *A. thaliana* and in other plants. Our results demonstrated that the method is reliable, sensitive, reproducible and accurate. In comparison to previously published methods, this method has the major advantage of having employed standards for external calibration, in addition to the use of a high quality and specific isotope-labelled internal standard with the same chemical structure of GSH and PC<sub>n</sub>. The use of an internal standard normalizes the instrument signal, improves calibration linearity, compensates for thiol-peptide losses during sample preparation, and takes into account the matrix effect on analyte signals. All the above confers further strength to the validated method, allowing the achievement of a highly accurate quantitative

analysis, and enabling the minimization in the thiol-peptides loss. Additionally, it combines on the one hand a simple and fast extraction procedure, on the other high sensitivity and simultaneous determination of five classes of thiol-peptides within one run. In fact, this method allows to monitor all transitions of the thiol-peptides in 14 min during the same HPLC-ESI-MS-MS acquisition. Finally, the method is highly reproducible for quantifying γ-EC, GSH and PC<sub>n</sub> in plant samples.

## 4. Experimental

### 4.1. Chemicals and instrumentation

All nutrients for plant growth were purchased from Duchefa Biochemie (Haarlem, BH, Netherlands). Water (18.2 MΩ cm) was deionized using a Milli-Q system (Millipore Corp., USA). Acetonitrile (LC-MS grade), ultra-pure water (LC-MS grade), formic acid (FA, MS grade ≈ 98%), Tris (2-carboxyethyl) phosphine (TCEP, ≥ 99%), γ-L-glutamyl-L-cysteine (γ-EC; HPLC grade), reduced L-glutathione (GSH), glycine-<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-labelled glutathione (<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-GSH), the latter being used as an internal standard, were all supplied by Sigma-Aldrich (Saint Louis, MO, USA). Phytochelatin 2 (PC<sub>2</sub>), phytochelatin 3 (PC<sub>3</sub>), phytochelatin 4 (PC<sub>4</sub>) were purchased from AnaSpec Inc. (Fremont, CA, USA), whereas glycine-<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-labelled PC<sub>2</sub> (<sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-PC<sub>2</sub>), this latter used as an internal standard, was specifically synthesized on purpose by AnaSpec. 5-sulphosalicylic acid (SSA, ≥ 99%) and diethylenetriamine-pentaacetic acid (DTPA, ≥ 99%) were supplied by Sigma-Aldrich (Saint Louis, MO, USA).

The HPLC-ESI-MS-MS analyses were performed using an Agilent 1290 Infinity UHPLC (Santa Clara, CA, USA), with a thermostated autosampler, a binary pump, and a column oven, coupled to an AB Sciex API 4000 triple quadrupole mass spectrometer (Concord, ON, Canada), equipped with a Turbo-V ion spray source (Concord, ON, Canada). A ten-port switching valve (Valco Instruments Co. Inc., Huston, TX, USA) was used as a divert valve to discard both head and tail of the HPLC runs. The instrumentation also included a quaternary HPLC pump (Series 200, PerkinElmer, Boston, MA, USA), which supplied the mass spectrometer when the eluent from the column was diverted to waste. Chromatographic separation was performed by a reverse-phase Phenomenex (Torrance, CA, USA) Kinetex 2.6 μm XB-C18 100 Å, 100 × 3 mm HPLC column, protected by a C18 3 mm ID security guard ULTRA cartridge.

System control, data acquisition and processing were carried out by an AB Sciex Analyst<sup>®</sup> version 1.6.3 and Multiquant<sup>®</sup> version 3.0.2 software.

#### 4.2. Plant material and growth conditions

The material employed in this study consisted of axenically-grown *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) plants (Columbia ecotype). Seeds were sterilized with 70% (v/v) ethanol for 1 min, followed by 10% (v/v) NaClO for 13 min, then thoroughly rinsed with deionized water and incubated for 2 days in darkness at 4 °C. Germinated seeds were grown for 21 days in Petri dishes supplied with sterilized solid Gamborg's B-5 Basal Salt Mixture medium, supplemented with Gamborg's vitamin solution, sucrose (30 g l<sup>-1</sup>) and agar (7 g l<sup>-1</sup>). The pH of the final solution was adjusted to 5.8 before the addition of agar. The plant material was then kept under 16/8 h light/dark cycle, at 22 ± 1 °C and a photosynthetic photon flux density of 120 μmol m<sup>-2</sup> s<sup>-1</sup>, with a 60% relative humidity. On the 21st day, each plant was transferred to a single 15-ml centrifuge tube and supplied with 1 ml of liquid B-5 medium. From a total of 120 plants, 60 were treated with 20 μM Cd in the form of 3CdSO<sub>4</sub>·8H<sub>2</sub>O, whereas deionized water was added to the other 60 (controls), in identical volume to the Cd solution. After 72 h, all plants (Cd-exposed and controls) were carefully washed with deionized water and gently blotted dry with filter paper. Each plant was manually split into shoots and roots, both placed separately in a 2 ml Eppendorf tube. Each sample was weighed (100 mg fr. wt), frozen in liquid nitrogen and stored in darkness at -80 °C until further procedures were carried out.

#### 4.3. Extraction of thiol-peptides from plant shoots and roots

Plant samples (100 mg fr. wt), previously stored at -80 °C, were subjected to extraction according to Fontanini et al. (2018), with some minor modifications. Briefly, each sample (shoot and root, separately) was placed in liquid nitrogen and ground into a powder with a mixer mill (MM200, Retch, Haan, Germany), using two agate grinding balls (5 mm diameter) to facilitate mechanical cell lysis at a vibrational frequency of 30 Hz, for 1 min. The use of the mixer mill gave a higher and more reproducible yield than mortar extraction. Subsequently 300 μl of ice-cold prepared extraction buffer [containing 5% (w/v) SSA, 6.3 mM DTPA, 2 mM TCEP] was added to each sample, together with <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-GSH and <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-PC<sub>2</sub> internal standards (each internal standard at a concentration of 200 ng ml<sup>-1</sup>). Powder was resuspended using a vortex mixer for 30 s and homogenized again using the mixer mill for 1 min at 30 Hz. The suspension obtained was vortexed for 30 s, kept in an ice bath for 15 min and vortexed for 5 min. The extract was then centrifuged at 10,000 × g (Hermle, Z 300 K, Wehingen, Germany) for 20 min at 4 °C. Each supernatant was filtered by a Minisart RC4 0.45 μm filter (Sartorius, Goettingen, Germany) and then stored at -80 °C until analysis.

**Table 6**  
Program for HPLC pumps.

Step	HPLC Quaternary Pump				HPLC Binary Pump			
	Time (min)	Flow rate (μl min <sup>-1</sup> )	Methanol (%)	Water (%)	Time (min)	Flow rate (μl min <sup>-1</sup> )	Solvent A (%)	Solvent B (%)
0	0.0	100	50	50	0.0	300	2	98
1	14.0	100	50	50	5.0	300	2	98
2					9.5	300	44	56
3					10.5	300	95	5
4					11.5	300	95	5
5					11.7	300	2	98
6					14.0	300	2	98

#### 4.4. Preparation of standard and control solutions

Stock solutions of thiol-peptides were separately prepared at a 1 mg ml<sup>-1</sup> concentration, by weighing appropriate amounts of the respective standards and dissolving them in a water solution containing 1 mM TCEP to prevent oxidation. All solutions were stored in darkness at -80 °C until use. A working solution, used for the preparation of both standard and control solutions (CSs), containing each thiol-peptide at a concentration of 2 μg ml<sup>-1</sup>, was freshly prepared by diluting the stock solution with an appropriate quantity of TCEP (2 mM). Calibration curves were built daily using ten standard solutions with a concentration ranging from 1 ng ml<sup>-1</sup> to 1000 ng ml<sup>-1</sup> (1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng ml<sup>-1</sup>). Each solution was prepared by serial dilution of the working solution with 2 mM TCEP, adding proper amounts of either <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-GSH or <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-PC<sub>2</sub>, in order to achieve a final concentration of 200 ng ml<sup>-1</sup> for both. <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-GSH was used as an internal standard to assay γ-EC and GSH, while <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-PC<sub>2</sub> was the internal standard for PCn. Three different sets of solutions were prepared to evaluate accuracy, precision and matrix effect of the method. Set 1 was prepared using neat solutions of the analytes in the extraction buffer, in order to obtain three control solutions with analytes at a concentration of 15 ng g<sup>-1</sup> fr. wt. (CS<sub>1</sub>1), 150 ng g<sup>-1</sup> fr. wt. (CS<sub>1</sub>2), 600 ng g<sup>-1</sup> fr. wt. (CS<sub>1</sub>3). Internal standards were added in appropriate amounts to each, to achieve a final concentration of 200 ng ml<sup>-1</sup>. Set 2 was prepared by spiking three different batches of shoot and root extracts with suitable amounts of working solution and internal standards, in order to obtain CS<sub>2</sub>s containing the analytes at four different concentration levels, as well as <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-GSH and <sup>13</sup>C<sub>2</sub>, <sup>15</sup>N-PC<sub>2</sub> at a final concentration of 200 ng ml<sup>-1</sup>. In detail, each extract was divided into four subsamples, which were then treated as follows: one of them was added with no working solution (CS<sub>2</sub>0), while the others with given amounts of working solutions, so that the final concentrations of the added analytes were 15 ng g<sup>-1</sup> fr. wt. (CS<sub>2</sub>1), 150 ng g<sup>-1</sup> fr. wt. (CS<sub>2</sub>2), and 600 ng g<sup>-1</sup> fr. wt. (CS<sub>2</sub>3). The latter set, Set 3, was prepared by adding the internal standards to three batches of shoot and root samples before extraction, in order to take them to a final concentration level of 200 ng ml<sup>-1</sup> (CS<sub>3</sub>0).

#### 4.5. HPLC conditions

The HPLC separation was carried out using acetonitrile with 0.1% v/v FA, as a solvent A, and water with 0.1% v/v FA, as a solvent B, under the gradient conditions shown in Table 6. Flow rate and column oven temperature were set to 300 μl min<sup>-1</sup> and 30 °C, respectively. The samples were maintained at 10 °C in the autosampler tray and the sample injection volume was set to 20 μl. Taking into account that the thiol-peptides of interest usually have significantly different endogenous concentrations, three methods able to discard different eluate portions of the HPLC runs, by suitable divert valve timings, were used in order to ensure that the analytical method was suitably robust, and to prevent a progressive worsening of the instrumental sensitivity. Briefly, in the first method (used for the standard solutions) the mass

spectrometer was supplied with the column eluate in the time-range 1.5–10.0 min; in the second one, used for  $\gamma$ -EC and GSH analysis, in the time-range 1.8–3.0 min; in the third one, used for PCn analysis, in the time-range 6.0–9.0 min.

#### 4.6. ESI-MS-MS conditions

Since ESI-MS-MS sensitivity is very dependent on the structural properties of chemical compounds, in order to maximize the signal-to-noise ratio, the tuning parameters were optimized for  $\gamma$ -EC, GSH and PC<sub>2-4</sub> via direct introduction of standard solutions (10  $\mu\text{g ml}^{-1}$ ) of each thiol-peptide in different buffers. This was achieved using a syringe pump. Both ESI positive and negative ion modes were tested, exploiting the amphoteric structure of thiol-peptide compounds with at least one acidic (carboxyl group) and basic group (amino group). Positive ion mode offered significantly higher responses for all analytes, so that the final method was based on just such a polarity. Making use of optimized de-clustering potential (DP), collision energies (CEs), and collision exit potentials (CXPs), three transitions were monitored for each compound; in particular, one of them was used as a quantifier (Q) according to its favorable S/N ratio, and the other two as qualifiers (q). Ion ratio was evaluated in standard solutions and calculated by the formula [(peak area of qualifier)/(peak area of quantifier)]. The selected reaction monitoring (SRM) transitions and the related parameters for all the analytes are listed in Table 7. Further operative parameters were set as follows: IonSpray voltage (IS), 5.5 kV; Gas Source 1 (GS1), 45 arbitrary units; Gas Source 2 (GS2), 35 arbitrary units; source Temperature (TE), 600 °C; Collision Gas (CAD) Nitrogen, 6.3 mPa, Curtain Gas (CUR) 10 arbitrary units; Entrance Potential (EP), 10 V; Prefilter (ST), –15.4 V; Focusing Lens 1 (IQ1), –10.6 V.

#### 4.7. Assessment of the method performances

Data reliability, reproducibility and robustness have been ensured by validating the method with reference to sensitivity, accuracy, precision, reproducibility and stability. Recovery and matrix effect were

**Table 7**  
Mass spectrometry operative parameters.

Operative Parameters						
Analyte	SRM transition	Ion ratio	r.s.d. (%)	DP	CE	CXP
$\gamma$ -EC	251.1 $\rightarrow$ 83.8 (q)	0.91	5.25	42	36	5.2
	251.1 $\rightarrow$ 121.8 (Q)	1.0	0.00		17	8.7
	251.1 $\rightarrow$ 129.9 (q)	0.52	3.32		23	10.2
GSH	308.1 $\rightarrow$ 75.9 (q)	0.53	7.82	57	35	5.8
	308.1 $\rightarrow$ 162.0 (q)	0.53	3.66		25	12.4
	308.1 $\rightarrow$ 178.8 (Q)	1.00	0.00		18	16.1
<sup>13</sup> C <sub>2</sub> , <sup>15</sup> N-GSH	311.1 $\rightarrow$ 75.9 (q)	0.43	5.87	57	35	5.8
	311.1 $\rightarrow$ 165.0 (q)	0.55	2.81		25	12.4
	311.1 $\rightarrow$ 181.8 (Q)	1.00	0.00		18	16.1
PC <sub>2</sub>	540.1 $\rightarrow$ 308.0 (q)	0.72	2.55	88	31	8.1
	540.1 $\rightarrow$ 336.1 (Q)	1.00	0.00		30	9.4
	540.1 $\rightarrow$ 411.3 (q)	1.29	3.81		25	12.3
<sup>13</sup> C <sub>2</sub> , <sup>15</sup> N-PC <sub>2</sub>	543.1 $\rightarrow$ 311.0 (q)	0.43	5.87	88	31	8.1
	543.1 $\rightarrow$ 337.1 (Q)	0.55	2.81		30	9.4
	543.1 $\rightarrow$ 414.3 (q)	1.00	0.00		25	12.3
PC <sub>3</sub>	772.2 $\rightarrow$ 232.9 (Q)	1.00	0.00	121	52	19.3
	772.2 $\rightarrow$ 540.1 (q)	0.53	7.75		37	16.1
	772.2 $\rightarrow$ 643.3 (q)	0.65	9.79		34	9.7
PC <sub>4</sub>	1004.3 $\rightarrow$ 233.0 (Q)	1.00	0.00	125	68	6.5
	1004.3 $\rightarrow$ 540.2 (q)	0.54	6.73		48	15.8
	1004.3 $\rightarrow$ 875.2 (q)	0.33	8.85		41	14.7

also studied (Matuszewski et al., 2003).

The linearity of the method was evaluated within the calibration curve range and tested from 1 to 1000 ng ml<sup>-1</sup> of analyte concentrations. The calibration curves were built by plotting the peak area ratio (analyte/internal standard) against the concentration ratio (analyte/internal standard) with five replicates for each standard, and were expressed by the general equation  $y = mx + n$ , whose coefficients were calculated by linear regression with weighting of  $1/x$ .

The instrumental sensitivity was evaluated by measuring the thiol-peptides' limits of detection (LODs) and quantification (LOQs), making use of standard solutions containing the analytes at different concentration levels. The concentration levels providing signal-to-noise (S/N) ratios close to 3 and 10, were assumed as LODs and LOQs, respectively.

The method accuracy was evaluated by either Set 1 or Set 2. In particular, in Set 2 the spiked concentration of the thiol-peptides of interest was assessed by subtracting the endogenous concentration values measured in CS<sub>2</sub>0 to the measured concentrations in CS<sub>2</sub>1, CS<sub>2</sub>2 and CS<sub>2</sub>3. Accuracy % for both sets was then calculated by the formula [(mean observed concentration)/(nominal spiked concentration) x 100].

The method precision was determined by three replicate injections of the three CSs from Set 2, and it was expressed in terms of relative standard deviation (r.s.d. %). Both accuracy and precision were evaluated by analyzing three batches of shoots and roots three times within 24 (intra-day assay) h and in 3 different days (inter-days assay), respectively.

In order to estimate the method's ability to recover the analytes through the sample preparation, recovery was evaluated by comparing the peak area of the internal standards in CS<sub>3</sub>0 and CS<sub>2</sub>0, in relation to the three different batches of *A. thaliana* roots and shoots. Recovery was expressed in percentage with [(Peak area of IS spiked in CS<sub>3</sub>0/Peak area of IS in CS<sub>2</sub>0) x 100].

Matrix effect, which significantly affects the electrospray ionization efficiency and, as a consequence, the performances of HPLC-MS-MS, is deeply influenced by the matrix composition and is particularly effective in complex matrices such as plant samples. It was roughly estimated by comparing the analyte peak areas from the CS<sub>2</sub>1, CS<sub>2</sub>2, and CS<sub>2</sub>3, subtracted by those relative to the endogenous concentrations measured in CS<sub>2</sub>0, to the peak areas from CS<sub>1</sub>1, CS<sub>1</sub>2, and CS<sub>1</sub>3. Matrix effect was then calculated for each analyte at the three concentration levels: (Peak area of the added analyte in CS<sub>2</sub>/Peak area of the analyte in CS<sub>1</sub>) x 100, where CS<sub>1</sub> stands for CS<sub>1</sub>1, CS<sub>1</sub>2, and CS<sub>1</sub>3, while CS<sub>2</sub> stands for CS<sub>2</sub>1, CS<sub>2</sub>2, and CS<sub>2</sub>3.

#### 4.8. Statistical analysis

Data analysis was performed by Graph-Pad Prism 6.0 statistical program (GraphPad Software Inc., San Diego, CA, USA). Data were reported as the mean  $\pm$  s.d. (standard deviation).

#### Declaration of interest

None.

#### Funding

This work was supported by the Italian Ministry of University and Scientific Research (MIUR-PRIN 2015) funds (prot. 20158HTL58, PI L. Sanità di Toppi).

#### Abbreviations

FA	Formic acid
TCEP	Tris(2-carboxyethyl) phosphine
$\gamma$ -EC	$\gamma$ -L-glutamyl-L-cysteine

GSH	L-glutathione in reduced form
PC2	Phytochelatin 2
PC3	Phytochelatin 3
PC4	Phytochelatin 4
SSA	5-Sulphosalicylic acid
DTPA	Diethylenetriamine-pentaacetic acid
ESI	Electrospray ionization
fr. wt	Fresh weight
CS	Control solution
SRM	Selected reaction monitoring
DP	Declustering potential
CE	Collision energy
CXP	Collision exit potential
Q	Quantifier
q	Qualifier
IS	Internal standard;
GS1	Gas source 1
GS2	Gas source 2
TE	Temperature
CAD	Collision Gas
EP	Entrance potential
ST	Pre-filter
IQ1	Focusing lens 1 potential

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phytochem.2019.05.007>.

## References

- Berlich, M., Menge, S., Bruns, I., Schmidt, J., Schneider, B., Krauss, G.-J., 2002. Coumarins give misleading absorbance with Ellman's reagent suggestive of thiol conjugates. *Analyst* 127, 333–336. <https://doi.org/10.1039/B110988J>.
- Bräutigam, A., Wesenberg, D., Preud'homme, H., Schaumlöffel, D., 2010. Rapid and simple UPLC-MS/MS method for precise phytochelatin quantification in alga extracts. *Anal. Bioanal. Chem.* 398, 877–883. <https://doi.org/10.1007/s00216-010-3970-7>.
- Cao, Z.-Y., Sun, L.-H., Mou, R.-X., Zhou, R., Zhu, Z.-W., Chen, M.-X., 2015. A novel method for the simultaneous analysis of seven biothiols in rice (*Oryza sativa* L.) using hydrophilic interaction chromatography coupled with electrospray tandem mass spectrometry. *J. Chrom. B* 976–977, 19–26. <https://doi.org/10.1016/j.jchromb.2014.11.007>.
- Chace, D.H., Millington, D.S., Terada, N., Kahler, S.G., Roe, C.R., Hofman, L.F., 1993. Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. *Clin. Chem.* 39, 66–71.
- Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. *Annu. Rev. Plant Biol.* 53, 159–182. <https://doi.org/10.1146/annurev.arplant.53.100301.135154>.
- Degola, F., De Benedictis, M., Petraglia, A., Massimi, A., Fattorini, L., Sorbo, S., Basile, A., Sanità di Toppi, L., 2014. A Cd/Fe/Zn-responsive phytochelatin synthase is constitutively present in the ancient liverwort *Lunularia cruciata* (L.) Dumort. *Plant Cell Physiol.* 55, 1884–1891. <https://doi.org/10.1093/pcp/pcu117>.
- El-Zohri, M.H.A., Cabala, R., Frank, H., 2005. Quantification of phytochelatin in plants by reversed-phase HPLC-ESI-MS-MS. *Anal. Bioanal. Chem.* 382, 1871–1876. <https://doi.org/10.1007/s00216-005-3331-0>.
- Fontanini, D., Andreucci, A., Ruffini Castiglione, M., Basile, A., Sorbo, S., Petraglia, A., Degola, F., Bellini, E., Bruno, L., Varotto, C., Sanità di Toppi, L., 2018. The phytochelatin synthase from *Nitella mucronata* (Charophyta) plays a role in the homeostatic control of iron(II)/(III). *Plant Physiol. Biochem.* 127, 88–96. <https://doi.org/10.1016/j.plaphy.2018.03.014>.
- Grill, E., Winnacker, E.-L., Zenk, M.H., 1991. Phytochelatin. *Methods in Enzymology, Metallobiochemistry Part B Metallothionein and Related Molecules*. Academic Press, pp. 333–341. [https://doi.org/10.1016/0076-6879\(91\)05115-C](https://doi.org/10.1016/0076-6879(91)05115-C).
- Grill, E., Winnacker, E.-L., Zenk, M.H., 1985. Phytochelatin: the principal heavy-metal complexing peptides of higher plants. *Science* 230, 674–676. <https://doi.org/10.1126/science.230.4726.674>.
- Gupta, D.K., Vandenhove, H., Inouhe, M., 2013. Role of phytochelatin in heavy metal stress and detoxification mechanisms in plants. In: Gupta, Dharmendra K., Corpas, F.J., Palma, J.M. (Eds.), *Heavy Metal Stress in Plants*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 73–94. [https://doi.org/10.1007/978-3-642-38469-1\\_4](https://doi.org/10.1007/978-3-642-38469-1_4).
- Gupton-Campolongo, T., Damasceno, L.M., Hay, A.G., Ahner, B.A., 2013. Characterization of a high affinity phytochelatin synthase from the Cd-utilizing marine diatom *thalassiosira pseudonana*. *J. Phycol.* 49, 32–40. <https://doi.org/10.1111/jpy.12022>.
- Huang, Y.-Q., Wang, Q.-Y., Liu, J.-Q., Hao, Y.-H., Yuan, B.-F., Feng, Y.-Q., 2014. Isotope labelling – paired homologous double neutral loss scan-mass spectrometry for profiling of metabolites with a carboxyl group. *Analyst* 139, 3446–3454. <https://doi.org/10.1039/C4AN00312H>.
- Kawakami, S., Gledhill, M., Achterberg, E., 2006. Determination of phytochelatin and glutathione in phytoplankton from natural waters using HPLC with fluorescence detection. *Trac. Trends Anal. Chem.* 25, 133–142. <https://doi.org/10.1016/j.trac.2005.06.005>.
- Liu, P., Cai, W.-J., Yu, L., Yuan, B.-F., Feng, Y.-Q., 2015. Determination of phytochelatin in rice by stable isotope labeling coupled with liquid chromatography–mass spectrometry. *J. Agric. Food Chem.* 63, 5935–5942. <https://doi.org/10.1021/acs.jafc.5b01797>.
- Matuszewski, B.K., Constanzer, M.L., Chavez-Eng, C.M., 2003. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS. *Anal. Chem.* 75, 3019–3030. <https://doi.org/10.1021/ac020361s>.
- Minocha, R., Thangavel, P., Dhankher, O.P., Long, S., 2008. Separation and quantification of mono thiols and phytochelatin from a wide variety of cell cultures and tissues of trees and other plants using high performance liquid chromatography. *J. Chromatogr. A* 1207, 72–83. <https://doi.org/10.1016/j.chroma.2008.08.023>.
- Mou, R., Cao, Zhao-yun, Lin, X., Wu, L., Cao, Zhen-zhen, Zhu, Z., Chen, M., 2016. Characterization of the phytochelatin and their derivatives in rice exposed to cadmium based on high-performance liquid chromatography coupled with data-dependent hybrid linear ion trap orbitrap mass spectrometry: characterization of the phytochelatin and their derivatives. *Rapid Communications in Mass Spectrometry*. <https://doi.org/10.1002/rcm.7669>.
- Orwar, O., Fishman, H.A., Ziv, N.E., Scheller, R.H., Zare, R.N., 1995. Use of 2,3-naphthalenedicarboxaldehyde derivatization for single-cell analysis of glutathione by capillary electrophoresis and histochemical localization by fluorescence microscopy. *Anal. Chem.* 67, 4261–4268. <https://doi.org/10.1021/ac00119a010>.
- Sanità di Toppi, L., Gabbriellini, R., 1999. Response to cadmium in higher plants. *Environ. Exp. Bot.* 41, 105–130. [https://doi.org/10.1016/S0098-8472\(98\)00058-6](https://doi.org/10.1016/S0098-8472(98)00058-6).
- Schröder, P., Collins, C., 2002. Conjugating enzymes involved in xenobiotic metabolism of organic xenobiotics in plants. *Int. J. Phytoremediation* 4, 247–265. <https://doi.org/10.1080/15226510208500086>.
- Simmons, D.B.D., Hayward, A.R., Hutchinson, T.C., Emery, R.J.N., 2009. Identification and quantification of glutathione and phytochelatin from *Chlorella vulgaris* by RP-HPLC ESI-MS/MS and oxygen-free extraction. *Anal. Bioanal. Chem.* 395, 809–817. <https://doi.org/10.1007/s00216-009-3016-1>.
- Sneller, F.E.C., Heerwaarden, L.M.V., Kraaijeveld-Smit, F.J.L., Bookum, W.M.T., Koevoets, P.L.M., Schat, H., Verkleij, J.A.C., 1999. Toxicity of arsenate in *Silene vulgaris*, accumulation and degradation of arsenate-induced phytochelatin. *New Phytol.* 144, 223–232.
- Wood, B.A., Feldmann, J., 2012. Quantification of phytochelatin and their metal(loid) complexes: critical assessment of current analytical methodology. *Anal. Bioanal. Chem.* 402, 3299–3309. <https://doi.org/10.1007/s00216-011-5649-0>.
- Zenk, M.H., 1996. Heavy metal detoxification in higher plants - a review. *Gene* 179, 21–30. [https://doi.org/10.1016/S0378-1119\(96\)00422-2](https://doi.org/10.1016/S0378-1119(96)00422-2).