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# Validation of the 2,3-dihydroxi-propionyl group in selenium speciation by chemical synthesis and LC-MS analyses

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## Abstract

2,3-dihydroxi-propionyl (2,3-DHP) group is a specific residue detected in selenized yeast that forms numerous stable and highly abundant Se species in several different yeast strains and fermentation batches. The conjugated form of 2,3-DHP-selenocysteine and glutathione is one of the most abundant species, found in nearly all selenized yeast. In order to overcome the commercial unavailability of this compound, its synthesis was carried out through the active ester formation of pentachlorophenyl glycerate with selenocystine, followed by the redox conjugation with glutathione. The optimization process of the synthesis was utilized for the production of three other Se-yeast specific compounds as well, namely the conjugate of glutathione and selenocysteine, the conjugate of 2,3-DHP-selenocysteine and selenocysteine, and di-N-2,3-dihydroxy-propionyl-selenocysteine. The upstream and clean-up procedures were supported and monitored with HPLC-UV, -ICP-MS, and -ESI-QQQMS set-ups, while the identification was done with HPLC-ESI-QTOFMS. The synthesized 2,3-DHP-selenocysteine/selenocysteine conjugates possessed identical fragmentation patterns to literature data.

## Introduction

In the last decade selenium-speciation and molecule structure identification has been gravely hindered by the lack of standards. Numerous articles have been published introducing newfound se-containing compounds, in many cases with ESI-MS identification completed with fragmentation patterns and proposed molecule structures.[1-3] While during peptide synthesis ESI-$MS^n$ based structure identification counts as routine, there is rarely if ever

enough of the target selenium-containing components to verify the suggested molecule structure. Fragmentation pattern may be enough for identification for molecules with already known S-analogues, or if the structure can be predicted from biological pathways, but when neither is an option, the only and ultimate way is through standards. However the number of commercially available or synthetically described Se species of plant and mammal metabolism has been increasing,[4-14] the availability of yeast specific Se species is extremely limited.[15] Taking into account that almost 70 Se species have been identified from selenized yeast [1, 16] and the fact that Se-yeast is the only natural (that is, not a synthetic compound based) and approved source for human selenium supplementation in the EU, the list of lacking (~ 60) selenium standards is more than remarkable.

One of the highly abundant and commercially unavailable Se-species is the conjugate of glutathione and 2,3-dihydroxy-propionyl-selenocysteine (CAS No. 1006377-09-8; $C_{16}H_{27}O_{11}N_4SSe^+$ $[M+H]^+$, m/z 563.05568). This Se-yeast specific compound was reported first by McSheehy et al. [17] and Goenega-Infante et al.,[18] while its structure was tentatively identified in 2008 based on high resolution ESI-MS data.[19] Since that time this compound has been detected and cited continuously from several producers and yeast strains.[1, 20, 21] While glutathione is highly concentrated in yeasts and occurs ubiquitously in eukaryotic and prokaryotic cells, the glyceroyl acid amine residue has only been previously reported either in antibiotics[22] or – interestingly – in selenium-containing conjugates from yeast and, recently, from black mustard (*Brassica nigra*).[23] Indeed, the metabolic role and origin of the 2,3-dihydroxy-propionyl (2,3-DHP; incorrectly referred also 2,3-dihydroxy-1-oxopropyl, 2,3-DOP) group has not been elucidated yet, which is especially interesting as no sulphur analogues of any of the Se containing species (of this group) have been found.

The ultimate goal of our study was to work out the synthesis of this compound in order to provide more reliable structure identification than standardless high resolution ESI-$MS^n$ data derived elucidation. The greatest challenges of the synthesis were the commercial unavailability of any active ester form of glycerate and the tendency of polyols for condensation/polymerization. The intermediate step of the synthesis, the coupling of the active ester to selenocystine results in two yeast-specific Se-compounds, the conjugate of 2,3-DHP-selenocysteine and selenocysteine (CAS 1246200-50-9 + 3614-08-2; $C_9H_{17}O_7N_2Se_2^+$ $[M+H]^+$, m/z 424.93634),[1] and di-N-2,3-DHP-selenocysteine (CAS 1357479-85-6; $C_{12}H_{21}O_{10}N_2Se_2^+$ $[M+H]^+$, m/z 512.95211).[21] The last step of the synthesis includes the oxidative conjugation of glutathione and a modified selenocysteine residue to form a S-Se bridge. Similarly to this step, with the conjugation of glutathione and native selenocysteine

another Se-species detected in yeast (CAS No. 188609-44-1; $C_{13}H_{23}O_8N_4SSe^+$ $[M+H]^+$, m/z 475.03963)[21] could be also synthesized and purified. The clean-up procedures were monitored with HPLC-UV, -ICP-MS, and -ESI-QQQ-MS detection, while the identification was based on HPLC-ESI-QTOFMS characterization.

Since all the four Se species are Se-yeast specific to the best of our knowledge, their availability might offer an important tool in the quantitative characterization and quality control of Se-yeast production.

## Experimental

### Reagents and standards

Acetonitrile (ACN; far UV HPLC grade), methanol (far UV HPLC grade) and Dowex 50WX4 cation-exchange resin (200-400 mesh) were bought from Fisher Scientific (Loughborough, Leicestershire UK). Ammonium acetate (a.r.), tris-hydroxymethyl-aminomethane (TRIS; a.r.), HCl (37 m/m %) and NaI (a.r.) were purchased from Reanal (Budapest, Hungary). Activated charcoal (4-14 mesh, granular, Norit® PK 3-5), Whatman Grade 1 filter paper, HCOOH (~98%, puriss), dithiothreitol (DTT), 4-methylmorpholine (NMM; 98.0%), N,N'-dicyclohexylcarbodiimide (DCC; 99.0%), pentachlorophenol (PCP; 98%), selenocystine ($Sec_2$45 97%), DL-glyceric acid hemicalcium salt hydrate (≥98%), reduced (≥98.0%) and oxidized (≥98%) glutathione stocks were purchased from the Sigma-Aldrich group (Schnelldorf, Germany). N,N-dimethylformamide (DMF; 99%) and $H_2O_2$ (a.r., 30 m/m%) were ordered from Merck (Darmstadt, Germany).

Milli-Q water (18.2 MΩ*cm, Merck-Millipore, Molsheim, France) was used throughout the experiments.

### Instrumentation

Inductively coupled plasma mass spectrometry (ICP-MS) Agilent 7500cs (Agilent, Santa Clara, CA, USA) was used to monitor the isotopes of $^{77}$Se and $^{82}$Se during the chromatographic clean-up processes if applicable. The instrument was coupled to an Agilent 1200 high-performance liquid chromatography (HPLC) system. Intermediate products of syntheses were also monitored with an HPLC – ESI-MS coupling where a QTRAP 3200 triple quadrupole – linear ion trap mass spectrometer (ESI-QQQ-MS; Applied Biosystems/Sciex; Foster City, CA, USA) was used either in the Enhanced Q3 Single MS (EMS) mode for the full-scan experiments with an integration time of 1 s or in Enhanced

Product Ion (EPI) mode for MS/MS analyses. The related instrumental parameters are described in the supplementary material.

For the identification of selenium species an Agilent 6530 Accurate-Mass ESI-QTOF-MS was used with an Agilent 6220 derived dual ion spray source. The instrument was coupled to an Agilent 1290 HPLC system. The operating parameters of the ESI-QTOF-MS can be seen in the supplementary material.

## Methods

### Desalting of glyceric acid

Glyceric acid hemicalcium salt was converted to the free acid form according to Berens and Scharf [24] by dissolving 465 mg glyceric acid salt in 25 ml 50 V/V% methanol-water solution, and then 19.0 g Dowex 50WX4 cation-exchange resin was added during stirring. After 20 minutes of incubation the resin was removed by filtration, afterwards the solution was filtered first through 2.0 g activated charcoal then through a filter paper, concentrated to about 5 ml using a vacuum rotary evaporator at 26 °C, and then strained using 0.45 μm PTFE filter. The leftover water content was removed on 55 °C using a vacuum rotary evaporator. 210 mg glyceric acid was acquired that was stored at -23° until used.

### Synthesis and clean-up of PCP-glycerate

106 mg (1.0 mmol) glyceric acid, 237 mg (1.14 mmol) DCC, and 291 mg (1.1 mol) PCP were dissolved in 3 ml of DMF, placed in icy water bath and stirred for 24 hours, when another 339 mg (1.64 mmol) DCC and 412 mg (1.54 mmol) PCP in 3 ml DMF were added, and left to incubate for 24 more hours. The product was dried in a vacuum rotary evaporator and dissolved in the mixture of 4.5 ml ACN and 3 ml DMF. The solution was centrifuged at 4000 *g* for 10 min, decanted and filtered through a 0.45 μm PTFE filter.

Clean-up of PCP-glycerate was executed with fraction collection based on semi-preparative reversed phase HPLC-UV separation and was verified with ESI-QQQ-MS. The relevant instrumental parameters can be seen in Tables 1 and 2 in the supplementary material. The corresponding fractions were pooled and lyophilized. 13.2 mg dry matter was acquired with a yield of 3%.

**Coupling of PCP-glycerate and $Sec_2$**

$Sec_2$ was solubilized in DMF according to the procedure described by Dernovics et al.[25] 4.0 mg PCP-glycerate (11 µmol) was dissolved in 4 ml DMF and placed in an ice bath under Ar current and with continuous stirring, 50 µl $Sec_2$ solution (9 µmol) and 2 µl (18 µmol) NMM were added. To keep the pH of the solution between 7 and 8, 2 µl NMM was added three more times at 15 minute intervals, and then it was incubated at room temperature for 48 hours with constant pH monitoring. The final product was lyophilized and dissolved in 4.0 ml 10 mM ammonium acetate buffer (pH=5.5). The solution was centrifuged at 4000 *g* for 10 min, decanted and filtered through 0.45 µm PTFE filters. The formation of di-2,3-DHP-Sec and 2,3-DHP-Sec-Sec was monitored with analytical scale strong anion exchange (SAX) HPLC-ICP-MS set-up, while their clean-up was carried out with sequential semi-preparative SAX-HPLC-ICP-MS and RP-HPLC-ICP-MS based fraction collections. The relevant parameters can be seen in Table 1 in the supplementary material. The column flow was split both cases to provide adequately low flow rate for the nebulizer of the ICP-MS. The corresponding fractions were pooled and lyophilized.

**Conjugation of $Sec_2$ with glutathione**

For this step 0.1 M TRIS buffer (pH=8.6) was used. 3.4 mg (10 µmol) $Sec_2$ was reduced and dissolved in 3 ml buffer containing 26.1 mg (169 µmol) DTT. 106 mg (173 µmol) oxidized glutathione dissolved in 4.0 ml buffer was added to the solution, then 3.8 mg NaI (25 µmol; as catalyst)[26] and 24 µl (160 µmol) $H_2O_2$ were added. The solution was incubated for 2 hours at room temperature.

The screening of reaction products was done with analytical scale SAX-HPLC-ICP-MS, while the clean-up of the selenocysteine-glutathione conjugate was done with semi-preparative SAX-HPLC-ICP-MS (Table 1 in the supplementary material). The reaction solution was 1+3 (V/V) diluted with 10 mM ammonium acetate buffer (pH=5.5) prior to injection. Fractions were pooled, lyophilized, dissolved in 300 µl 10% (V/V) ACN-$H_2O$ solution, and injected to HPLC-ESI-QTOF-MS system for characterization. The relevant instrumental parameters can be seen in Tables 1 and 3 in the supplementary material.

**Conjugation of (2,3-DHP)-Sec-Sec and di-N-2,3-DHP-Sec with glutathione**

The pooled (2,3-DHP)-Sec-Sec and di-N-2,3-DHP-Sec compounds acquired from the semi-preparative SAX-HPLC-ICP-MS clean-up were dissolved in 2.0 ml of 0.1 M TRIS buffer (pH=8.6). First 2.5 mg (16 µmol) DTT was added, followed by 150 mg (244 µmol) oxidized

glutathione, then 100 µl 16 mg/ml NaI solution (11 µmol) and finally 24 µl (160 µmol) $H_2O_2$ were mixed to the solution. The solution was incubated for 2 hours at room temperature.

The clean-up and the HPLC-ESI-QTOFMS characterization of the 2,3-DHP-selenocysteine-glutathione conjugate was carried out the same manner as it was done with the selenocysteine-glutathione conjugate.

## Results and discussion

### Conjugation of $Sec_2$ with glutathione and the characterization of selenocysteine-glutathione

From the family of selenium containing glutathione conjugates Sec-glutathione is one of the least complex compounds and it occurs in nearly all batches and strains in selenized yeast,[21] however its concentration does not exceed that of 2,3-DHP-Sec-glutathione. Apart from very low abundant $Sec_2$ and $Sec_2$ species,[1, 27] this compound is unique in terms of containing a non-modified Sec residue conjugated through either a S-Se or a Se-Se bond.

The difficulty of the chemical synthesis of Sec-glutathione is the effective oxidative conjugation in the presence of the huge excess of DTT required for the solubilization of $Sec_2$,[28] which can be resolved by NaI catalyzed oxidation[26] and by the depletion of DTT with oxidized glutathione. As presented in Fig. 1a, the arising Sec-glutathione elutes between $Sec_2$ and oxidized/reduced glutathione on SAX-HPLC, thus providing adequate separation for chromatographic clean-up.

The purified Sec-glutathione was characterized with HPLC-ESI-QTOFMS and MS/MS experiments. Fig. 1b shows the TIC and EIC of the compound, Fig. 1c shows the full scan recorded at the apex of the related EIC ($C_{13}H_{23}O_8N_4SSe^+$ $[M+H]^+$, m/z 475.03959, Δ= -0.08 ppm) and Fig. 1d presents the MS/MS data (see also Table 4 and the pathway of synthesis in the supplementary material). Similarly to the fragmentation of the Se-containing glutathione family and $Sec_2$ in positive ion mode,[19, 29] the intense fragments arrive from the loss of Gly, γ-Glu residues and neutral losses of $NH_3$ and HCOOH, while the S-Se bond is hardly fragmented and the intact glutathione and Sec residues are only low abundant.

It is important to mention that the synthesis of Sec-glutathione was addressed both as an optimization step and as an independent method for the synthesis of a commercially unavailable compound.

**Synthesis and clean-up of PCP-glycerate**

The use of pentafluorophenol (PFP) might be preferred over PCP, as PFP esters react faster and the removal of PCP may be difficult.[30] However in our research it was found that the reaction of glyceric acid with PFP didn't yield any detectable amount of ester (results not shown); therefore the step was repeated with the use of PCP.

PCP renders to the PCP-glycerate hydrophobic properties, thus providing the possibility for a reversed phase HPLC based clean-up. Fig. 2a presents the relevant HPLC-UV chromatogram where the compound eluting at 14.3 min was identified with ESI-MS/MS as PCP-glycerate after preparative scale fraction collection. The compound could be identified due to its unique isotopic pattern containing five chlorine atoms and it could be characterized with the same fragmentation mechanism during both the ionization process in the ion source (Fig. 2b) and the MS/MS fragmentation (Fig. 2c), i.e., the arising of pentachlorophenyl anion (m/z 351.0 $[C_9H_4Cl_5O_4]^-$ → m/z 264.8 $[C_6Cl_5O]^-$). The low yield of synthesis can be partly attributed to the polyolic structure of glyceric acid that facilitates the formation of by-products, e.g, PCP glycosides, and partly to the need for water-containing HPLC eluent.

**Coupling of PCP-glycerate to $Sec_2$ and the characterization of the (2,3-DHP)-Sec-Sec and di-N-2,3-DHP-Sec species**

Active ester coupling to $Sec_2$ yields a mixture of non-derivatized, single and double derivatized species,[25] thus requiring a clean-up step. As the free $-NH_2$ groups are bound in the reaction with PCP-glycerate the resulting species will show anionic properties even at slightly acidic pH, which enables the SAX-HPLC based purification.

Fig. 3a presents the HPLC-ICP-MS chromatogram of the synthesized products, where three selenium containing peaks could be observed: $Sec_2$ arriving close to the dead volume, and the hypothetic (2,3-DHP)-Sec-Sec and di-N-2,3-DHP-selenocysteine species in the order of elution, respectively. The latter two compounds were cleaned-up and characterized with HPLC-ESI-QTOFMS analyses. Fig. 3b shows the TIC and the EICs of the two compounds extracted at the theoretical m/z values.

Fig. 3c presents the full scan recorded at the related EIC of the m/z 424.93 compound. The accurate mass ($C_9H_{17}O_7N_2Se_2^+$ $[M+H]^+$, m/z 424.93607, Δ= -0.64 ppm), isotopic distribution and MS/MS fragments (see Fig. 3d) match those of reported by Arnaudguilhem et al.[1] Concerning di-N-2,3-DHP-selenocysteine, the data presented on Fig. 3e ($C_{12}H_{21}O_{10}N_2Se_2^+$

$[M+H]^+$, m/z 512.95203, Δ= -0.16 ppm) are in agreement with those published by Casal et al.,[21] while the MS/MS fragments have been presented here for the first time (Fig. 3f).

The suggested fragmentation pathways of the two compounds and the pathway of synthesis are included in the supplementary information. It should be highlighted that the fragmentation of both species results in the abundant appearance of the couple of m/z 255.97 – m/z 167.95 fragments that is also characteristic of the conjugate of 2,3-DHP-selenocysteine – glutathione (m/z 563.05).

Taking into account the low efficiency of the 2,3-DHP coupling process, both /single and double/ derivatized compounds were purified and pooled in order to increase the yield of the following conjugation step with glutathione.

**Conjugation and characterization of 2,3-DHP-Sec-glutathione**

Combining the optimized process of Sec-glutathione conjugation and the clean-up of 2,3-DHP-containing $Sec_2$ species were the prerequisites to arrive at a detectable amount of 2,3-DHP-Sec-glutathione. However this compound is slightly retained on special reversed phase HPLC columns intended for use with eluents with low organic solvent content,[18] therefore a more robust clean-up technique with SAX-HPLC was chosen.[19]

The SAX HPLC-ICP-MS chromatogram of the reaction products can be seen in Fig. 4a. The first Se-containing compound, eluting at 500 s, was identical to the conjugate of Sec-glutathione that was formed in the reaction of non-derivatized Sec residues. The HPLC-ESI-QTOFMS characterization of the more intense second peak, eluting at 720 s, is presented in Fig. 4b. The targeted search for m/z 563.05568 resulted in an EIC of a single peak with the full scan shown on Fig. 4c and MS/MS fragmentation data shown on Fig. 4d. Both the MS ($C_{16}H_{27}O_{11}N_4SSe^+$ $[M+H]^+$, m/z 563.05546 $[M+H]^+$, Δ= -0.39 ppm) and MS/MS data (see Table 4 and the pathway of synthesis in the supplementary material) correspond to the previously reported information on this compound,[19] which indicates the synthesized compound matches the genuine, Se-yeast specific 2,3-DHP-Sec-glutathione conjugate.

While some (e.g., the γ-Glu specific) of the MS/MS fragments of Sec-glutathione and 2,3-DHP-Sec-glutathione are shared, the majority of the fragments are different. The most significant difference is the high abundance of the Sec residue that appears both in native (m/z 167.95) and 2,3-DHP-derivatised (m/z 255.97) forms during the fragmentation of 2,3-DHP-Sec-glutathione but appears only as a minor fragment during the fragmentation of Sec-glutathione. This great difference in fragmentation pattern is unusual, as the two compounds share their basic structure. Indeed, the addition of the 2,3-DHP residue, that can be broken off

during fragmentation at the amide bond, could stabilize the Sec residue and increase its abundance while affecting the bond strength of the S-Se bridge.[17] The high fragmentation event of the S-Se bridge in positive ion mode together with the abundant appearance of the Sec residue is a unique feature of 2,3-DHP-containing glutathione derivatives and it is reported exclusively in such structures.[1, 31]

## Conclusions

Both the quality control and the quantitative characterization of selenized yeast batches require standards to monitor stability and to identify sample origin. As non-Se-yeast-specific selenium compounds (namely, selenomethionine, selenocysteine and inorganic selenium species) specified by the Commission Regulation (EC) No 1170/2009 cannot provide customized options for these purposes, the newly synthesized 2,3-DHP containing species and the conjugate of selenocysteine – glutathione may offer a viable solution. On the other hand, the more than 50 Se-species discovered during the last five years from plant and yeast samples call attention to the evident lag in the number of available standards that may be caught up stepwise with an approach similar to our method, that is, with a grouped batch of synthesis.

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**Figure captions**

**Figure 1** (a) Analytical scale SAX-HPLC-ICP-MS chromatogram of the products arising from the oxidative conjugation of $Sec_2$ and glutathione. Dashed line indicates the selenium signal, while the continuous line refers to sulphur. The compound eluting before reduced glutathione at 520 s was collected for further characterization with preparative scale SAX-HPLC. (b) HPLC-ESI-QTOF-MS total ion chromatogram (TIC) of the compound collected from SAX-HPLC. The inset presents the extracted ion chromatogram (EIC) for m/z 475.0396. (c) Full scan spectrum recorded near the apex of the EIC for m/z 475.0396. The inset shows the selenium pattern of the target compound. (d) MS/MS spectrum and structure of the compound at m/z 475.0396.

**Figure 2** (a) Preparative scale reversed phase (RP) HPLC-UV chromatogram of the products resulting after the coupling of PCP and glyceric acid. The compound eluted at 14.3 min was collected for further characterization and synthesis. (b) ESI-QQQ-MS full scan spectrum of the compound collected from RP-HPLC. The inset presents the theoretical (left) and experimental (right) isotopic pattern of PCP-glycerate. (c) MS/MS spectrum of the compound at m/z 351.0, together with the proposed fragmentation event.

**Figure 3** (a) Analytical scale SAX-HPLC-ICP-MS chromatogram of the products arising from the coupling of PCP-glycerate and $Sec_2$. The two peaks marked with the hypothetical compound structures eluting at 605 s and 1230 s were pooled for further characterization and synthesis with preparative scale SAX-HPLC. (b) HPLC-ESI-QTOF-MS based TIC of the compounds collected from SAX-HPLC. The inset presents the EICs for m/z 424.9370 and m/z 512.9532. (c) Full scan spectrum recorded near the apex of the EIC for m/z 424.9370. The inset shows the selenium pattern of the target compound. (d) MS/MS spectrum of the compound at m/z 424.9370. (e) Full scan spectrum recorded near the apex of the EIC for m/z 512.9532. The inset shows the selenium pattern of the target compound. (f) MS/MS spectrum of the compound at m/z 512.9532.

**Figure 4** (a) Analytical scale SAX-HPLC-ICP-MS chromatogram of the products arising from the oxidative conjugation of (2,3-DHP)-Sec-Sec and di-N-2,3-DHP-Sec with glutathione. The compound eluting at 720 s was collected for further characterization with preparative scale SAX-HPLC. (b) HPLC-ESI-QTOF-MS based TIC of the compound collected from SAX-HPLC. The inset presents the EIC for m/z 563.0554. (c) Full scan spectrum recorded near the apex of the EIC for m/z 563.0554. The inset shows the selenium pattern of the target compound. (d) MS/MS spectrum and structure of the compound at m/z 563.0554.

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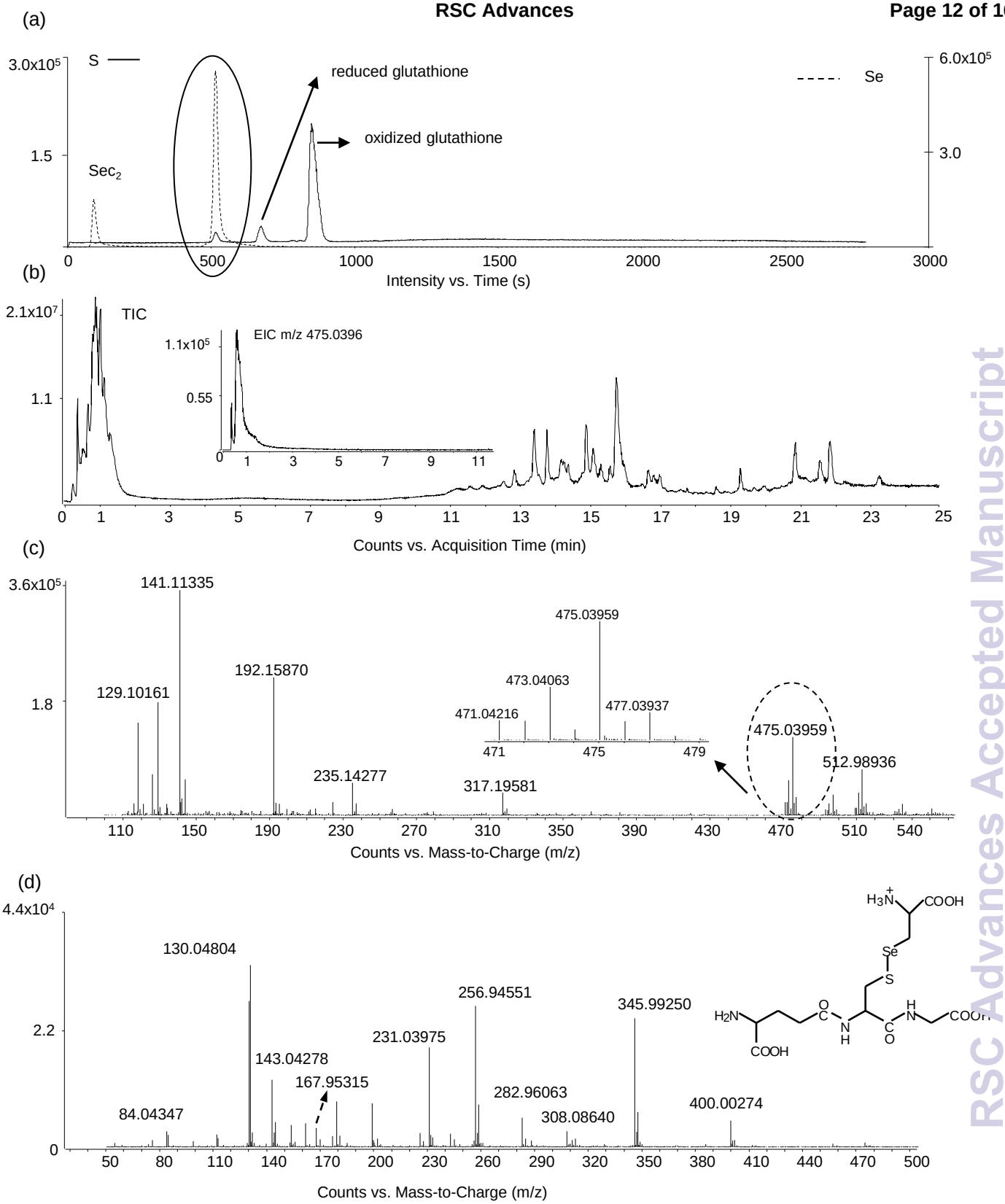
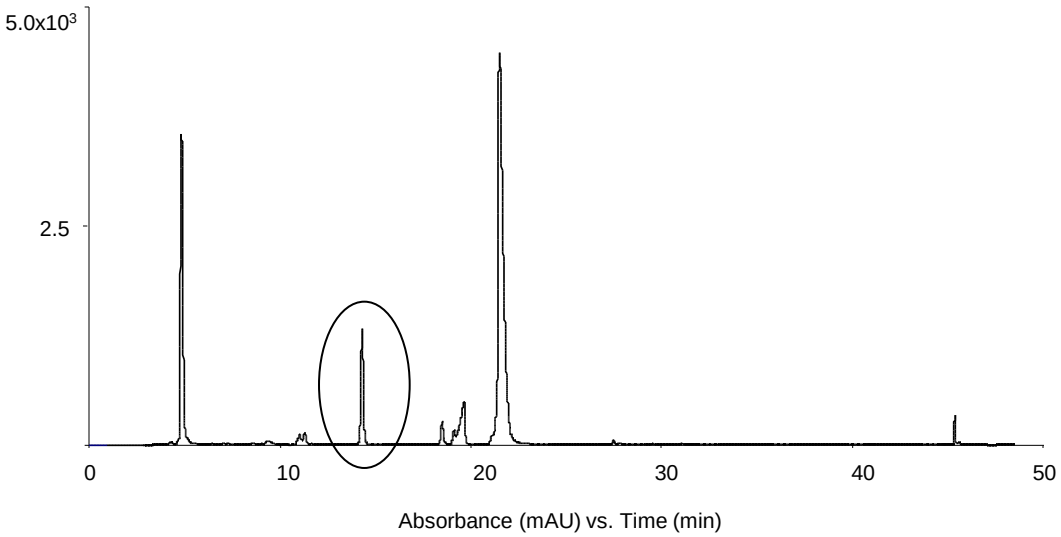
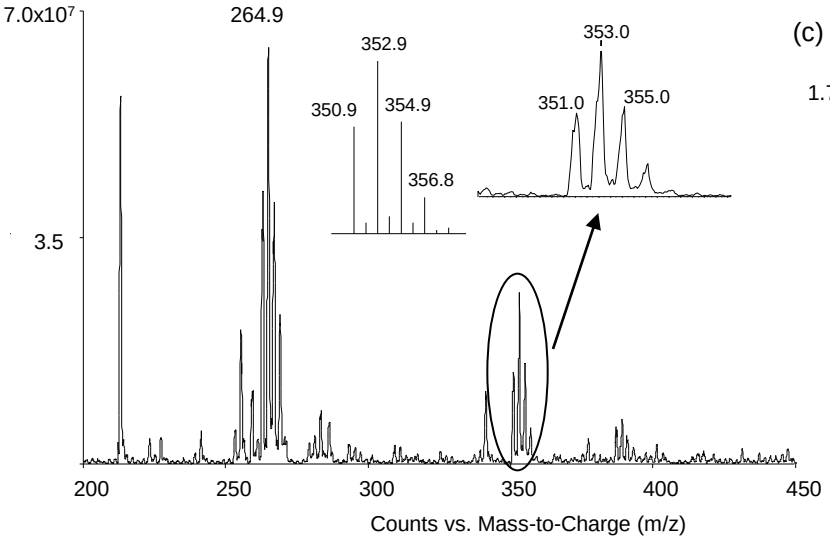


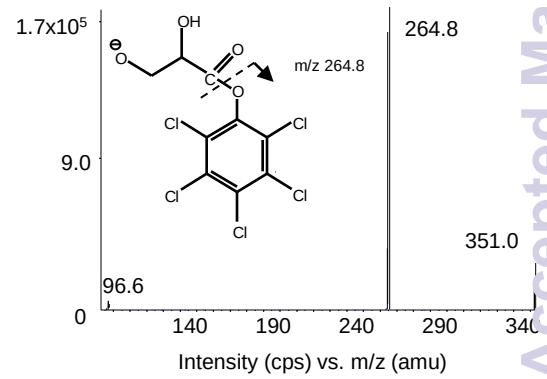
(a)

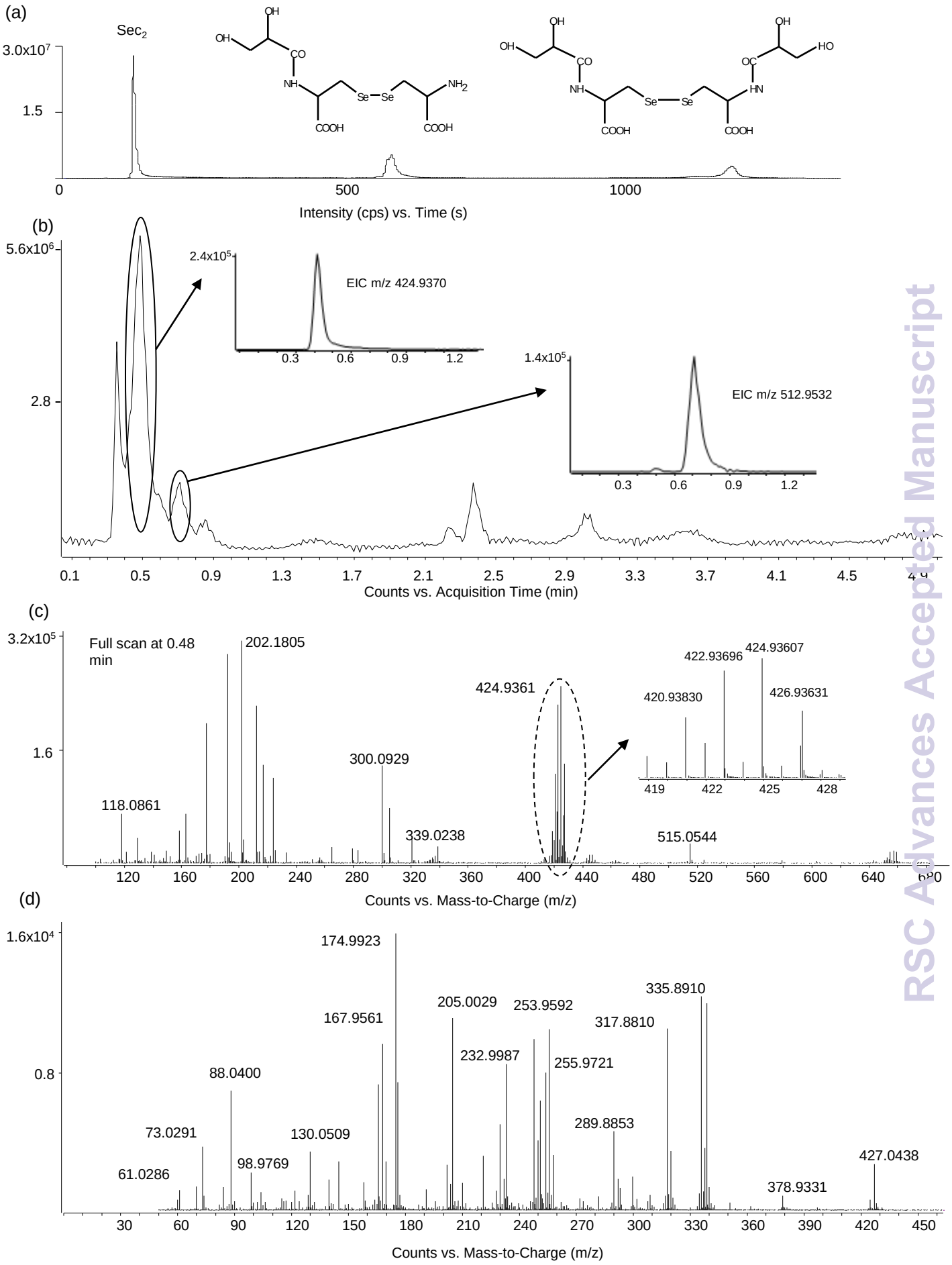



(b)

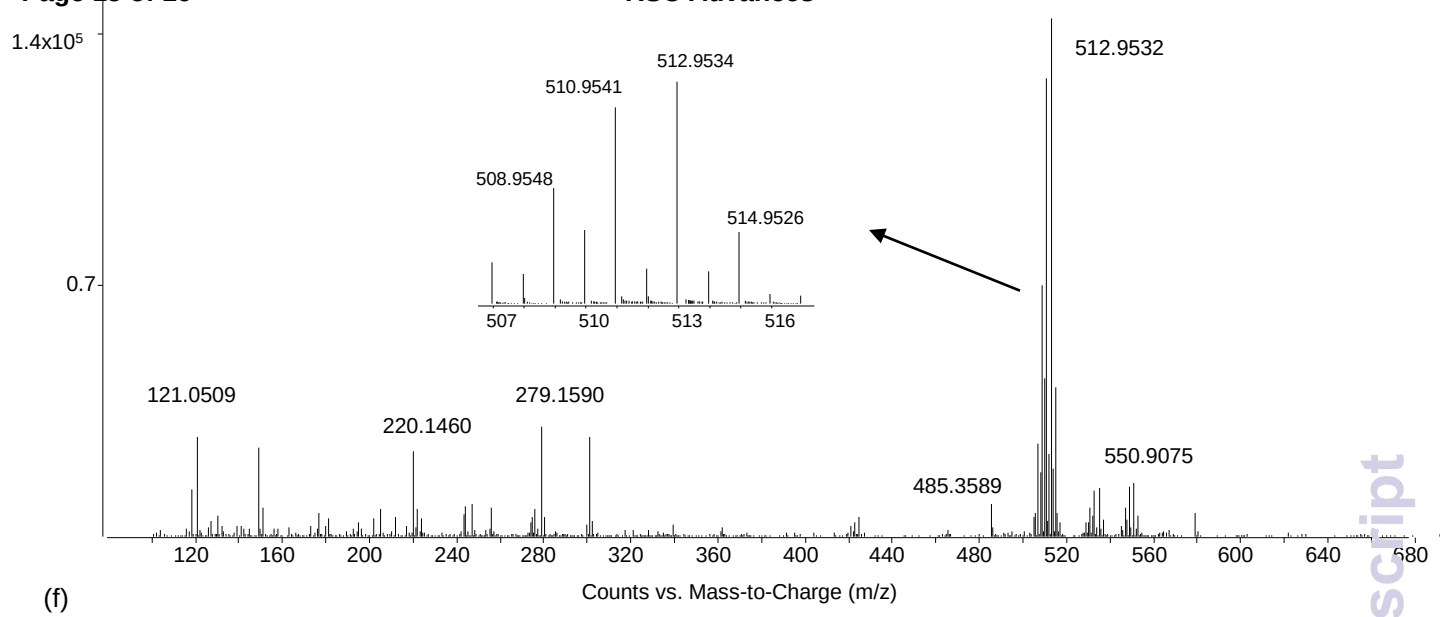



(c)





(e)




(f)