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Review

Metallo-thiolomics: Investigation of thiol peptide regulated metal homeostasis in plants and fungi by liquid chromatography-mass spectrometry

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ABSTRACT

This review gives an overview and critical evaluation about modern LC-MS techniques for identification and quantification of thiol peptides and their metal complexes in seed plants, algae and fungi. In analogy to other research fields we propose the term metallo-thiolomics for the global study of the entirety of thiol peptides and their metal complexes (metallo-thiolome) aiming at the elucidation of the thiol peptide regulated metal homeostasis. Off-line and on-line approaches based on liquid chromatography (LC) and electrospray tandem mass spectrometry (ESI-MS/MS) were mainly used for identification and quantification of thiol peptide apo-forms. The complementary application of inductively coupled plasma mass spectrometry (ICP-MS) has demonstrated to be useful for the investigation of native metal–thiol peptide complexes. Furthermore, new quantification strategies via the element signal in ICP-MS will be presented. We critically discuss the impact of these approaches on the progress in plant biochemistry and highlight future trends and developments.

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1. Introduction

Seed plants, algae and fungi have to cope with toxic or surplus of essential metals in the environment derived mainly from mining and other industries [1,2]. Deficiencies of micronutrients seriously disturb normal growth and development – excess of metals, especially at toxic intracellular concentrations, severely affects metabolic reactions and physiological processes. Thus, plants and fungi have developed adaptive stress responses to environmental changes. Tolerance to metals (i) is a particular aspect of cellular homeostasis and (ii) needs a distinct cross-talk between signalization pathways as consequence of interference with the essential metal homeostasis. Extensive biochemical and analytical research is needed in order to elucidate and understand the versatile mechanisms in metal sensing and metal stress response.

Intracellular thiol peptides are of utmost significance for life, containing the major active form of sulfur (thiol or sulfhydryl group: –SH, oxidation state: -2), which itself is one of the most versatile elements in life [3]. The tripeptide glutathione (GSH, γ-Glu-Cys-Gly) is the most prominent constituent of plant cellular redox buffering system [4,5] and serves as intracellular metal binding thiol ligand in seed plants [1,6,7], algae [7,8] and fungi [9,10]. GSH derived canonic phytochelatins (PC) of the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2$–6) are synthesized under metal stress by phytochelatin synthase in plants and fungi (Fig. 1) [1,11]. Besides canonic PC different isoforms containing altering terminal amino acids (CysPC_n, desGlyPC_n, $\text{CysPC}_n\text{desGly}$) or substituted Gly residues were found in seed plants [1] and green algae [8,12].

PC form metal complexes playing an essential role in intracellular cadmium storage and detoxification [7]. Several metals (Cd, Pb, Hg, and Ag) and metalloids (As) are considered as non-nutrient elements, since they have not known function in plant metabolism (except for a Cd-carbonic anhydrase of marine diatoms [13]). Even if cadmium toxicity is still not completely understood it might result from high affinity of sulfhydryl ligands and chemical similarity to zinc [14]. Cd is the preferential inductor for PC synthesis [1], cause regulatory events in sulfate assimilation [1,15] and thus alterations in Cys synthesis as well as thiol peptides.

Elucidation and understanding of thiol peptide regulated metal homeostasis demands a global analysis of all thiol peptides and their metal complexes (metallo-thiolome) in plant samples (Fig. 2). A variety of different analytical methods is proposed for the separation, identification, and quantification of thiol peptides in biological samples [16]. Detection can be carried out by electrochemical techniques (polarography, voltammetry, amperometry), UV–vis and fluorescence spectrometry, and mass spectrometry (MS). In many cases thiol peptide detection is preceded by a multiple-step procedure including sampling, thiol peptide extraction, reduction and derivatization, and by chromatographic or electrophoretic separation [16]. Reversed phase liquid chromatography (RP LC) is the most established separation technique combined with thiol-specific spectrophotometric detection. Therefore sulfhydryl groups of GSH and phytochelatins are usually derivatized post-column with Ellman's reagent (5,5′-dithio-bis-(2-nitrobenzoic acid), DTNB) for specific UV–vis detection at $\lambda = 410$ nm [17,18]. For specific fluorescence detection thiols can also be derivatized pre-column with monobromobimane (mBrB) [19]. The advantage of mBrB towards DTNB is the higher sensitivity and stability of the derivatives [20,21]. But the derivatization efficiency of both reagents decreases with increasing chain-length of the PCs. This should be taken into account when thiol quantification has to be carried out. The decrease of mBrB-derivatization efficiency is much stronger and therefore it is not capable for PC-quantification [22] which limits this method to the quantification of small thiols such as cysteine, γ-glutamylcysteine and GSH. On the other hand the classic method of RP LC and DTNB post-column derivatization becomes difficult if low abundant PCs and isoPCs have to be identified and quantified. In order to overcome these limitations mass spectrometry was proposed for highly sensitive thiol peptide detection, their unequivocal identification and accurate quantification.

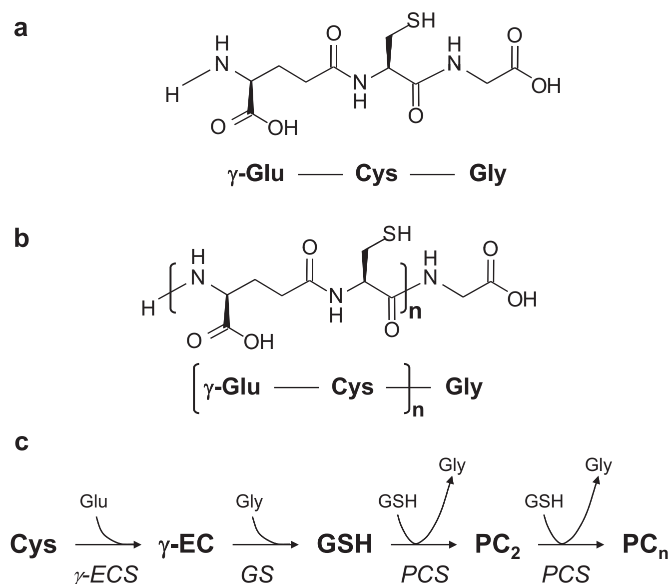

Fig. 1. (a) Chemical structure of glutathione (GSH); (b) chemical structure of canonic phytochelatins ($n = 2$–6); (c) pathway for the synthesis of canonic phytochelatins from Cys, Glu and Gly via γ-Glu-Cys (γ-EC) and GSH. Involved enzymes are γ-EC synthetase (γ-ECS), glutathione synthetase (GS), and phytochelatin synthase (PCS).

The coupling of liquid chromatography with mass spectrometry has been already realized more than 30 years ago [23]. Few years later miniaturized chromatographic columns of inner diameters less than 300 μm (capillary LC) were coupled to mass spectrometry with an electrospray ionization (ESI) source [24]. Determination of the exact molecular mass in MS as well as fragmentation of the molecule of interest in a collision cell followed by mass spectrometric analysis of the fragments (tandem mass spectrometry, MS/MS) enable structural identification. Nowadays LC-MS/MS systems using nanoLC and capLC are powerful, indispensable tools for peptide sequencing and quantification in modern proteomics approaches [25]. Also in plant metabolomics studies LC-MS/MS techniques are meanwhile widely used [26] and applied to the determination of thiols such as glutathione (GSH) and oxidized glutathione (GSSG) [27]. As for protein and peptide analysis the combination of reversed phase liquid chromatography with electrospray (ESI)-MS is the predominant technique used for thiol peptide studies.

Phytochelatins form intracellular complexes with metals. Therefore several research groups introduced element mass spectrometry (inductively coupled plasma mass spectrometry, ICP-MS) as complementary technique for specific metal detection and quantification in plant metabolism studies. ICP-MS is highly sensitive (down to attogram levels), enables multielement and multi-isotope detection, shows a large dynamic range up to nine orders of magnitude, and the ionization process is practically compound- and matrix-independent, [28]. Nevertheless, some matrix components can lead to interferences requiring either collision/reaction cells or high resolution sector field instruments. ICP-MS allows the specific detection of metals and other heteroelements (e.g. sulfur) in metallo-biomolecules such as metal–thiol peptide complexes [29]. However, for these studies ICP-MS cannot be used as stand-alone technique as it is not able to provide structural information. Therefore, many modern approaches for the investigation of metallo-biomolecules are based on the combination of

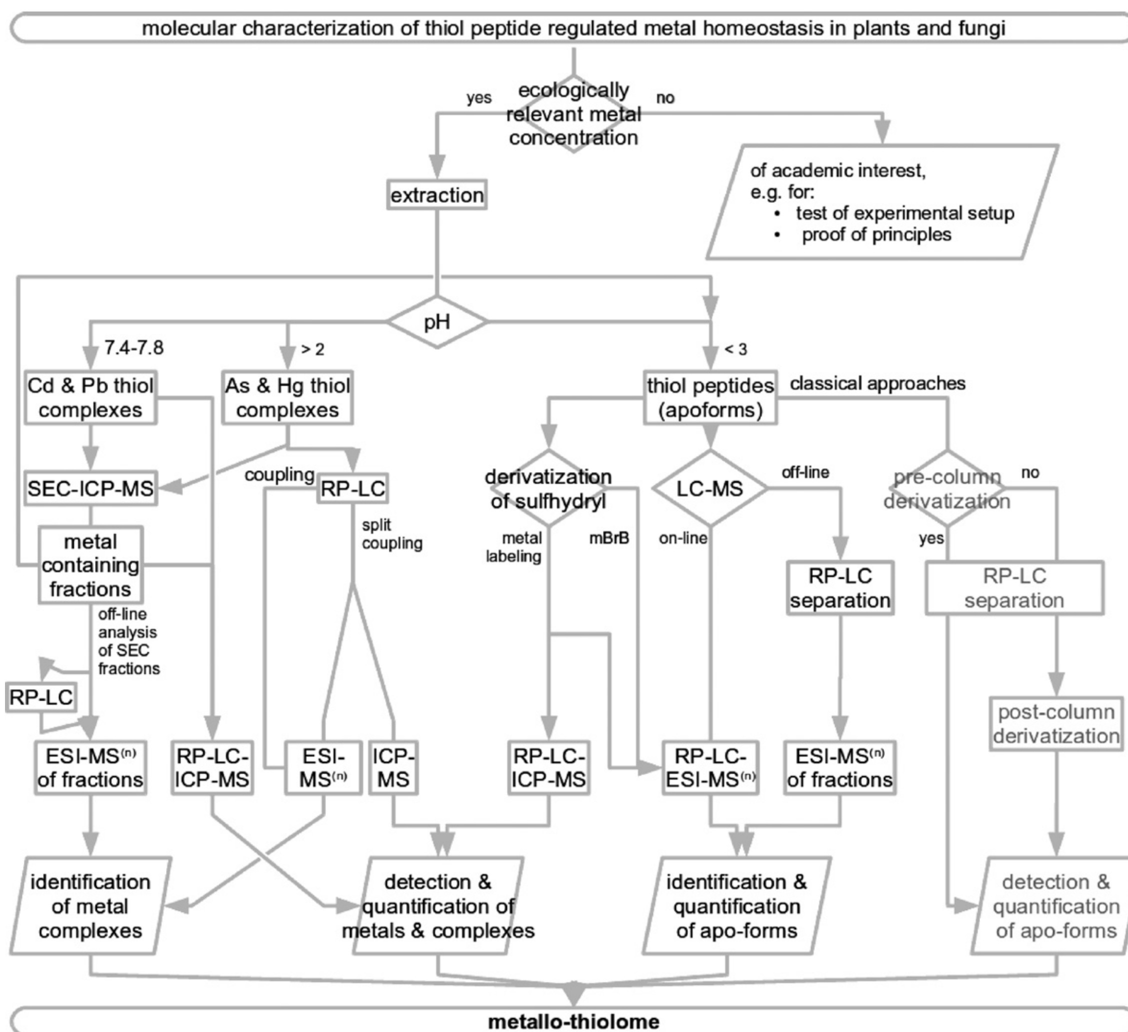

Fig. 2. Analytical strategies for the investigation of the metallo-thiolome in seed plants, algae and fungi for molecular characterization of thiol peptide regulated metal homeostasis.

chromatographic separation with element specific (ICP-MS) and molecule specific (ESI-MS) detection [30].

The objective of this review is to give an overview about modern LC-MS techniques for identification and quantification of thiol peptides and their metal complexes in plants, algae and fungi. In analogy to other research fields we propose the term metallo-thiolomics for the global study of the entirety of thiol peptides and their metal complexes (metallo-thiolome) aiming at the elucidation of the thiol peptide regulated metal homeostasis. Different approaches will be critically compared and evaluated. Their contribution to the scientific progress in plant biochemistry will be discussed.

2. Extraction of thiol peptides

In order to investigate thiol peptide regulated metal homeostasis in plants, metal exposure should be in ecological relevant concentration ranges [1]. Studies using up to four orders of magnitude higher than ecological relevant metal concentrations are rather interesting to show a proof of principle or to test instrumental and methodological developments.

Extraction conditions of thiol peptides from plants depend on the objective of the particular study. In order to extract apoforms (metal-free) of thiol peptides from seed plants firstly their cell walls have to be destructed by homogenization in liquid nitrogen and subsequent harsh alkali conditions (pH > 14) and secondly metal–thiol peptide complexes are decomposed under strong acidic conditions (pH 1) [31–34]. The first step can be avoided if the cell walls are fragile as e.g. those of the green algae *Clamydomonas rheinhardtii* [8] and *Stigeoclonium tenue* [12]. Addition of reducing agent such as sodium borohydride [31,32,34], TCEP (tris(2-carboxyethyl) phosphine) [8,35], or DTT (dithiothreitol) [12,33] avoids possible oxidation of sulfhydryl groups.

For extraction intact metal–thiol complexes milder conditions have to be applied. In this case cell walls can be destructed preferentially under liquid nitrogen. Subsequently, cadmium and lead thiol complexes are proposed to be extracted by buffer solutions at pH 7.4–7.8 [36–40]. Thiol peptide complexes of arsenic and mercury are stable over a wider pH range down to pH 2 and often extracted with formic acid [41,42] or water [34]. A further problem is the possible oxidation of thiol groups leading to a decomposition of the metal complex. Wei et al. proposed precautions such as a non-oxidizing (N_2) preparation environment and sample storage at −85 °C [37].

3. Identification of thiol peptides by reversed phase LC-ESI-MS/MS

3.1. Off-line approaches

First approaches for identification of metal-free thiol peptides by electrospray mass spectrometry employed a purification of the

plant extract by reversed phase LC [43,44]. The elution times of thiol peptide standards were previously determined with DTNB derivatization and UV–vis detection. Then the sample was injected to LC and one or several thiol peptide containing fractions including phytochelatins were collected and concentrated. This off-line approach is widely used until today in many studies [8,12,32]. The advantages are solvent evaporation and exchange avoiding possible incompatibilities with the ESI source, the pre-concentration of the analytes in the sample, and the long analysis time available for MS and MS/MS experiments allowing e.g. in nanoESI-MS the detailed investigation of a fraction during 0.5–2 h [8]. In contrast, in on-line LC-MS/MS the analysis is limited to the elution time of an analyte peak (about 0.5–2 min). A disadvantage is, however, the likewise concentration of matrix components which could suppress the ionization of thiol peptides and render the detection of low-abundant PCs difficult.

From the rich literature on thiol peptide identification by this approach few examples are selected for illustration purposes. Pawlik–Skowronska investigated two ecotypes (metal sensitive and tolerant) of the green alga *S. tenue* under lead exposure [12]. Phytochelatins were identified in ESI-MS according to their molecular mass. While the sensitive form showed canonic phytochelatins $PC_{2–5}$ the tolerant ecotype induced $CysPC_{2–5}$ as major thiol peptides. Similar finding showed the recent work of Bräutigam et al. on the cadmium exposed green alga *Chlamydomonas reinhardtii* where $CysPC_{2–5}$ were the main thiol peptides induced, too [8]. In this study all thiols were unambiguously identified by their accurate mass in nanoESI-QTOF MS and additionally by their b and y fragments in MS/MS (Fig. 3). A further example demonstrated that this approach could also successfully be applied to fungi where PC_2 was identified in the aquatic hyphomycete *Heliscus lugdunensis* [32].

3.2. On-line approaches

Some of the authors worked with the previously described off-line approach proposed consequently also an on-line LC-ESI-MS coupling for thiol peptide analysis [31,45]. Even few papers described the use of a capillary electrophoresis–ESI-MS coupling [44,46] but this more difficult technique has not been furthermore established in this field. In contrast, the widely accepted reversed phase LC-ESI-MS coupling allowed in recent years the identification of thiols in many plant metabolism studies. Representative examples are the characterization of glutathione and phytochelatins after cadmium exposure in wheat (*Triticum aestivum*) [47] and in different brown and red seaweed species [45]. In the first study peptide fragmentation in tandem mass spectrometry allowed the confirmation of phytochelatin and also iso-phytochelatin structures while in the second work identification rely only on molecular mass determination. The latter case demonstrates that identification of glutathione and the canonical phytochelatins, which have distinct, well-defined masses, did not necessarily require high mass resolution and tandem MS if the analytes are sufficiently concentrated.

An interesting work combined pre-column mBrB derivatization of sulfhydryl groups and fluorescence detection with identification of the derivatized PCs by LC-MS/MS [48]. The mass spectra showed singly and doubly charged ions and the fragmentations in MS/MS were more complex. Nevertheless, the data demonstrated that each cysteine residue in $PC_{2–4}$ could be derivatized with mBrB. An advantage of this approach was that derivatization protected the thiol peptides from oxidation and allowed thus their detection at low concentration levels in roots of the hyperaccumulator *Sedum alfredii*. The presence of PC in this plant was queried in previous papers [49,50].

Due to the acidic conditions in reversed phase LC in almost all studies only the apo-forms of thiol peptides were identified by the described off-line and on-line LC-MS approaches. However, in contrast to cadmium and lead the higher stability of mercury–phytochelatin complexes at moderate acidic conditions enables also the analysis of entire Hg-thiol peptides. This was demonstrated by Chen et al. who identified Hg–$PC_{2–4}$ complexes by RP LC-ESI MS/MS in *Brassica chinensis* [34].

4. Quantification of thiol peptides by reversed phase LC-ESI-MS/MS

While LC-MS has been established for thiol peptide identification, many research groups used the conventional thiol derivatization with Ellman's reagent for their quantification in spite of the known disadvantages such as incomplete derivatization and misleading signals from coumarins which affect the accuracy of the quantification result [31]. Nevertheless, derivatization can also be advantageous because it prevents thiols from oxidation.

To date only few papers describe the use of reversed phase LC-ESI-MS/MS for quantification of thiol peptide apo-forms. One of the reasons is probably that expensive PC standards are needed for calibration. Special care has to be taken to avoid oxidation of the non-derivatized sulfhydryl groups during the analytical process. However, LC-MS/MS allows highly specific and sensitive quantification in the multiple reaction mode (MRM) by monitoring transitions between precursor molecular ions and their principle product ions.

El-Zhori et al. described an LC-MS/MS method for GSH and $PC_{2–3}$ quantification in beans (*Vicia faba*), with special emphasis made on disulfide reduction [33]. The authors achieved for each analyte quantification limits (LOQ) of 0.2 nmol g^{-1} plant tissue corresponding to about 33 nM. However, the total run time for one LC analysis was 45 min. Sarry et al. used a coupling technique for relative PC and iso-PC determination, too [51]. Thirteen PCs induced in *Arabidopsis thaliana* after Cd exposure were chromatographically separated during approximately 25 min. Simmons et al., who focused on oxygen free thiol peptide extraction from the green alga *Chlorella vulgaris*, achieved LODs of 81 nM for GSH, 440 nM for PC_2 and 120 nM for PC_3 [35]. Run time of HPLC was 30 min. Recently Bräutigam et al. reported for the first time reproducible quantification of six thiol peptides (GSH, CysGSH, PC_2, PC_3, $CysPC_2$ and $CysPC_3$) in crude extracts of the green alga *C. reinhardtii* at concentrations between 15 and 198 nmol g^{-1} fresh weight (FW) [52]. The rapid Ultra-Performance Liquid Chromatography (UPLC)-MS/MS method allow baseline separation of all thiol peptides within 7 min.

5. The complementary use of ICP-MS and ESI-MS for the study of metal–thiol peptide complexes

5.1. LC-ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) can be a valuable complementary technique to electrospray MS in order to study metal complexes with thiol peptides. ICP-MS allows highly sensitive metal specific detection after chromatographic separation. Hence, LC-ICP-MS enables a screening of the metal speciation in a plant extract. A general challenge is the preservation of the native metal–phytochelatin complex. Classic reversed phase LC for thiol peptide separation at low pH is in most of the cases not suitable because metal–phytochelatin complexes decompose under acidic conditions. Exceptions are arsenic–PC and mercury–PC complexes where the As–S and Hg–S bonds show higher stability [34,53]. To date there are only few reports applying RP LC-ICP-MS to separation and metal-specific detection of entire cadmium and lead–phytochelatin complexes, for example cadmium–$PC_{2–4}$ complexes could be eluted with an acetate buffer (pH 7.3) [36]. In

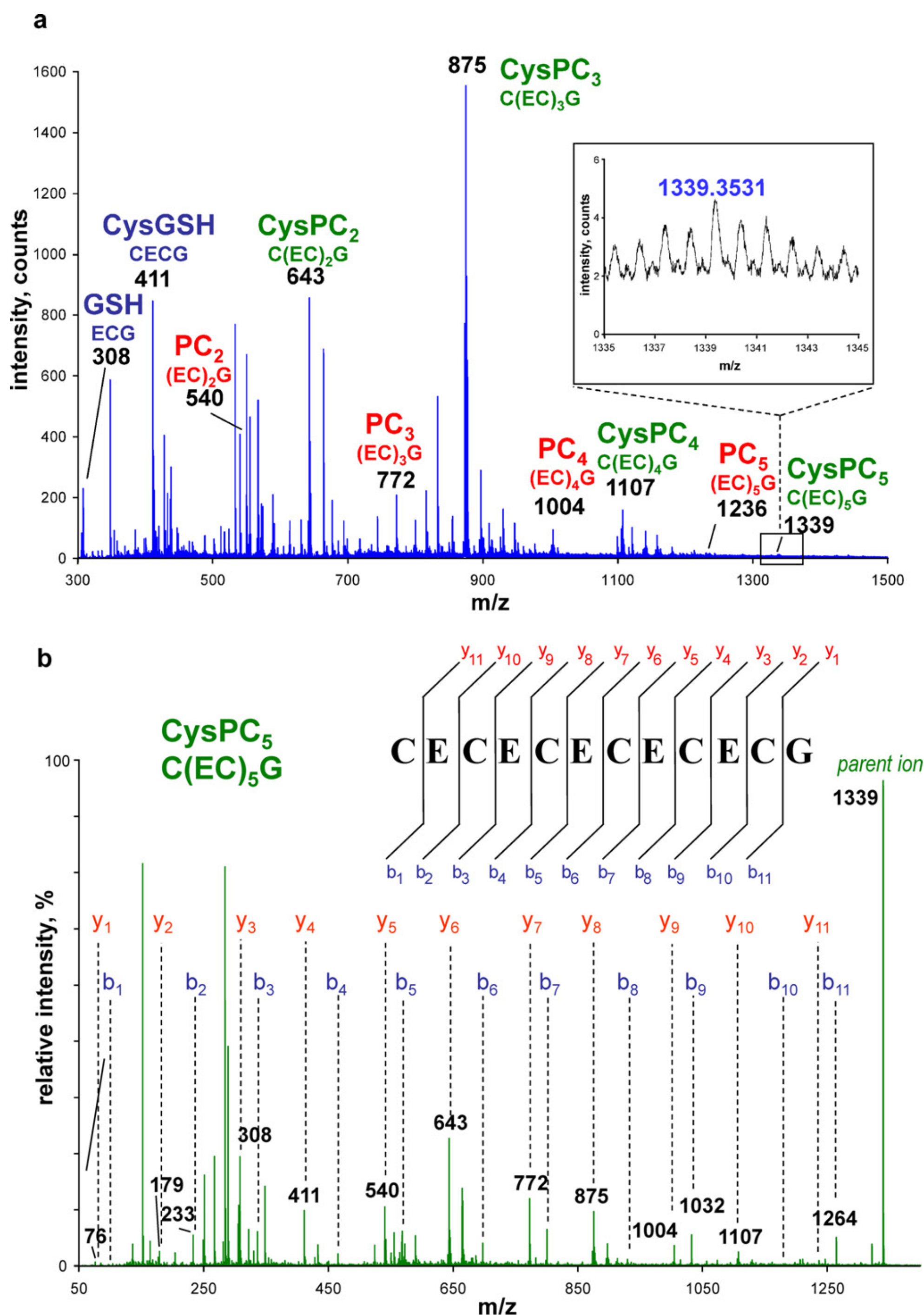

Fig. 3. (a) Detection of canonic PC and CysPC in the cadmium exposed green alga *C. reinhardtii* by nanoESI-QTOF MS, insert: zoom of the CysPC$_5$ signal; (b) unambiguous identification of CysPC$_5$ by the b and y fragments in MS/MS [8].
With kind permission from Springer Science + Business Media: Anal. Bioanal. Chem., "Analytical approach for characterization of cadmium-induced thiol peptides–a case study using *Chlamydomonas reinhardtii*", 395, 2009, pages 1744 and 1745, A. Bräutigam, D. Schaumlöffel, G.-J. Krauss, D. Wesenberg, Figs. 5 and 6, Copyright Springer-Verlag 2009.

this case the identification of a Cd–PC$_2$ complex in *A. thaliana* was possible by comparison of retention time with help of a standard.

The majority of research groups were using the coupling of size exclusion chromatography (SEC) to ICP-MS with an eluent buffered at around pH 7.5 in order to analyse plant extracts for the presence of metal complexes (for example: [37,54,55]). SEC is regarded as a soft separation method not disrupting the integrity of metal–bioligand complexes. However, this statement is not always true and has to be applied with caution. This was demonstrated by Schaumlöffel et al. for nickel complexes in *Sebertia accuminata* which were decomposed on the SEC column while nickel was adsorbed by the stationary phase [56]. Therefore a mass balance comparing the injected and eluted total mass of the metal is recommended in order to detect possible metal adsorption on the column. Furthermore, the chromatographic resolution of SEC is much inferior to RP LC and do not allow baseline separation of each metal complex. In many studies only one to three not well resolved peaks are observed [39,55,57]. Therefore, the analysis of plant extracts

by SEC-ICP-MS allows only the detection of fractions containing metals which are potentially coordinated by bioligands. A parallel ICP-MS detection of sulfur can give an indication that among these bioligands are thiols [58,59].

5.2. Fractionation by SEC

For the reasons pointed out in the previous paragraph size exclusion chromatography is in many studies only used as first purification step in an analytical approach for thiol peptide analysis in plants. In this strategy SEC-ICP-MS is used for screening of plant extracts for metal containing fractions. These fractions are then isolated for subsequent mass spectrometric identification of the thiol peptide apo-forms [57,60]. In a variation of this approach extracts were firstly screened and fractionated by SEC-ICP-MS and then phytochelatin apo-forms were subsequently identified by RP LC-ESI-MS in *A. thaliana* [39] and in the fungus *Boletus edulis* [61]. Other research groups were using simply SEC-ICP-MS for metal screening and LC-ESI-MS in parallel e.g. for phytochelatin identification in pea (*Pisum sativum*) [40]. The week point of a combination of these analytical strategies is that the link between metals and thiol peptides cannot be proved. However, few studies demonstrated even the observation of entire Cd–PC complexes, e.g. in the cadmium hyperaccumulator *B. chinensis* [38] and in Indian mustard (*Brassica juncea*) [58], applying ESI-MS/MS after SEC fractionation at pH 7.8. Although in both studies synthetic Cd complexes showed clear signals in MS and MS/MS, the data for complexes isolated from plant samples were rather weak. Another example is the investigation of arsenic–thiol peptide complexes in *B. juncea* [58]. Their higher stability allowed the introduction of a second fractionation step by RP LC after SEC for better purification, but only a weak signal for an arsenic–glutathione complex was observed in ESI-MS. Recently, the same group employed successfully two-dimensional chromatography (SEC-ion pairing RP LC) with ICP-MS for detection of Pb–thiol peptide complexes in roots and shoots of *B. juncea* and *Sesuvium portulacastrum* [59]. The simultaneous detection of lead and sulfur in the peak suggested the presence of thiol complexes. Although the complementary identification of the lead complexes by MALDI-TOF-MS failed, an apo-form of $CysPC_2$ was found in both plants.

5.3. Parallel RP LC–ICP-MS/ESI-MS/MS coupling.

A critical issue for accurate characterization of metal–thiol complexes in plants is their instability during the analytical process including extraction, chromatographic fractionation and lyophilization. Blümlein et al. demonstrated that the off-line approach of SEC fraction collection and freeze drying prior to mass spectrometric analysis negatively affected the stability of arsenic–PC complexes [53]. After this procedure only 2% of the total arsenic was still bound to thiol peptides. Therefore Feldmann and co-workers introduced an on-line approach using reversed-phase LC with a splitting of the chromatographic effluent and its parallel introduction into ICP-MS and ESI-MS/MS [41,42,53]. In contrast to Cd–PC complexes it was found that As–PC complexes were stabilized at low pH of a mobile phase containing 0.1% formic acid and thus they were amenable to RP LC separation. During the on-line RP LC-ICP-MS/ESI-MS measurement 83% of arsenic was bound to thiols. Applying this approach several As–thiol complexes such as $As\text{-}PC_{2-4}$ were identified in *Holcus lanatus* [41], *Pteris cretica* [41], *Thunbergia alata* (Fig. 4) [53,62] and sunflower (*Helanthus annuus*) [42,63]. Krupp et al. used the same technique for the identification of mercury–phytochelatin complexes in rice (*Oryza sativa*) and *Marrubium vulgare* [64]. In contrast to previous works applying 200 μM [34] and 1 mM [65] mercury, respectively, in this new study plants were only exposed to a mercury concentration of 50 μM in order to induce Hg-PCs. However, such mercury contamination is probably rare in natural soils.

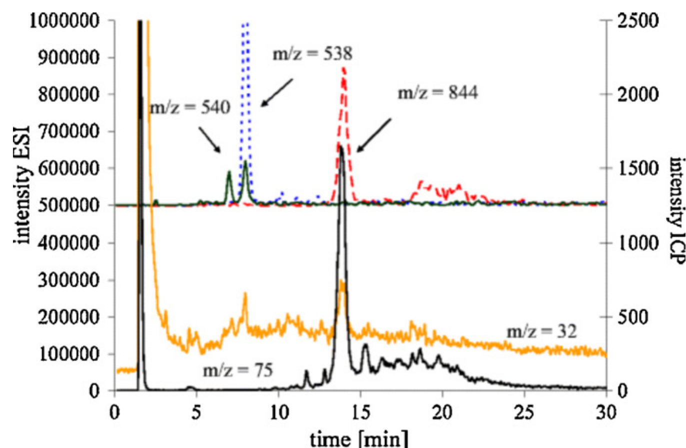

Fig. 4. Detection of PC_2 and $As\text{–}PC_3$ complex in *T. alata* with a parallel RP LC-ICP-MS/ESI-MS coupling. PC_2 is detected in ICP-MS via the sulfur signal at *m/z* 32 and in ESI-MS at *m/z* 540; $As\text{–}PC_3$ at *m/z* 32 (S) and 75 (As) in ICP-MS and at *m/z* 844 in ESI-MS [62].
With kind permission from Springer Science + Business Media: Anal. Bioanal Chem. "Can we trust mass spectrometry for determination of arsenic peptides in plants: comparison of LC-ICP-MS and LC-ES-MS/ICP-MS with XANES/EXAFS in analysis of *Thunbergia alata*", 390, 2008, page 1745, K. Bluemlein, A. Raab, A. A. Meharg, J. M. Charnock, J. Feldmann, Fig. 4, Copyright Springer-Verlag 2007.

6. Thiol peptide quantification strategies based on element detection

Beside thiol peptide identification in plant extracts their accurate quantification is a prerequisite to establish reliable biochemical models. Classic quantification approaches basing on derivatization were extensively reviewed [66,67]. An interesting alternative can be the quantification via element detection in LC-ICP-MS [29]. Possible elements are the sulfur of the cysteine residues in thiol peptides, metals in their complexes with thiol peptides, and metals artificially introduced by derivatization. An advantage of this approach is that calibration can be carried out with a generic element standard and no expensive synthetic peptides are needed as for LC-ESI-MS quantification. However, the varying organic solvent introduction during gradient elution in LC-ICP-MS has an influence on the sensitivity of the regarded metal (gradient effect) which has to be balanced, e.g. by addition of an internal standard [68]. Especially high organic solvent rates in reversed-phase LC and hence high organic solvent load of the plasma decrease the sensitivity.

6.1. Thiol peptide quantification via sulfur detection

Among those elements which can be detected by ICP-MS, sulfur is the common element in all thiol peptides. However, sulfur detection in ICP-MS is not very sensitive due to interferences, high background and low ionization efficiency in the plasma [69]. ICP-MS equipped with a sector field or with a collision/reaction cell were necessary in order to allow the detection PCs in physiological relevant concentrations [62]. Thus, examples for thiol peptides quantification via sulfur detection are rather scarce [53,62].

6.2. Quantification of metal–thiol peptide complexes

Not only the quantification of thiol peptides apo-forms but also quantification of their intact metal complexes is mandatory for an

elucidation of metal homeostasis in plants. Accurate quantification by LC-ICP-MS requires that the complexes are stables during sample preparation and chromatographic separation. Furthermore a high chromatographic resolution is needed for baseline separation of each individual species to be quantified. LC-ICP-MS enables *a priori* only quantification of the metal in the complexes. Therefore their structures and stoichiometrical composition have to be characterized, e.g. by ESI-MS, in order to conclude from the metal concentration to the concentration of the metal–thiol peptide complex.

Up to now only few papers report on quantification of intact metal–thiol peptide complexes. Sadi et al. compared the relative quantities of Cd–PC_2 in roots and shoots of wild-type and transgenic *A. thaliana* based on the Cd signal in LC-ICP-MS [36]. Quantification revealed that the cadmium complex was translocated in the shoots and the genetically modified plant showed a higher accumulation rate. Feldmann and coworkers used the previously described LC-ICP-MS/ESI-MS/MS split coupling for absolute quantification of As–thiol peptide complexes is *H. annuus* [42,63] and *T. alata* [53,62]. This allowed the study of uptake, translocation, transformation and stability of arsenic thiol peptide complexes. The system was calibrated with a single arsenic compound and the gradient effect has been compensated by an internal standard. Parallel ESI-MS/MS identification of the As-complexes gave information on their stoichiometries and thus allowed their quantification via the As signal in ICP-MS.

6.3. Thiol peptide quantification via metal labels

Recently, new biomolecule quantification strategies based on derivatization with metal labels were developed [68]. In this approaches metal-labeled peptides and proteins are quantified with LC-ICP-MS via quantification of the metal attached to the biomolecule by derivatization. The high sensitivity of ICP-MS allows detection limits down to the attomole level depending on the metal used for labeling. However, accurate quantification can only be possible under the condition that (i) derivatization is specific, complete, reproducible, and stable during separation, (ii) the stoichiometry of the metal-labeled biomolecule is known, and (iii) all labeled molecules are baseline-separated in LC.

To date only two papers have been published using this new metal-labeling approach for quantification of phytochelatin apoforms. Bramanti et al. were derivatizing thiol groups in PCs with *p*-hydroxymercurybenzoate leading to stable mercury tagged thiols with Hg–S bonds [70]. Mercury was sensitively and specifically detected by cold vapour generation atomic fluorescence spectrometry and thus Hg-labeled PC_{2-6} could be quantified after calibration with only one mercury standard.

The first report on phytochelatin quantification by LC-ICP-MS after iron labeling was published by Bräutigam et al. [71]. Thiol groups of cadmium induced PCs in *C. reinhardtii* were derivatized with ferrocene for iron specific quantification in ICP-MS. The quite complex chromatogram demonstrated that not only PCs were labeled in the raw alga extract. In order to identify the phytochelatin peaks and to balance the gradient effect of the eluent on the iron sensitivity, labeled phytochelatin standards were used for calibration. This, however, negated the advantage of the approach to use only one generic metal standard for calibration. This study allows a direct comparison of the labeling approach with the UPLC-ESI-MS/MS method because both methods were applied for PC quantification in the same Cd-exposed algal sample [52,71]. Only PC_2, CysPC_2 and CysPC_3 could be quantified by iron labeling and HPLC-ICP-MS with an analysis time of over 90 min. Moreover, only PC_2 quantification was in good agreement with results obtained with the UPLC-MS/MS method. In contrast, the quantities of CysPC_2 and CysPC_3 were much overestimated with the ferrocene approach. The UPLC-MS/MS method showed much simpler sample preparation without derivatization step and was fast, highly molecule-specific and sensitive. It allowed the quantification of six thiol peptides in *C. reinhardtii* in only 7 min which enabled high-throughput analysis for physiological investigations [72]. Furthermore, detection limits of the UPLC-MS/MS were 3 orders of magnitude lower than for detection of ferrocene-derivatized PCs via the iron signal in ICP-MS. The comparison of both approaches demonstrates the advantage of UPLC-MS/MS quantification. In spite of its high potential the metal-labeling approach is still at the developing stage and not yet suitable for routine analysis.

7. Conclusions—the impact of LC-MS approaches on plant biochemistry

Regarding the LC-MS based approaches introduced in recent years the question arises how these developments contributed to scientific progress in plant biochemistry notably to the investigation of thiol peptide regulated metal homeostasis. It is obvious, that the well-established classic approaches are limited to the detection and quantification of apo-forms. Moreover, the accuracy of the result can be negatively influenced by misleading signals in UV–vis and fluorescence detection [31] and by the lack of chromatographic resolution. Only mass spectrometry can unambiguously identify and accurately quantify thiol peptides eluting from a chromatography column. MS enabled furthermore the discovery of a number of iso-phytochelatins and their quantification. This led, for example, recently to new insights in metal homeostasis in *C. reinhardtii* [72]. A new biochemical mechanism was suggested where the synthesis of CysPCs prevents the organism to produce canonic PCs with long chain length.

An important issue which can only be solved by mass spectrometry based approaches is the maximum number n of γ-Glu-Cys units in phytochelatins. Most papers up to now cite in their introduction the work of Zenk and co-workers published between 1986 and 1989 [18,73–77]. These are the only papers which claimed to have found a maximum number of $n = 7$, 8 and 11 based on classical methods such as amino acid analysis and Edman degradation but these experimental data were only presented for PC_{2-5}. Longer PCs were assigned to peaks in LC, e.g. the presence of PC_{7-11} was suggested in *Rauvolfia serpentina* only by comparing retention times with shorter thiol peptides but the longer PCs were not structurally identified [77]. Other early publications on phytochelatins during the same time period reported solely on $n = 2–4$ [78–81]. Back in 1986, Steffens et al. used already tandem mass spectrometry for the identification of phytochelatins in tomato cells (*Lysopersicon esculentum*) [81]. This study reported only on PC_3 and PC_4 but not on longer PCs. Interestingly, all subsequent papers up to now which employed mass spectrometry methods identified mainly PC_{2-5}; few studies propose PC_6 but these data were often weak (for example: [8]). The occurrence of longer phytochelatins was never described any more. Moreover, in a recent study intermolecular oxidation products between PCs were identified by MS/MS, e.g. PC_3–PC_4, which could pretend the detection of longer PCs in LC by classical methods [8]. In consequence, basing on a plenty of new mass spectrometric data published, the chain length of phytochelatins should be revised. Therefore, we suggest the general structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$ ($n = 2–6$) for canonic phytochelatins until new mass spectrometric data will give evidence for longer PC chain lengths.

The global study of the metallo-thiolome demands not only the characterization of the thiol peptide apo-forms but also their native metal complexes. The latter point is still a challenge for analytical chemistry and has only been achieved in few studies including LC-ICP-MS approaches. In many cases the structure and stabil-

ity of intracellular metal–thiol peptide complexes are unknown. Therefore the description of biochemical mechanisms of metal homeostasis including native metal–thiol peptide complexes is still an unsolved problem requiring novel analytical approaches for metallo-thiolomics.

8. Future trends and developments

The future needs for metallo-thiolomics show which analytic-chemical developments will be most important in order to spur on scientific progress in the elucidation of metal homeostasis in seed plants, algae and fungi. Two main areas for future trends can be identified which are interrelated: (i) accurate identification and quantification of metal–thiol peptide complexes in their native forms and in their natural environment, and (ii) description of the metallo-thiolome with high spatial resolution, ideally in individual biological cells. These aims demand for example new separation methods for metal-complexes. New approaches could be based e.g. on hydrophilic interaction liquid chromatography (HILIC) which has already been applied to metabolomic studies [82]. Furthermore, miniaturized couplings are required to handle small sample volumes, e.g. a recently developed nanoHPLC-ICP-MS system which is able to analyse only 10 nL [83]. These systems have to be combined with methods for single cell sampling [84]. However, in future mass spectrometric based approaches will not be the only ones for metallo-thiolomics studies. Synchrotron-based micro-X-ray fluorescence (μSXRF), which uses a X-ray beam with high flux produced by third generation synchrotron and focused down to the submicrometer scale (∼0.9 μm), allows chemical imaging at the cellular level with detection limit in the $\mu g\,L^{-1}$ range [85]. Micro-X-ray absorption techniques (micro-extended X-ray absorption fine structure, μEXAFS, and micro-X-ray absorption near edge structure, μXANES) have the potential to identify main ligands (e.g. sulfhydryl groups) of metals at the cellular level [86]. Highest spatial resolution down to 30–50 nm can be achieved with nano-secondary ion mass spectrometry (nano-SIMS) for imaging of elements in cells [87]. Finally, it can be expected that a combination of these modern analytical techniques will approach further the aim of elucidation of the metallo-thiolome and the thiol peptide regulated metal homeostasis.

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