Biological sulphur-containing compounds – analytical challenges

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Abstract: This review covers analytical methods applied to the determination of none volatile sulphur-containing biological compounds. The classes of S-compounds include amino acids, proteins, lipids, carbohydrates and sulphur-containing metabolites. Techniques covered include element specific detectors as well as molecular specific detectors from X-ray absorption near-edge structure (XANES) to elemental and molecular mass spectrometers. The major techniques used are inductively coupled plasma mass spectrometry (ICP-MS) and high-resolution electrospray mass spectrometry (ESI-MS) in their various forms. Both techniques either individually or combined require the sample to be present in liquid form and therefore involve sample preparation usually extraction and depending on sample and molecular class studied potentially also derivatisation in addition to generally requiring chromatographic separation. Over recent years, detection limits achieved by elemental methods and computational methods to extract signals of sulphur-containing compounds out of the mass of data produced by molecular high-resolution mass spectrometers made significant gains. Still the determination of sulphur-containing compounds is challenging, but nowadays the methods have been developed well enough to allow application to real samples for absolute quantification of biomolecules such as proteins or lipids.

1. Introduction

Sulphur is one of the more abundant elements used in biology. It probably was involved in form of metal-sulphide catalysts during the evolution of life. Its low redox potential and its ability to bind either directly to carbon atoms or via an oxygen-bridge is widely used by nature. Sulphur-containing compounds exist in nature among amino acids, carbohydrates, lipids and secondary metabolites and are involved in major biochemical reactions as illustrated in Figure 1.

Thiolates (HS-C group) can act as metal-ligands, base in enzymatic reactions (protease), in group-transfer reactions (CoA transfer); they form also the base of disulphide bridges and control the redox potential (glutathione). Sulphide ($C-S_2-C$ group) on the other hand acts as redox group (lipoic acid) and metal ligand (Fe_nS_n clusters). Thioester (H_3C-S-C group), like methionine, act as methyl donor, ligand in enzymes and form cross-links (Cyt C). Some coenzymes are also sulphur-containing, like thiamine and biotin. The sulphate-group ($C-O-SO_3^-$ group) and sulphonate-group ($C-SO_3^-$) modify in nature compound behaviour, e.g solubility of lipids.

One would think given its high abundance and wide distribution in biological tissues that the determination (identification and quantification) of sulphur-containing compounds is well developed and relative straight forward, but that is not the case. Identification of sulphur-containing compounds by traditional molecular discovery methods like nuclear magnetic resonance spectroscopy (NMR) and molecular mass spectrometry (MS) can be as difficult as the quantification of sulphur in organic compounds by elemental mass spectrometry (ICP).

In this review, we try to show where in our opinion the challenges in the determination of sulphur-containing biological compounds are from the point of view of an elemental analytical chemist. Part one of the review consists of a short description of sulphur-containing compounds as examples of the wide variety encountered in living cells. Part two covers analytical techniques and analytical challenges when analysing sulphur-containing compounds.
Due to the wide variety and the use of different analytical methods, we do not cover volatile sulphur-containing compounds and their detection and quantification methods.

2. Classes of sulphur-containing compounds

This part gives a short summary of sulphur-containing compounds present in biological matrices in addition to the ubiquitous sulphate ions. The major principal structures in which sulphur can occur in nature are summarised in Table 1. Sulphur is essential to life in all its forms. It is involved in its variable molecular species in among others all major redox-processes, synthesis of proteins, carbohydrates, secondary metabolites (Figure 1) with bacteria forming the basis of the biological sulphur cycle.

2.1. Amino acids, peptides and proteins

The main sulphur-containing amino acids methionine and cysteine are present in most proteins [1]. Methionine itself is essential for human beings, whereas cysteine can be synthesised in a five-step process from methionine. Cysteine is redox active and often binds thiophilic metal-ions in the catalytic centre of proteins. It is also often involved in stabilising the three-dimensional structure of proteins by formation of disulphide bridges [2]. Oxidation of cysteine can lead to sulfenic, sulfinic and sulfonic acid, especially the oxidation to sulfenic acid is an important reversible post-translational protein modification [3,4]. Methionine in contrast is mostly the start codon for protein synthesis in addition to being part of the sequence. A post-translational protein modification is the *in vivo* oxidation of specific methionine residues forming methionine sulfoxide [5]. For more details of the sulphur-containing amino acids see [6].
Human serum albumin, the most abundant protein in blood serum, for example contains in addition to the initiator methionine seven methionines in the sequence and 35 cysteine residues per molecule 34 of them forming intra-molecular disulphide bridges [7]. A tryptic digest of excreted reduced human serum albumin without miss-cleavage contains 22 different sulphur-containing peptides.
One of the dominant sulphur-containing peptides in eukaryotic cells is glutathione (GSH) a tripeptide containing an unusual γ-glutamyl-bond and cysteine. It is important among others for the homoeostatic maintenance of the redox potential in cells and can bind to thiophilic metals (like arsenic [8] and mercury [9]) and xenobiotics [10,11]. The related phytochelatins in plants [12,13,14], nematodes [15] and worms [16] are, in contrast, mostly produced in response to metal-induced stress.
A non-proteogenic sulphur-containing amino acid is taurine. It is synthesised from cysteine or methionine by humans, but not by all mammals. Taurine contributes to the osmolyte pool and is involved in several other physiological processes like nerve cell development and it can be incorporated into lipids (structure of an example taurolipid see Figure 2 B3) [17,18].

2.2. Sulphur-containing carbohydrates

There are very few sulphur-containing carbohydrates *per se* known (examples see Figure 2 A1 and A2). The major ones are the carrageenans, which are linear sulphated polysaccharides

95 in red seaweed important in the food and pharmaceutical industry [19,20]. They are a class of
96 polysaccharides containing galactose, with the main difference between them being the
97 position and number of sulphate-group(s) and the presence or absence of 3,6
98 anhydrogalactose [20,21**Error! Bookmark not defined.**]. They contain between 22 and 35
99 w% sulphate groups, which have a strong influence on solubility and gelling properties [20].
100 Secondary metabolites and glycolipids contain also sulphur-containing carbohydrates.
101 Predominant among the lipids are the sulfoquinovones (as in Figure 2 A2 showing the
102 carbohydrate moiety, see next paragraph) [22].
103

104 **2.3. Sulphur-containing lipophilic compounds**

105 Living cells contain a wide variety of sulphur-containing lipids. They predominantly contain
106 a sulphate or a sulfono-group (SO_4^- or SO_3^--group) bound to the sugar-moiety of a glycolipid
107 and are highly anionic. An important member of this family is sulfoquinovosyl diacylglycerol
108 (SQDG, 6-deoxy-6-sulfo-glucose), which is part of photosystem II and the cytochrome b6f
109 complex [22]. It occurs in photosynthetic organisms and some bacteria [22]. In them sulphur
110 is directly bound to carbon as C-SO_3^- (as in Figure 2 A2). Environmental conditions influence
111 SQDG levels in plants [23].
112 Another member of the sulphonate-containing lipids are the sulphatides (example structure
113 Figure 2 B1), which are sphingolipids important among others in the myelin sheath of nerve
114 cells (4-7 % of the lipids present) and in the brain [24,25]. Members of this family are
115 widespread in tissues and play important roles in a variety of biological processes (nervous
116 system, immune system, haemostasis, thrombosis, kidneys) [26]. In them, a sulphonate-group
117 is bound to a galactose moiety [26]. The other major sulfoglycolipid family are the
118 seminolipids containing the same galactose moiety (example structure Figure 2 B2) [24].
119 Other sulphur-containing lipids include taurine-containing lipids (a fatty acid conjugated to
120 taurine, Figure 2 B3) and present in animal tissue especially in brain. Some fatty acids
121 containing a sulphate-group, for example caeliferin found in grasshopper, are known to
122 influence plant response to grazing [27]. In some bacteria and marine invertebrates, a number
123 of unusual sulphur-containing fatty acids are present, containing two sulphate groups and a
124 variable number of chloride atoms with unknown biological function [28].
125

126 **2.4. Secondary metabolites**

127 Sulphur-containing secondary metabolites are widespread and occur in a variety of forms.
128 They are derived either from one of the sulphur-containing amino acids (mostly cysteine) or
129 contain a sulphate, sulphonate-group or a thiol-ring containing structure. Plants especially
130 produce a whole range of sulphur-containing metabolites, which often have defensive
131 properties. Members of the *Allium* genus for example produce an abundance of compounds
132 related to alliin [29]. These compounds are the main taste producing compounds in *Alliums*
133 whether it is alliin for garlic or iso-alliin for onion (example structure see Figure 2 C2) [30].
134 In these compounds sulphur is bound directly to carbon and forms a disulphide bridge with
135 another carbon-bound sulphur, either sulphur atom in these structures can be oxidised or
136 dioxidised. Alliin, its precursors and their relatives are thought to have, among others,
137 antifungal properties helpful for plant survival [29].
138 Members of the *Capparales* order (among others Broccoli), in contrast, produce
139 predominantly glucosinolates (example structure Figure 2 C1), which are responsible for their
140 specific smell when cut and the bitter taste [31,34]. These are molecules containing beside a
141 sulphate-group a thioglucose group and variable side-chains of an amino acid, to date more
142 than 200 members of this class are known [32]. Specific enzymes in plant cells, when
143 released, transform these compounds into (iso)-thiocyanate [33,34]. These molecules are also

144 thought to be part of the plant's defence mechanism [34]. Ingested in small amounts these
145 compounds may contribute to the health promoting properties of *Bassica* vegetables [33,35].
146 An essential sulphur-containing metabolite for mammals is thiamine (Vitamin B1, Figure 2
147 C4). Phosphorylated thiamine is a co-factor for several enzymes in the energy-metabolism
148 and carbon-metabolism [36]. In this case, sulphur is bound in a thiazole ring.
149 There is a wide variety of other sulphur-containing secondary metabolites known. Among
150 others, a diterpenoid (serofendic acid, structure see Figure 2 C3) isolated from foetal calf
151 serum with neuroprotective activity [37].
152

153 ## 3. Techniques for isolation, separation, identification and quantification
154 ## of sulphur-containing compounds

155 ### 3.1. Sample preparation
156 One of the major considerations during sample preparation is the stability of sulphur-
157 containing compound(s). Most sulphur-containing compounds are not only redox-sensitive,
158 but can also form artificial polymers or breakdown products [38].
159 Especially redox-sensitive are all thiol-containing compounds. Rao et al. [39] studied the
160 stability of some none derivatised thiolic and non-thiolic species in serum over time using
161 ESI-MS. Their results showed that thiol-containing compounds are unstable over time and
162 derivatisation is required for stabilisation. A large variety of reagents is available for this step
163 with the choice depending on the subsequent detection method. When detection by UV or
164 fluorescence spectrometry is required, UV or fluorescence active compounds are used. For
165 derivatisation of thiols determined by molecular mass spectrometry, normally small agents
166 like iodoacetamide (preferred in proteomics) or iodoacetic acid are used. Mester et al. [39]
167 compared a number of derivatisation agents and considered iodoacetic acid is the suitable
168 reagent for derivatisation of reduced thiol-groups using dithiothreitol (DTT) as reductant for
169 oxidised thiols.
170 Important in this step is the quantitative derivatisation of the thiol-groups, since incomplete
171 derivatisation results in additional (undesirable) detector signals, which may or may not have
172 different retention times during high-performance liquid chromatography (HPLC) separation.
173 Derivatisation is also used to introduce an additional elemental tag, like arsenic [40], onto a
174 thiol-group to improve quantification by ICP-MS. Tagging with selenium-containing
175 compounds, which improves identification of the compound(s) by molecular mass
176 spectrometry due to its specific isotope pattern can also be used [41]. Another possibility is
177 the introduction of ICAT (isotope coded affinity tag, eg. biotin coupled to iodoacetic acid) or
178 MeCAT (containing a metal complexed to a thiol-reactive group like iodoacetic acid) [42]
179 onto cysteine residues, which can introduce enriched stable metal isotopes to allow relative
180 quantification by ESI-MS or absolute quantification by ICP-MS for elemental tags suitable
181 for peptide quantification. Hansen et al. [43] wrote an authorative review of methods for the
182 derivatisation of thiols and Klencsar et al. [44] summarised suitable functional groups for
183 detection by ICP-MS.
184 Reduced thiol-containing compounds can be enriched using affinity chromatography.
185 Different affinity resins have been developed over the years based on covalent-binding and
186 the formation of a disulphide-bond (eg thiopropyl sepharose) [45], on gold nanoparticles [46],
187 arsenic [47,48],mercury-compounds [49] and others [50,51].
188 The study of secondary metabolites, like alliin-derivatives and glucosides, requires often the
189 deactivation of specific cellular enzymes during extraction to allow the determination of the
190 intracellular compound(s), since these often react with enzymes upon cell damage to other
191 compounds used in cellular defence. Depending on the enzyme in question, these reactions

192 can be avoided using acidic extraction conditions, specific enzyme inhibitors or boiling of the
193 sample before homogenisation and extraction [31,38,52,53].
194 Sulphur-containing lipids can be extracted using standard lipid extraction methods, the
195 difficulties start when other non-sulphur containing lipids have to be removed before
196 analysis. The main extraction protocols are the procedures by Blight, Dyer and by Folch [54].
197 Since sulphur-containing lipids are more polar than tri-glycerides, they extract mostly
198 together with the polar phospholipids. For enrichment of sulfo-lipids sample clean-up by SPE
199 can be useful as this allows the separation into different lipid classes simplifying detailed
200 analysis [54].

201 **3.2. Purity of reagents**

202 Important for the determination of sulphur-containing molecules are considerations about the
203 purity of all chemicals used during sample preparation and analysis. The sulphur content is
204 normally not evaluated in e.g. derivatisation chemicals. The manufacturer does generally not
205 determine sulphur-content of solvents and the use of HPLC-MS grade solvents for example
206 does not guarantee low sulphur-background. As experience in our laboratory shows, the
207 sulphur content can vary significantly from batch to batch. The same possible source of
208 contamination can come from the use of MilliQ water, which can contain significant amounts
209 of some sulphur-compound(s). Before use, it is therefore advisable to check solvents and
210 other reagents for their sulphur-content, when using ICP-MS as detector. Another potential
211 contamination source can be the high-purity gases (predominantly argon and oxygen) used
212 during ICP-MS analysis.

213 **3.3. Separation**

214 Reversed-phase HPLC separates most sulphur-containing compounds, separation of different
215 lipid classes can be done using normal-phase HPLC, other separation modes like hydrophilic
216 interaction liquid chromatography (HILIC) are suited for more polar compounds and anion or
217 cation exchange chromatography are useful for specific ionic target molecules. Separation
218 does not present a problem *per se* for sulphur-containing compounds.
219 Normal-phase HPLC is mostly used in complex lipid analysis. The problem with this
220 separation method is beside its relative low separation power, the difficulties in combining it
221 with either elemental or molecular mass spectrometers for identification and quantification of
222 the eluting compounds. For example, the eluents are not particularly well suited for ESI-MS.
223 The compounds are difficult to ionise, due to their low polarity and therefore require
224 atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization
225 (APPI) for ionisation.
226 HILIC, which separates polar lipids [55] and other compounds [56] well, is a better choice,
227 especially using acetonitrile/water as eluent is well suited for ESI-MS. Combining HILIC
228 with ICP-MS detection can be problematic for plasma stability due to very high amount of
229 organic solvent required for HILIC. The columns also generally show a better peak capacity
230 and therefore separation power than normal-phase columns.
231 The major separation technique used for sulphur-containing compounds in biological samples
232 is reversed-phase chromatography using C18-columns. This technique not only separates
233 small sulphur-containing molecules, like alliin and GSH, but is also suitable for larger
234 peptides after tryptic digest of proteins. Using C4-columns the technique is applicable to
235 small to medium sized proteins [104]. Reversed-phase chromatography is perfectly suited for
236 direct combination with molecular mass spectrometers and can be connected to elemental
237 mass spectrometers without undue difficulties using a torch with a small inner diameter
238 injector and platinum sampler and skimmer cone as well as adding oxygen to the plasma gas
239 to prevent carbon built-up on the sampler cone..

240 Small ionic sulphur-containing compounds, like taurine, have been successfully separated
241 using anion exchange columns or mixed mode-columns from other amino acids [57]. Taurine
242 and sulphate in urine and serum were quantified by ICP-MS after separation with an anion
243 exchange column [58]. For analytes such as these, the relative low separation power of a
244 normal ion exchange column is sufficient. Ion exchange columns can directly be coupled to
245 conventional ICP-AES or MS as elemental detector, but can create difficulties due to the
246 required use of (often non-volatile) buffers such as phosphate for molecular mass
247 spectrometry. For ion exchange chromatography to be coupled to ESI-MS the separation
248 methods have to be modified to use more volatile buffers such as formic acid or ammonium
249 carbonate.
250 Over recent years, significant developments took place with regard to column material; The
251 particle size of the packing material for most chromatographic columns decreased resulting in
252 improved separation efficiency. Additional packing materials especially in reversed-phase
253 chromatography were introduced among these are the core-shell materials or non-porous
254 column materials. These developments improved the separation efficiency of modern
255 columns significantly. But is it good enough for complex biological samples? The difficulties
256 start when all the sulphur-containing compounds have to be baseline separated, since
257 biological samples often contain large numbers of structurally similar compounds. Shallots
258 for example (Figure 3a-c) contains at least 44 different sulphur-containing compounds with a
259 concentration range of four orders of magnitude (some of which are shown in Figure 3b and
260 c). A tryptic digest of pure albumin contains 22 sulphur-containing peptides without
261 considering potential protein modifications. For detection by molecular mass spectrometry,
262 baseline separation of the compounds may not be mandatory for quantification, but it is
263 essential for absolute quantification of any given compound via ICP-MS [59]. Therefore, the
264 columns used for separation have to be high-resolution columns. In some instances, the
265 combination of reducing agent for disulphides and the derivatisation agent used can influence
266 the peak shape [39]. For methionine-containing peptides, the possibility of multiple
267 compounds occurring has to be considered, since oxidation of methionine (producing two
268 stereoisomers) can result in chromatographically resolved peaks depending on amino acid-
269 sequence and column [60]. Alternatively, 2D-chromatography either on-line or off-line by
270 collecting fractions can simplify the samples to a point where base-line separation is possible.

271 ### 3.4. Detection

272 ***UV or fluorescence - probes***
273 Sulphur-containing groups are not *per se* UV active, but depending on the structure, sulphur-
274 containing compounds are directly detectable by UV-absorption spectrometry. For example
275 quantitative identification without derivatisation of sulphur-compounds in allium species can
276 be achieved at wavelength between 205 and 254 nm [61-65].
277 Derivatisation with thiol-specific probes, either for improved detection by UV or for
278 detection by fluorescence spectrometry is also widely used. Ellmann' reagent (5,5′-Dithiobis-
279 2-nitrobenzoe acid, DTNB), monobrombimane [66,67] and a range of other reagents were
280 developed to specifically label reduced thiol-groups [68]. Depending on the fluorophore the
281 distribution and quantity of reduced thiols in live cells can be studied using confocal
282 microscopy with cell-wall penetrating agents and therefore the influence of environmental
283 factors on the reductive cell environment [69,70]. All compounds used for labelling form
284 thioether bonds with free thiol-groups. Excitation and emission wavelengths depend on the
285 fluorophore; some labelling compounds can be compound-specific [70].
286 Quantification of thiols by UV and fluorescence spectrometry requires species-specific
287 standards and compound(s) to be baseline separated.
288

289 ***Molecular mass spectrometry***
290 Molecular mass spectrometry, especially with ESI, APCI, APPI or matrix assisted laser
291 desorption ionisation (MALDI), allows the determination of the molecular mass and when
292 using high-resolution instruments the molecular composition of organic compounds can be
293 deduced. For the calculation of the molecular composition of sulphur-containing compounds
294 the small mass defect of sulphur isotopes ($\Delta m^{32}S$ = -0.0279) and the isotopic pattern of
295 sulphur distinct from that of carbon, nitrogen and hydrogen are important and can be used in
296 algorithms to identify sulphur-containing compounds.
297
298 The major distinction is the ^{32}S to ^{34}S ratio (95.0 to 4.2 %), whereas the major carbon,
299 nitrogen and hydrogen isotopes are spaced one *m/z* apart. Nevertheless, it can be difficult to
300 identify sulphur-containing compounds based on their isotopic pattern alone. As can be seen
301 in Figure 4.A for a low molecular mass sulphur-containing compound the difference between
302 the theoretical sulphur-containing pattern and a non-sulphur-containing pattern is relative
303 large (Figure 4.A). For a high-molecular mass compound, the difference in the isotopic
304 pattern between a sulphur-containing and a non-sulphur-containing compound is significantly
305 smaller (Figure 4.B). Highly accurate isotope patterns as for example achievable using
306 Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) are required to
307 identify sulphur-containing compounds confidently by mass spectrometry alone [71,72].
308 Most programs used for data analysis allow the search for specific isotope clusters (taking
309 into account isotope spacing and intensity). For elements like chlorine and bromine with their
310 very distinct isotopic pattern, this method works very successfully for the identification of
311 unknown compounds. For chlorine the abundance distribution between ^{35}Cl and ^{37}Cl is 75.8
312 to 24.2 %, in contrast the abundance distribution of sulphur is 95.0 to 4.2 %. To improve this
313 Andersen et al. [73] recently developed a program (Dynamic Cluster Analysis, DCA) which
314 improves the applicability of isotopic pattern analysis in the search for unknown sulphur-
315 compounds. The method uses A+1 and A+2 isotope cluster spacing of high-resolution / high
316 mass accuracy spectra to evaluate the spectrum automatically for sulphur-containing
317 candidates. They checked their A+1 and A+2 algorithm among others against the Marinlit
318 natural products database. Results indicate that the majority of sulphur-containing
319 compounds below a molecular mass of 800 can be identified with higher confidence than
320 when using the A and A+2 algorithm of other programs [73]. No confident identification of
321 sulphur-containing compounds larger than that is possible by molecular mass spectrometry
322 due to the low abundance of ^{34}S compared to ^{32}S (4.2 % compared to 95.0 %).
323 Another option to improve identification of reactive thiol-containing compounds is the group-
324 specific derivatisation of these compounds allowing the use of tandem mass spectrometry for
325 the detection of specific fragments as used e.g. by Liem-Nguyen et al. for the detection and
326 quantification of thiol-containing compounds in natural waters [74].
327 Cysteine (Cys) and especially the intramolecular Cys-Cys bridges play an important part in
328 the 3-D structure of proteins. The position of Cys-Cys bridges can be studied using partial
329 reduction and alkylating the Cys differently at each step, depending on labelling conditions
330 the location of the disulphide bridge (surface / interior of protein) can be estimated [75-79].
331 The general matrix sensitivity of HPLC-MS applies also to the detection and quantification of
332 sulphur-containing compounds. An overview and descriptions to overcome the problem can
333 be found in Trufelli et al. [80] and Chambers et al. [81]. Signal intensity in molecular mass
334 spectrometry is compound and matrix dependent, absolute quantification therefore requires
335 species-specific standards (often isotopically labelled) preferably combined with the standard
336 addition method. Relative quantification (comparing two or more different biological states,
337 cell treatments for example) is often used in proteomics using thiol-reactive tags enriched in
338 deuterium (ICAT) or MeCAT [82,83]. Another option for quantification is the use of species-

339 specific isotope dilution mass spectrometry (SS-IDMS). For this approach, the compound is
340 synthesised using enriched isotopes (^{13}C, ^{2}H, ^{34}S or others) and a known amount spiked to the
341 sample before any sample treatment. The changing intensities of the isotope pattern are then
342 used for quantification [11]. In Table 2 some detection limits for specific metabolites using
343 HPLC-MS are shown.
344
345 Another feature of sulphur-containing compounds is the potential for in-source oxidation in
346 MS, which can hamper quantification when it is non-reproducible. In-source reactions can
347 vary depending on mass spectrometer and source-settings. In-source oxidation at methionine
348 needs to be considered when using MS as quantitative detector for peptides, but when using
349 relative quantification like ICAT this may be of no consequence since different samples can
350 be assumed to be similarly affected.
351

352 ***Elemental Detectors***

353 Elemental detectors used for the determination of sulphur-containing compounds are
354 predominantly inductively coupled argon-plasmas (ICP) either coupled directly to optical
355 emission spectrometer (OES) or mass spectrometers (MS).
356 Conductivity detectors, gravimetric or volumetric methods [84] are only suitable for very
357 specific sulphur compounds like sulphate or sulphide. X-ray fluorescence or X-ray absorption
358 spectroscopy (XANES and EXAFS) are able to identify the oxidation state (XANES) and
359 bond length to the near neighbours (EXAFS), but are not suitable for coupling with
360 separation methods and require access to specific accelerator beamlines at synchrotron
361 centres. The advantage of these techniques is the possibility to measure samples "in situ"
362 without any sample preparation, thereby avoiding artefact formation [85]. Another advantage
363 is the possibility to create high-resolution two or three -dimensional maps of tissues with
364 information of oxidation state and nearest atomic neighbours [86].Distinction between
365 different sulphur-containing compounds is however difficult, when oxidation state and
366 nearest neighbours are the same and minor compounds cannot be identified [87].
367
368 ICP with argon plasma is for most elements a highly efficient excitation and ionisation
369 source. Sulphur with is high ionisation potential (10.357 eV) compared to metallic elements
370 has a significantly lower ionisation efficiency. Nevertheless, it is possible to use ICP coupled
371 to either an OES or a MS as sulphur detector. Both detectors allow compound independent
372 quantification of sulphur since in both cases the actual compound present does not influence
373 signal intensity, which is solely depending on the amount of element (sulphur in this case)
374 present. This allows compound independent quantification (when the stoichiometry of the
375 compound is known) using any known stable compound as standard without having the
376 actual compound(s) present in the sample available as calibrants [88]. However, when
377 coupling HPLC to an ICP-detector the eluent composition can influence signal intensity. This
378 is especially important under gradient conditions using organic solvents, as methanol and
379 acetonitrile, for the separation of sulphur-containing compounds create variable plasma
380 conditions during the gradient due to the eluent dependent carbon enhancement effect
381 [59,85,89]. One option to compensate for the solvent effect is to mathematically correct it
382 [90], another is the use of isotope dilution analysis (IDA) when using ICP-MS [59,89] or the
383 introduction of a "counter-gradient" post-column [91-93]. Recently a new method requiring
384 an additional mass flow controller for compensation of gradient effects using methane or
385 carbon dioxide addition to the plasma [94] was introduced. The different methods were so
386 far, not directly compared therefore the merits of one method over to others are not known.
387 For the time being, the use of a specific method will depend mostly on the technical

feasibility in the laboratory of the user. All these gradient compensation techniques require manual application as so far none of the ICP-MS data analysis programs can automatically apply any of them.

Sulphur-containing compounds can be detected by ICP-OES at 180.676 nm (the most intense emission line) requiring a spectrometer capable to measure in the UV-range (usually achieved by flushing the spectrometer with additional nitrogen to remove interfering gases absorbing in the UV).
More sensitive and therefore nowadays preferred is the ICP-MS. Determination of sulphur by ICP-MS was for many years limited to either determination of $^{34}S^+$ with single quadrupole instruments or the use of high-resolution instruments due to the strong molecular interference of $^{16}O^{16}O^+$ on the main sulphur isotope of *m/z* 32.
High-resolution sector field instruments are able to resolve the molecular interferences of oxygen, nitrogen and hydrides (for example $^{16}O^{16}O^+$ on $^{32}S^+$). These instruments together with their multi-collector relatives allow the determination of different sulphur isotopes on mass and in the case of multi-collector instruments simultaneously with high precision [95]. Until about 2000 they were the instruments of choice for low level sulphur determination. Significant improvements of quadrupole instruments (ICP-qMS), after the introduction of reaction / collision cell technology, allowed the low-level determination of sulphur using these more widespread and technically simpler instruments. Mason et al. were one of the first to study the effect of different reaction / collision gases on the determination of sulphur and sulphur isotope ratios by ICP-qMS [96]. The use of xenon as collision gas allowed accurate determination of $^{32}S/^{34}S$ ratios [96]. Bluemlein et al. [85] compared the use of xenon as collision gas with oxygen as reaction gas (formation of $^{32}S^{16}O^+$) in an ICP-qMS with the use of a high resolution ICP-MS as sulphur detector for HPLC. Triple-quad ICP-MS (ICP-MS/MS), introduced in 2012, very quickly proved an excellent instrument choice for low-level sulphur determination [97,98]. Using ICP-MS/MS interferences are efficiently removed from the sulphur-signal using oxygen as reaction gas, by isolating the individual sulphur isotopes before reaction (eg. removal of $^{48}Ti^+$ from $^{32}S^{16}O^+$). A tutorial review about ICP-MS/MS can be found here [99]. A detailed review about sulphur determination by ICP-MS is available from Giner Martínez-Sierra et al. [100].
Detection limits for sulphur given in the literature vary greatly (Table 2). The main reason for these highly variable detection limits beside instrument type is the problem of sulphur-background in the gases and solvents used as mentioned above. One normally does not think that argon, argon/oxygen mixtures or oxygen used in ICP-MS may be contaminated with sulphur-compounds, but experience in our laboratory shows that especially with argon/oxygen mixtures, used when organic solvents are introduced into the ICP-MS, the sulphur background can be considerable due to the presence of SO_2, H_2S or other volatile sulphur-containing compound(s).
One advantage of ICP-MS, so far rarely used for sulphur, is the possibility to use stable isotopes not only for quantification of compound(s) either by species unspecific (IDMS) or species specific spiking (SS-IDMS), but to use enriched stable isotopes to quantitatively trace metabolic changes in cells over time [101].

Combined molecular and elemental mass-spectrometry

Both molecular and elemental mass spectrometers are not ideal for the identification and quantification of sulphur-containing compounds on their own. Either absolute quantification or identification is difficult. Combining both detectors off-line or on-line with HPLC

437 therefore is advantageous, especially for biological samples with their large number of
438 sulphur-compounds present. This allows the identification of the compound(s) and absolute
439 quantification using a mass balance approach. Identification of the compound is important
440 when the amount of compound and not the amount of sulphur has to be calculated from the
441 sulphur signal of the ICP-MS. Feldmann et al. recently published a review on the dual
442 detection system (ESI-MS + ICP-MS) for non-targeted analysis, including sulphur-containing
443 compounds [102].
444 Depending on the stability of the sulphur-containing compounds and the stability
445 (reproducibility) of the separation separate ICP-MS measurements for quantification and
446 molecular mass spectrometric measurements for identification can be successful. Advisable
447 for the mostly reactive sulphur-containing compounds is the on-line combination of both
448 detectors. This way any changes in the sample in the time between the determination of the
449 compounds with one detector and the other can be excluded as shown so clearly by Bluemein
450 et al. for arsenic-phytochelatin complexes [103]. The disadvantage of the parallel use of both
451 detectors is that nano-flow columns cannot be used and the instruments have to be physically
452 near to each other (a requirement not always easy to satisfy).
453 Prerequisites for the parallel quantification and identification (be it simultaneously or
454 separate) are separation conditions acceptable to both detectors, excluding the use of non-
455 volatile buffers (ESI-MS) and restricting, to some extent, the use of organic solvents (ICP-
456 MS). The use of a flow splitter is required for simultaneous determination by ICP-MS and
457 ESI-MS, preferably one developing some back-pressure to keep the flow rates to both
458 detectors stable over a gradient, but it can also be simple T-piece with different length and
459 inner diameter tubing. When the system is regularly put to different uses, the use of a sample
460 containing one known substance (or a few) giving good signals in both detectors is advisable
461 to determine the time-off set between the detectors. Last but not least, it is helpful for the
462 identification of compounds, when the data from the ICP-MS can be read by the program
463 used for data analysis of the ESI-MS or *vice versa*.
464 There are a number of publications where ICP-MS is used for quantification of sulphur-
465 containing compounds and the identification of the compounds is done off-line by ESI-MS or
466 MALDI-MS. For example off-line combination of elemental and molecular mass
467 spectrometry was successfully used for protein [104], peptides from tryptic digests [105] and
468 naturally occurring peptides (hepcidin) [106] with quantification via ICP-MS and
469 identification via ESI-MS. The same process was applied for the identification of drug
470 impurities and drug metabolites [107,108].
471 Fernández-Iglesias et al. [109] estimated the amount of protein bound to gold nano-particles
472 as the amount of sulphur relative to number and size of the gold nano-particles and combined
473 this approach with off-line gel electrophoresis for protein separation with protein
474 identification by ESI-MS after tryptic digest.
475 True parallel use of elemental and molecular mass spectrometry is so far rarely reported for
476 sulphur-containing compounds. Raab et al. reported the on-line combination of molecular and
477 elemental mass-spectrometry with a mass-balance approach for the quantification of sulphur-
478 containing compounds in garlic extract [38] and Bluemlein et al. [103] described the
479 quantification and identification of arsenic-phytochelatin complexes. Wesenberg et al. [110]
480 summarised the different techniques and combinations thereof for sulphur-containing
481 peptides, see also [14,111].
482 As this list shows quantification of sulphur by elemental detectors combined with
483 identification of the compound(s) by molecular mass spectrometry is a very useful
484 experimental approach for a wide variety of sample types and research questions.
485

4. Outlook

Sulphur is widespread in biological compounds. Over recent years, the analysis of these biologically important compounds made great strife. Improved or newly developed analytical methods permit nowadays the sensitive detection and quantification of sulphur-containing compounds in complex biological matrices. The application of these methods will allow more details about the complex biological sulphur-cycle to be discovered. Especially the combination of elemental and molecular mass spectrometry coupled with better-quality separation techniques will improve the understanding of the influence of the sulphur metabolism on health and disease. The major difficulties still to overcome are achieving reproducible low detection limits for elemental detection of sulphur and better peak capacity of HPLC columns to achieve enhanced separation of sulphur-containing compounds especially in complex protein or peptide mixtures.

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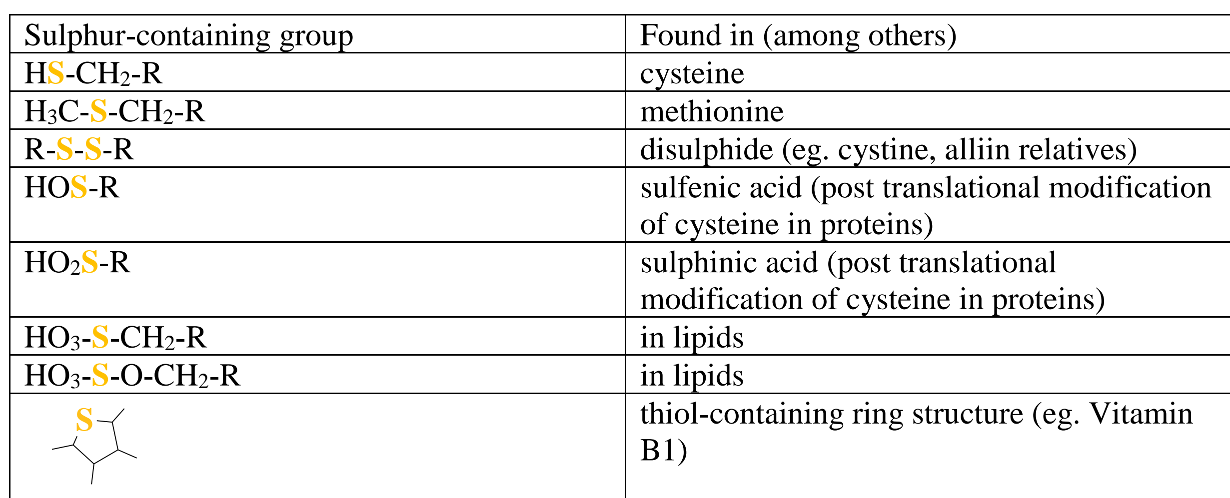
Table 1: principal structure of sulphur containing groups and their occurrence



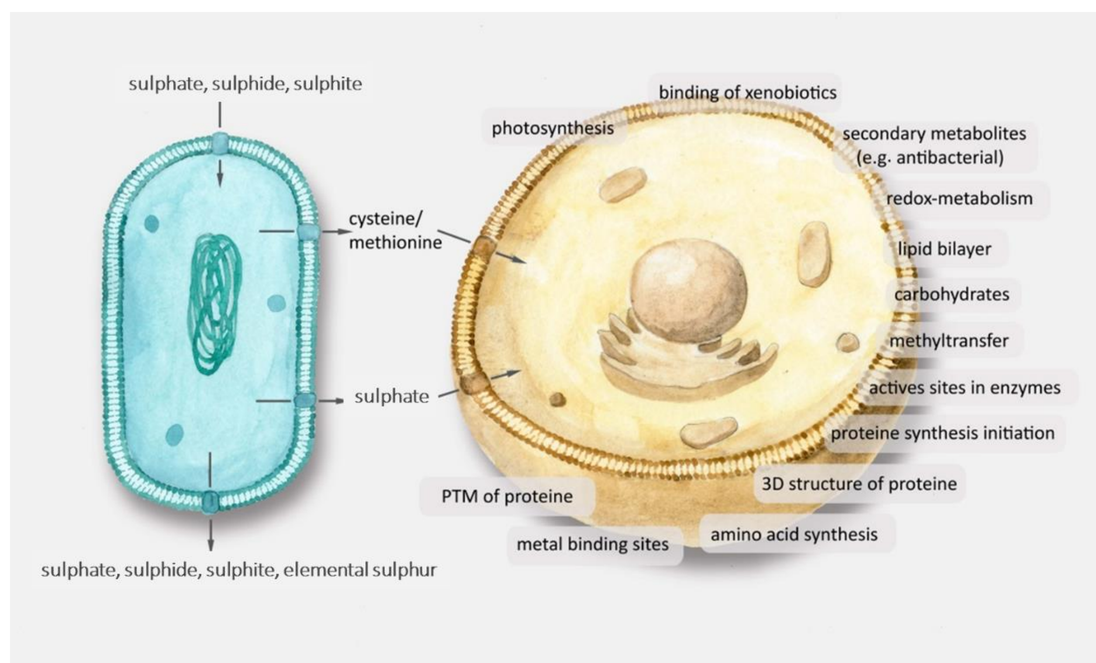
Sulphur-containing group	Found in (among others)
HS-CH_2-R	cysteine
H_3C-S-CH_2-R	methionine
R-S-S-R	disulphide (eg. cystine, alliin relatives)
HOS-R	sulfenic acid (post translational modification of cysteine in proteins)
HO_2S-R	sulphinic acid (post translational modification of cysteine in proteins)
HO_3-S-CH_2-R	in lipids
HO_3-S-O-CH_2-R	in lipids
S	thiol-containing ring structure (eg. Vitamin B1)

Table 1: Overview of limits of detection (l.o.d) for different analytical methods as cited in the literature

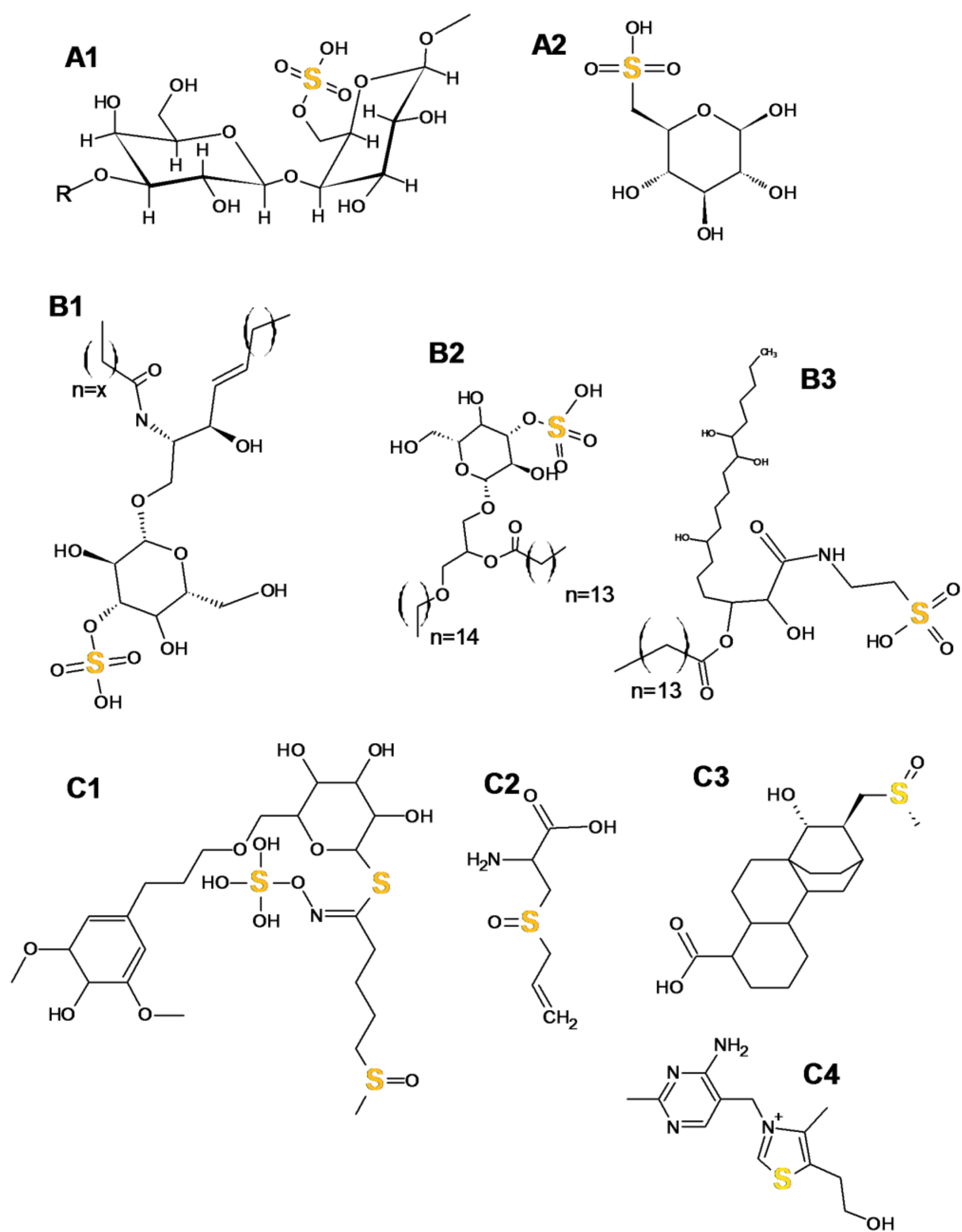
Instrument type	compound	l.o.d.
HPLC + High-resolution ICP-MS	sulphur	0.15 [85] to 5.46 [112] µM
High-resolution ICP-MS + membrane desolvatisation unit (total S)	sulphur	0.3 nM [113]
ICP-qMS	sulphur	3.11 mM (as ^{34}S) [85]
ICP-qMS + xenon	sulphur	0.62 [96] – 2.46 [85] µM
ICP-qMS tuned to high oxide	sulphur	0.41 [114] and 8.42 [115]

rates ($^{32}S^+$ -> $^{32}S^{16}O^+$)		µM
ICP-qMS + oxygen ($^{32}S^+$ -> $^{32}S^{16}O^+$)	sulphur	0.0062 [116] and 2.68 [85] µM
ICP-MS/MS ($^{32}S^+$ -> $^{32}S^{16}O^+$)	sulphur	0.094 – 0.19 µM [97]
ICP-MS/MS ($^{32}S^+$ -> $^{32}S^{16}O^+$)	sulphur	0.2 pM [117]
Preconcentration + ESI-MS/MS (SRM)	Small thiols in water	0.06 – 0.5 nM [74]
Fluorescence probe (imaging live cells)	thiols	0.35 to 2.3 µM [118]
ESI-MS/MS (MRM)	Various glucosinates	1-400 µg compound/L [11]
ESI-MS/MS (MRM)	Various glucosinates	30-360 µg compound /kg fresh plant material [52]
ESI-MS	Various glucosinates	10 – 50 µg compound /kg dry plant material [53]
ESI-MS/MS (SRM)	Various glucosinate metabolites in blood plasma / urine	0.03 to 1.1 µg/L [120]
HPLC with fluorescence detection (monobromobimane)	thiols	1.2 nM of SH [121]
HPLC with fluorescence detection (DTNB)	thiols	1.2 µM of SH [121]

511

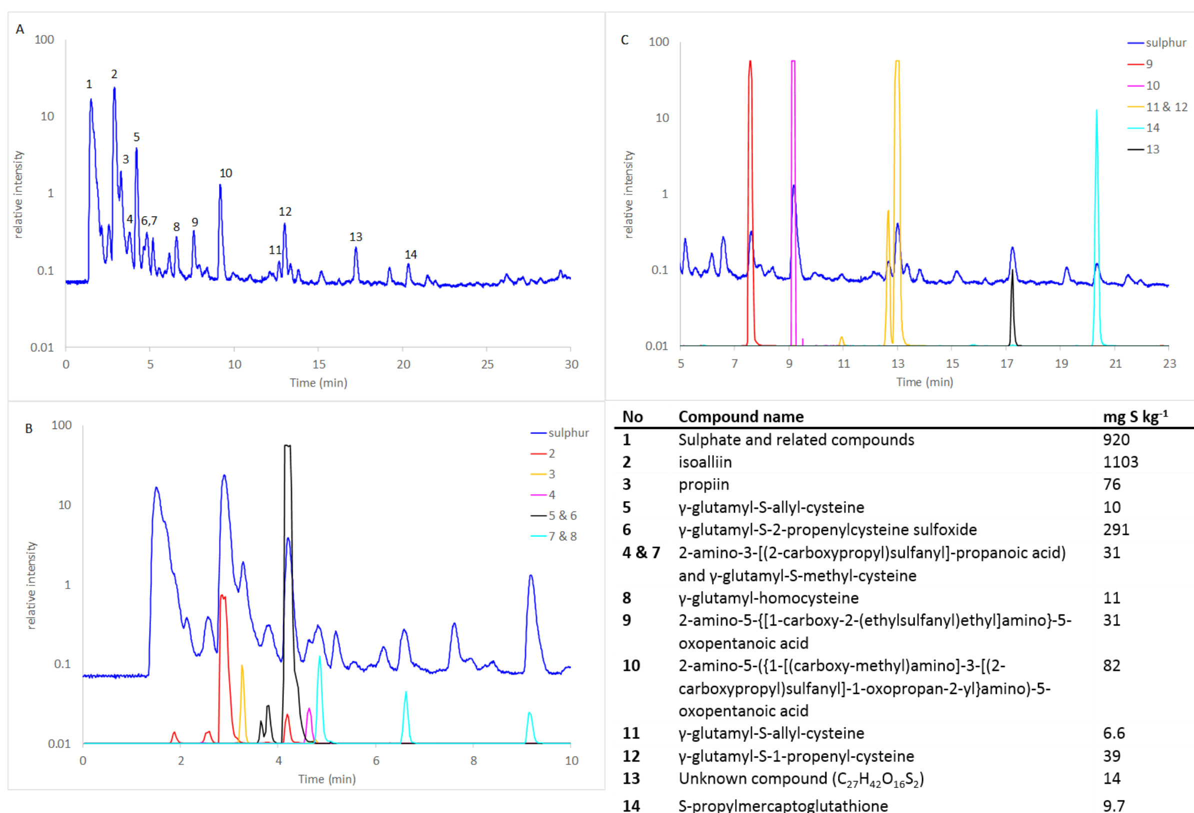


512
513
514 **Figure 1:** Sketch of metabolic involvement of sulphur containing compounds



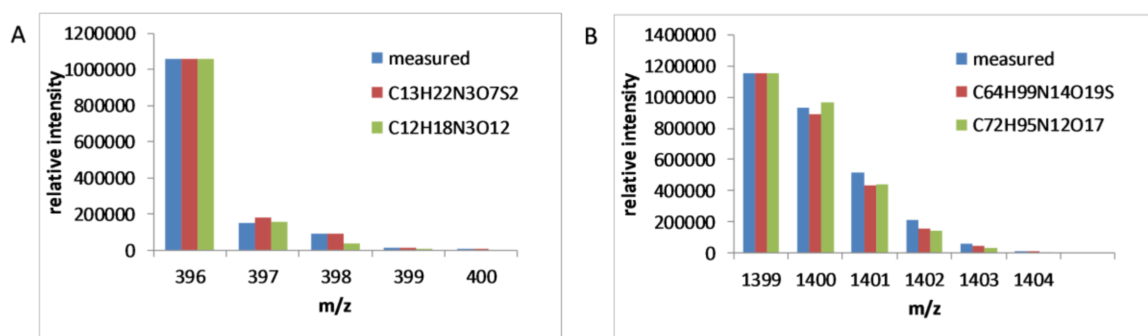
515

516 **Figure 2:** examples of sulphur-containing biological compounds: A1: γ-carrageenan, A2: 6-
517 sulfoquinovose, B1: sulfatide, B2: seminolipid, B3: taurolipid C, C1: glucosinolate, C2:
518 alliin, C3: serofendic acid C4: Vitamin B1



No	Compound name	mg S kg^{-1}
1	Sulphate and related compounds	920
2	isoalliin	1103
3	propiin	76
5	γ-glutamyl-S-allyl-cysteine	10
6	γ-glutamyl-S-2-propenylcysteine sulfoxide	291
4 & 7	2-amino-3-[(2-carboxypropyl)sulfanyl]-propanoic acid) and γ-glutamyl-S-methyl-cysteine	31
8	γ-glutamyl-homocysteine	11
9	2-amino-5-{[1-carboxy-2-(ethylsulfanyl)ethyl]amino}-5-oxopentanoic acid	31
10	2-amino-5-({1-[(carboxy-methyl)amino]-3-[(2-carboxypropyl)sulfanyl]-1-oxopropan-2-yl}amino)-5-oxopentanoic acid	82
11	γ-glutamyl-S-allyl-cysteine	6.6
12	γ-glutamyl-S-1-propenyl-cysteine	39
13	Unknown compound ($C_{27}H_{42}O_{16}S_2$)	14
14	S-propylmercaptoglutathione	9.7

Figure 3: separation of a shallot bulb extract using reversed phase HPLC separation and coupled to ICP-MS/MS as detector (m/z 48 for $^{32}S^{16}O^+$) (the intensity is in log-scale). The extract contains at least 44 sulphur-containing compounds. Panel A) Major identified and quantified compounds are labelled by number and correspond to the following compounds: Compound 1 (sulphate and related compounds) 35 mg S/ kg dry matter, compound 2 (alliin/isoalliin) 42 mg S/ kg dry matter, compound 3 (3-(Propylsulfinyl)-L-alanine, 180) 2.9 mg S/ kg dry matter, compound 4 (γ-Glutamyl-3-[(1-propen-1-ylsulfinyl]alanine 307) 11 mg S/ kg dry matter, compound 5a (γ-Glutamyl-homocysteine , 265) 1.2 mg S/ kg dry matter , compound 5b (γ-Glutamyl-S-methyl-cysteine , 265) 0.40 mg S/ kg dry matter, compound 6 (γ-Glutamyl-methionine, 279) 1.2 mg S/ kg dry matter, compound 7 (γ-Glutamyl-S-(2-carboxypropyl)—cysteine, 337) 9.3 mg S/ kg dry matter, compound 8a (γ-glutamyl-S-allyl-cysteine GSAC 291) 0.25 mg S/ kg dry matter, compound 8b (γ-glutamyl-S-1-propenyl-cysteine, 291) 1.5 mg S/ kg dry matter, compound 9 (687) 0.52 mg S/ kg dry matter, compound 10 (S-propylmercaptoglutathione 382) 0.37 mg S/ kg dry matter, compound 11 (? 21.6 min) 0.21 mg S/ kg dry matter, compound 12 (412) 0.29 mg S/ kg dry matter, Panel B shows extracted ion-chromatograms of compound 2 -6, Panel B shows extracted ion-chromatograms of compound 7 -12



	$C_{13}H_{22}N_3O_7S_2$ / $C_{12}H_{18}N_3O_{12}$	measured / $C_{13}H_{22}N_3O_7S_2$	measured / $C_{12}H_{18}N_3O_{12}$
M+1	1.17	0.94	1.10
M+2	2.58	0.95	2.46
M+3	3.64	1.02	3.73
mean difference	**2.46**	**0.97**	**2.43**

	$C_{64}H_{99}N_{14}O_{19}S$ / $C_{72}H_{95}N_{12}O_{17}$	measured / $C_{64}H_{99}N_{14}O_{19}S$	measured / $C_{72}H_{95}N_{12}O_{17}$
M+1	0.92	1.05	0.96
M+2	0.98	1.20	1.18
M+3	1.10	1.34	1.47
M+4	1.26	1.30	1.64
mean difference	**1.06**	**1.22**	**1.31**

Figure 4: examples of measured intensity for compounds compared to theoretical intensities for molecular composition containing either sulphur or not (data produced by MaXIS II, qTOF Bruker); panel A: compound at m/z 396 is S-1-propenylmercaptoglutathione oxidised, panel B: compound at m/z 1399 tryptic peptide of bovine serum albumin (TVMENFVAFVDK), in both cases the tables show the % intensity of the M+1 etc peaks relative to M.

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