Development and validation of extraction and analytical methods for speciation analysis of selenised yeast using hyphenated mass spectrometric techniques

By

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of *Doctor of Philosophy* is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____

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List of Abbreviations

AAS: Atomic absorption spectroscopy

Ab: Antibody

AES: Atomic emission spectroscopy

AFS: Atomic fluorescence spectroscopy

APCI: Atmospheric pressure chemical ionisation

atm: unit of atmospheric pressure

CIC: Compound independent calibration

CID: Collision-induced dissociation

CIR: Chiral inducing reagent

CNBr: Cyanogen bromide

CRM: Certified reference material

CSF: Cerebral spinal fluid

DCU: Dublin City University

DIW: Deionised water

DTT: Dithiothreitol

EC: European Commission

ECD: Electron capture dissociation

ECG: Electrocardiograms

EPA: Environmental Protection Agency

EPS: Enzymatic probe sonicator

ESI: Electrospray ionisation

EFSA: European Food Safety Authority

EU: European Union

FAAS: Flame atomic absorption spectroscopy

FT-ICR: Fourier transform ion cyclotron resonance

GC: Gas chromatography

GSH: Glutathione

GPx: Glutathione peroxidase

HG: Hydride generation

HPLC: High performance liquid chromatography

ICP: Inductively coupled plasma

ICP-OES: Inductively coupled plasma optical emission spectroscopy

Ig: Immunoglobulin

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

ISSC: Irish Separation Science Cluster

IUPAC: International Union of Pure and Applied Chemistry

KBD: Kashin-Beck disease

KD: Keshan disease

LC: Liquid chromatography

LGC: Laboratory of the Government Chemist, UK

LOD: Limit of detection

LOQ: Limit of quantification

MALDI: Matrix-assisted laser desorption ionisation

MAD-HG-AFS: Microwave-assisted digestion hydride generation atomic fluorescence spectroscopy

MAEE: Microwave assisted enzymatic extraction

MIP-MS: Microwave induced plasma-mass spectrometry

MMA: Mass measurement accuracy

MS: Mass spectrometry

MSFA: Methanesulfonic acid

NICB: National Institute for Cellular Biotechnology

NRC: National Research Council of Canada

ORS: Octopole reaction system

PMM: Peptide mass mapping

PTM: Post-translational modifications

RC: Regenerated cellulose

RF: Radio frequency

ROS: Reactive oxygen species

RPLC: Reversed phase liquid chromatography

rpm: revolutions per minute

Se: Selenium

SeCys: Selenocysteine

$SeCys_2$: Selenocystine

SEM: Scanning electron microscope

SeMet: Selenomethionine

Tris: Tris (hydroxymethyl) aminomethane

TMSe: Trimethylselenonium ion

UGA: Uracil Guanine Adenine

UPLC: Ultra performance liquid chromatography

USDA: United States Department of Agriculture

UV: Ultraviolet

WMD: White muscle disease

YCW: Yeast cell wall

YP1: Yeast Product 1

YP2: Yeast Product 2

YP3: Yeast Product 3

YP4: Yeast Product 4

YPA: Yeast Product A

YPB: Yeast Product B

YPC: Yeast Product C

YPD: Yeast Product D

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Ward P, Connolly C, Murphy R. Accelerated Determination of Selenomethionine in Selenized Yeast: Validation of Analytical Method. Biol Trace Elem Res. 2012;150(3 (2013)):446-50. doi:10.1007/s12011-012-9571-x.

Fagan S, Owens R, Ward P, Connolly C, Doyle S, Murphy R. Biochemical Comparison of Commercial Selenium Yeast Preparations. Biol Trace Elem Res. 2015:1-15. doi:10.1007/s12011-015-0242-6.

Ward P, Chadha M, Connolly C, Stalcup A, Murphy R. Detection of over 200 water-soluble selenium metabolites in commercial selenised yeast by reversed phase liquid chromatography-electrospray ionisation quadrupole time-of-flight mass spectrometry. Under review.

List of Poster Presentations

Biochemical profiling of commercially-available selenium-enriched yeast, *Patrick Ward, Cathal Connolly, Apryll Stalcup and Richard Murphy.* RSC BNAAS/TraceSpec Conference, University of Aberdeen, UK, 31st August – 4th September 2014.

Direct enantioseparation of selenomethionine in water-soluble fractions of commercially-available selenium-enriched yeast by HPLC-ICP-MS, *Patrick Ward, Cathal Connolly, Apryll Stalcup and Richard Murphy.* Irish Mass Spectrometry Society Annual Conference, Dublin, 10th May 2017.

List of Honours and Awards

Irish Research Council Employment Based Postgraduate Scholarship 2012 to fund a research M.Sc.

Irish Research Council Employment Based Postgraduate Scholarship 2014 to fund a research PhD.

Dedication

For my Parents

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To my parents, Marie and Michael, thank you for everything! I am here because of your unconditional support throughout my life and education. I could never repay the time and effort you have invested in me growing up over the years, you are the biggest inspirations in my life. A huge thanks to my sisters, Samantha and Anne, for their support, you always keep me grounded.

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Abstract

Selenium is an essential trace element for human and animal health. Numerous studies reported selenium to possess antioxidant effects, anticarcinogenicity and fertility improvements to name a few. The assimilation of selenium into the yeast via the sulfur metabolic pathway creates selenised yeast which is one of the primary sources of selenium supplementation.

In this thesis, novel approaches to the extraction and characterisation of commercial selenised yeast products are described. Extraction procedures were developed for selenomethionine determination. These methods involved both enzymatic and chemical extraction approaches and utilised microwave and ultrasonication energy for the purpose of liberating intracellular selenomethionine. Both the enzymatic and chemical extraction methods were subsequently validated for determination of the analyte.

A quantitative assay was also developed for the determination of chiral enantiomers of selenomethionine. The method was applied to commercially-available selenised yeast to characterise any differences in the water-soluble extracts of the products. Significant differences were evident, not only from chiral composition but also from the selenocompounds present in the water extract. Further screening of the water extract by HPLC-ICP-MS revealed numerous other selenocompounds. While coupling of HPLC to ICP-MS was sufficient as an investigative screening tool, selenocompound identification was not possible unless a standard of the analyte was available. To combat these issues other mass spectrometric techniques were investigated. The target water-soluble extracts were lyophilised and resuspended to increase analyte concentration and were analysed by liquid chromatography electrospray quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS). Selenium-containing species were confirmed by selenium's unique isotopic pattern. Determination of the elemental composition and proposed structure of some of these previously unreported selenocompounds was possible due to the accurate molecular mass from the first mass analyser and from MS^2 fragmentation analysis. Therefore, LC-ESI-QTOF-MS may be used as a fingerprint tool to make comparisons between commercially-available selenised yeast products.

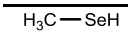
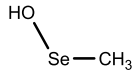
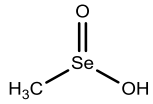
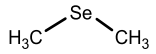
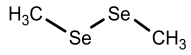
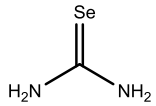
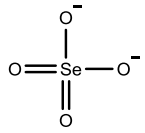
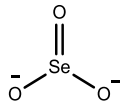
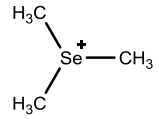
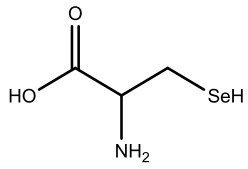
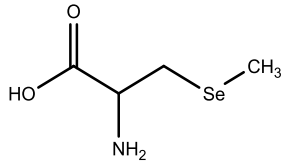
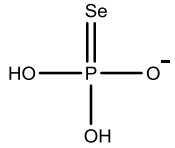
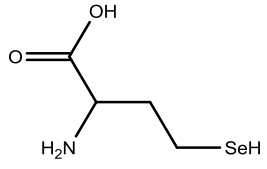
Chapter 1: Literature survey

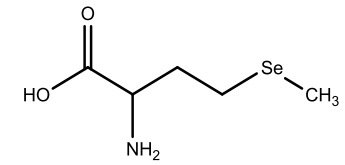
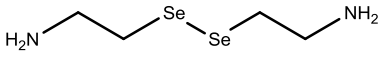
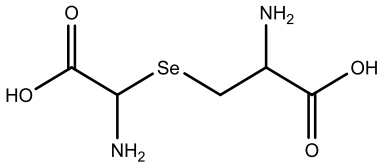
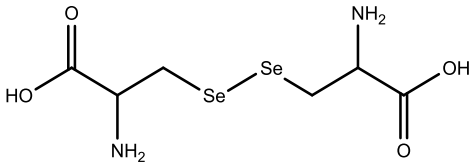
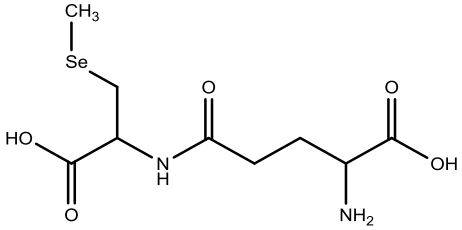
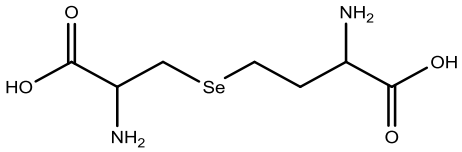
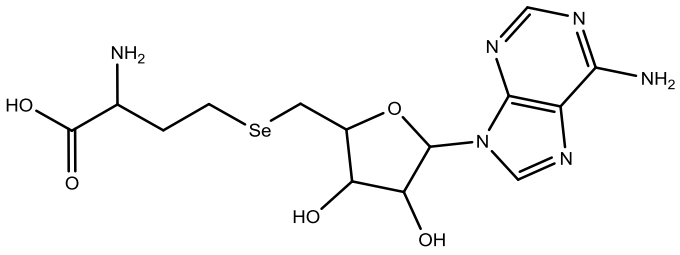
1.1 Selenium - General Introduction

Selenium (Se), has an atomic number of 34 and a relative atomic mass of 78.96 (Table 1.1). The isotopes considered to be stable for analysis are ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se [1]. The relative abundance of these isotopes is 0.89, 9.37, 7.63, 23.77, 49.61 and 8.73 % respectively [2]. This study utilises ICP-MS to monitor these isotopes and determine the selenium composition of selenium-enriched yeast.

Selenium was first discovered by a Swedish chemist, Jons Jakob Berzelius of the Karolinska Institutet in 1817 [3, 4]. Berzelius named selenium after the Greek goddess of the moon, Selene [5], due to the similarity of selenium's chemical properties to that of tellurium (Te) which was named after the Latin word for earth, Tellus. Selenium also shares similar chemical properties to that of sulfur [6] and the three elements (S, Se and Te) are found in group sixteen of the periodic table. This chemical similarity allows for selenium/sulfur competition in biological systems which can lead to the incorporation of selenium instead of sulfur. This incorporation can be specific or non-specific and will be discussed later. These chemical similarities between sulfur and selenium are exploited in the fermentation process to manufacture selenium-enriched yeast. The assimilation of selenium into the yeast via the sulfur metabolic pathway (Figure 1.1 [7]), creates a selenised yeast. These selenium-containing compounds include selenoamino acids such as selenomethionine (sulfur analogue-methionine), selenocysteine (sulfur analogue-cysteine) and selenocystine (sulfur analogue-cystine) as well as selenoproteins. However, numerous other selenocompounds were also detected by mass spectrometric techniques (Table 1.1) [8-11].

Table 1.1: Structures of some selenocompounds referred to throughout this thesis.

	Methylselenol
	Methylseleninate (MeSeOH)
	Methaneseleninic acid (MeSeA)
	Dimethylselenide
	Dimethyldiselenide
	Selenourea
	Selenate (selenic acid)
	Selenite (selenous acid)
	Trimethylselenonium ion
	Selenocysteine
	Selenomethylselenocysteine
	Monoselenophosphate
	Selenohomocysteine

	Selenomethionine
	Selenocystamine
	Selenocystathione
	Selenocystine
	γ-glutamyl-methylselenocysteine
	Selenocystathionine
	Selenoadenosylhomocysteine

Modified from Weekley *et al.*[8-11].

In 1973, glycine reductase and formate dehydrogenase were the first selenoproteins discovered in *Clostridium sticklandii* and *Clostridium thermoaceticum* cells, respectively [3]. Selenium has also been shown to be a co-factor for the glutathione peroxidase (GPx) antioxidants which eliminate cellular damaging hydrogen peroxides and lipid hydroperoxides (see Section 1.2.1.4 for more detail) [12]. Other benefits of selenium include anti-carcinogenicity [13] and enhanced fertility which will be discussed later (Section 1.2.1.1). Hence, selenium is considered to be an essential trace element not only for antioxidant defence mechanisms but also for the overall health of animals [14, 15].

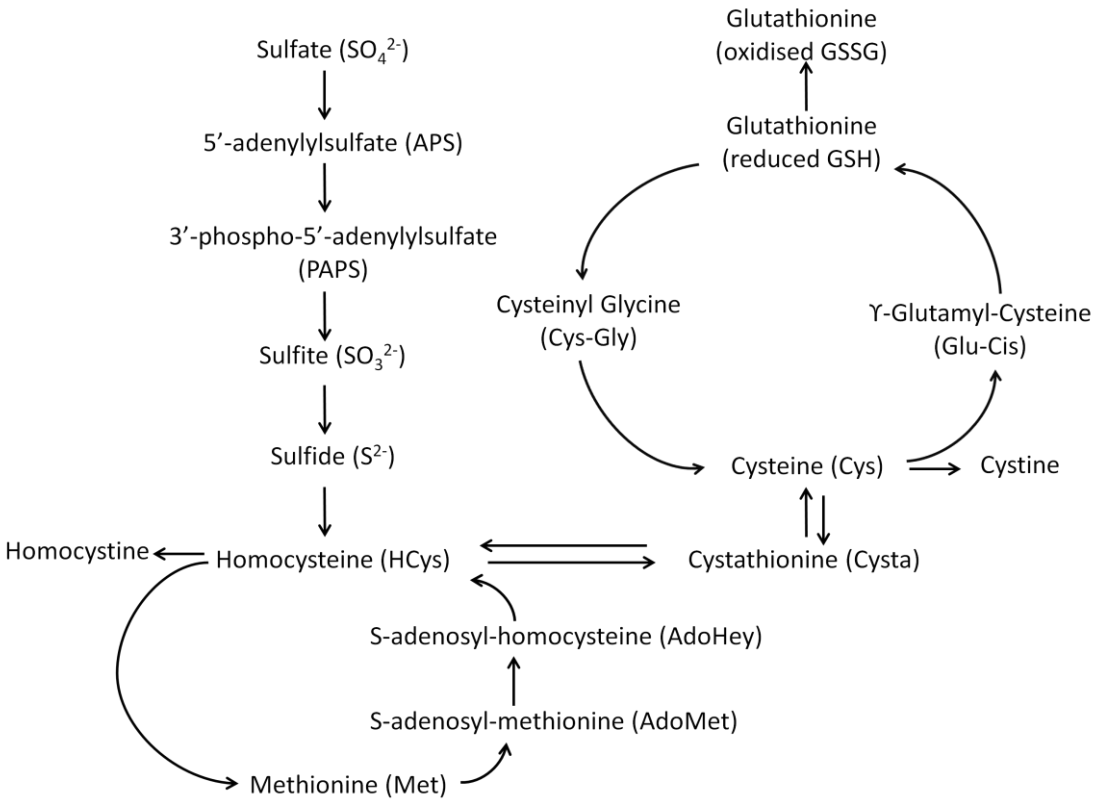


Figure 1.1: Sulfur metabolic pathway in yeast.
Taken from Rao *et al.* [7].

1.1.1 Production

Selenium, like many other elements, has numerous oxidation states such as its elemental form Se (0), selenite (IV; SeO_3^{2-}), selenate (VI; SeO_4^{2-}) and Se (-II) [16-18]. These different oxidation states allow for a broad range of selenium applications. Selenium is used for industrial [19], agricultural [20] and nutraceutical [21] purposes. The mining process of copper from its ore is carried out by electrolytic copper refining. This

produces high concentrations of selenium as a by-product which accounts for 90 % of global selenium production [19]. Indeed, 52 out of 56 copper refining plants around the world confirmed the presence of selenium in their anode slimes [19]. It was estimated that on average up to 25 % of this slime mass could be selenium. However, of this possible 25 % mass, only 50 % of the selenium was recovered on average from anode slimes of this refining process [19]. Effluent from mining activity, such as uranium milling in Saskatchewan, Canada, contains a high concentration of trace elements such as inorganic selenium [22]. Furthermore, elemental selenium is a byproduct from the refining of copper and is a general waste product in mining such as coal and phosphorite mining [23].

1.1.2 Geology

Geology is large factor in selenium toxicity or deficiency. While the accumulation of selenium in topsoil may also be due to human influences such as agricultural fertilisation, precipitation and irrigation [24], countries and regions within countries can naturally have higher selenium concentrations in their soils than others. This creates a selenium imbalance in the population, in food crops and animals. Worldwide selenium soil concentrations can vary from as low as 0.01 mg/kg to 1000 mg/kg [25-27]. Areas with high levels of selenium, if bioaccumulated in plants, can lead to selenium toxicity and thus selenosis [28] which will be discussed in more detail later. Selenium soil concentration variation is a worldwide concern, significant differences were found across North America as well as Ireland (see Figure 1.2 and 1.3). Ireland originally imported a lot of wheat and grains from the United States, (see Section 1.2.1), which is why it is included.

The U.S. Department of the Interior, U.S. Geological Survey measured the uneven selenium distribution of soil throughout North America which can be seen in Figure 1.2. This soil analysis was carried out by atomic absorption spectroscopy. The bright pink zones represent a total selenium concentration between 0.84 and approximately 5 mg/kg [26].

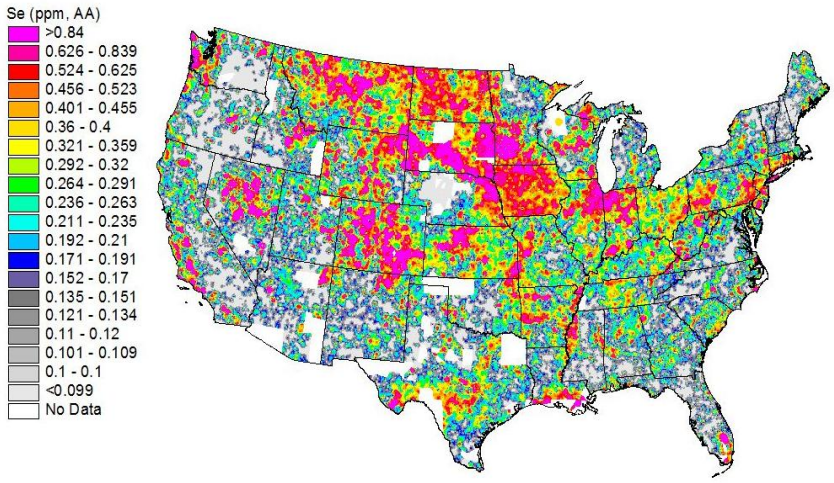


Figure 1.2: Map of selenium distribution in soil of the United States.
■ = High selenium areas [26].

Closer to home, the selenium levels in Irish soils are also highly variable. Seleniferous soils were found in poorly drained, low-lying, organic-matter-rich areas which had a mildly acidic to alkaline pH [29]. The primary source of selenium in Ireland is from Carboniferous black shales and limestones (e.g. Co. Meath). As the selenium leaches from these rocks into river flood plains or old lake beds, the selenium concentration of these soils will increase. In Ireland, these selenium toxic soils can have levels ranging from 5-1,000 mg/kg but the majority are between 10-50 mg/kg [27]. Areas of high selenium concentration were also found in the soils of west Limerick, with sporadic areas of South Tipperary and North Dublin also featuring elevated selenium concentration [27].

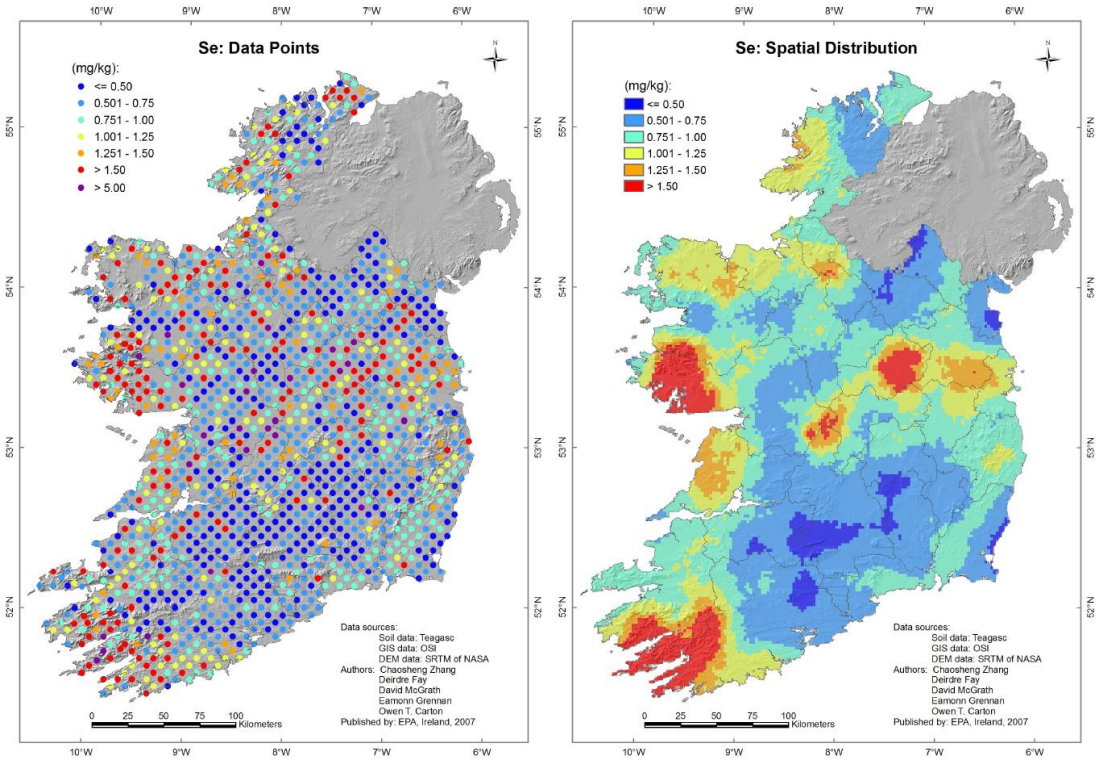


Figure 1.3: Map of selenium distribution in Irish soil.
Taken from Fay *et al.*[30].

However, the authors of 'Trace Elements and Heavy Metals in Irish Soils' duly note that because of sporadic selenium distribution these areas are not readily mapped at the scales usually employed [27]. The overall estimate of the area of selenium toxic soils (10-50 mg/kg Se) was in the order of 1,000 hectares. A breakdown of the selenium concentration across a seleniferous soil profile was carried out by Fleming and Walsh in 1957. A summary of these results is shown below.

Table 1.2: Selenium and organic matter in a seleniferous soil profile.

Soil depth (cm)	Organic matter	Se (mg/kg)
0 - 15	31.0	19.6
15 - 30	75.0	175.0
30 - 50	6.6	6.4
50 - 60	62.6	100.0
60 - 85	8.0	2.7

Adapted from McGrath *et al.* [27].

A more detailed study carried out by Teagasc and the Environmental Protection Agency in 2007 also found similar results. Fay *et al.* took a total of 1310 soil samples across the Republic of Ireland and analysed them for selenium concentration by atomic

fluorescence spectroscopy [30]. Low soil selenium levels are present in parts of Carlow, Cork, Tipperary, Waterford, Wexford and also in parts of East Galway [29, 30].

1.2 Biological role of selenium

Selenium is an essential trace element for the health of humans and animals alike. In humans, it is incorporated into enzymes and selenoproteins, that are involved in DNA synthesis, thyroid hormone production, antioxidant defence, reduction of inflammation, fertility and reproductive functions (Figure 1.4) [31]. Furthermore, selenium compounds have been regularly linked, investigated and reviewed for their anti-cancer properties [32-34].

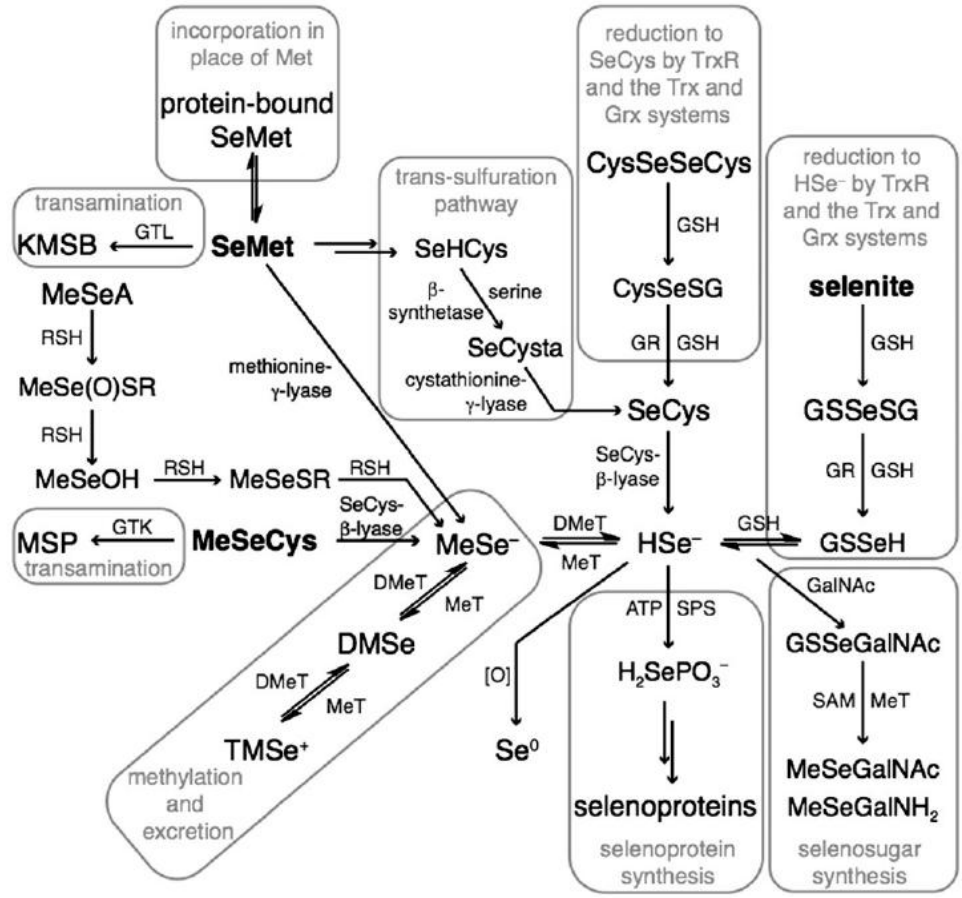


Figure 1.4: Metabolic and excretion pathways of dietary selenium compounds.
Taken from Mistry *et al.* [35] and Weekley *et al.* [11]. Includes metabolic pathway of selenomethionine (SeMet), elemental selenium (Se^0), selenite (SeO_3^{-2}) selenocysteine (SeCys), selenocystine ($SeCys_2$), selenohomocysteine (SeHCys), selenocystathionine (SeCysta), selenodiglutathione (GSSeSG), glutathionylseleno-N-acetylgalactosaminehydrogen (GSSeGalNAc), selenide (H_2Se), dimethylselenide ($(CH_3)_2Se$), trimethylselenonium ion ($(CH_3)_3Se^+$), glutathione (GSH), selenoglutathione (GS-SeH), Glutathione reductase (GR), glutamine transaminase K/L (GT K/L), dimethylselenide (DMSe), demethyltransfereases (DeMeT), methyltransferases (MeT), seleno-methylseleno-N-acetylglucosamine (MeSeGalNAc), seleno-methylselenogalactosamine (MeSeGalNH2), glutathionylseleno-N-acetylgalactosamine (GSSeGalNAc), selenophosphate synthetase (SPS), thioredoxin (Trx), thioredoxin reductase (TrxR), β-methylselenopyruvate (MSP), methylseleninic acid (MeSeA), sulfoadenosyl-L-methionine (SAM).

1.2.1 Selenium deficiency

The majority of Europe has always had poor soil selenium levels, but it is only in the last few decades that these low concentrations have started to affect the health of the human population. In the UK in the 1970's, adult selenium consumption was 60-63 µg/day; by the mid-1990's this had fallen to 29-39 µg/day [36, 37]. In Ireland, a selection of Irish foods, consumed by the majority of the population, was analysed for selenium content and compared to British, Canadian and American equivalents (Table 1.3).

Analysis of bread and flours used for bread making, clarify why there has been a drop in the selenium intake in the Irish and British population. The plains of North America contain higher levels of selenium in the soil than the UK and Europe, and the mid-Western plains are where most of the US wheat is produced. Therefore, this wheat contained higher concentrations of selenium and was exported to Europe for bread production [37, 38]. The reduced importation of wheat and flours (caused by import levies on Ireland and Britain when they joined the EU) containing higher concentrations of selenium from the USA and Canada has been replaced by lower selenium-containing produce [39, 40]. This reduction in selenium-enriched wheat and increase in the use of selenium-deficient home grown wheat has led to inadequate selenium intake. According to the European Food Safety Authority (EFSA), the recommended daily requirement of selenium for an adult is 55 µg regardless of gender. Children from 9-13 years of age have a lower requirement of 40 µg of selenium per day.

Table 1.3: Selenium levels in Irish, British, Canadian and American flours and bread.

	Ireland	**UK**	**Canada**	**USA**
	µg Se /100 g	**µg Se /100 g**	**µg Se /100 g**	**µg Se /100 g**
White Flours (plain, self-raising, bakers, and strong)	6.0-6.9	2.3 - 2.5	26.0-30.0	18.7-39.0
Wheat Flours	7.0-9.9	5.9	56.0-65.0	62.7-87.0
White Bread	6.6	4.3-4.4	42.0-67.0	27.4-32.0
Brown Breads	8.6-12.9	3.9-4.8	65.0-71.0	41.0-67.6

Adapted from Murphy *et al.* [39].

Deficiency of selenium is well documented in China. It is reported that a lack of selenium in the diet can lead to heart disease [11], hypothyroidism [41] and other

diseases such as Keshan disease (KD) (named after the Keshan district in China [42, 43]), and Kashin-Beck disease (KBD) [44]. A study of the prevalence of KD investigated 178 villages in 124 counties in 15 KD endemic provinces in China. Suspected KD patients, who had irregular electrocardiograms (ECG) results, were also examined using chest X-ray. Verification of KD diagnosis was based on the criteria for diagnosis of Keshan Disease [45]. Based on these findings, researchers estimated that there were ~1.7 million KD patients with approximately 0.4 million chronic KD patients and ~1.2 million potential KD patients [45]. The disease causes endemic osteochondropathy in people from regions of China with low selenium soil levels [44]. People with KBD suffer from impairment of the joints in the limbs. This consists of an enlargement of finger joints and dysfunction of wrists, elbows, knees and ankles but can also include the shoulders and hips [44]. Because selenium deficiency is acknowledged as the reason for the frequency of KBD, selenium supplementation was employed as a method for prevention of this disease. These supplementation methods include ingestion of selenium-enriched yeast, sodium selenite or selenate, or spraying vegetation with soluble selenium salts [45, 46]. The application of selenium supplementation in the majority of high prevalence KBD regions has been beneficial for the prevention of KBD [47].

In addition, selenium deficiency affects human reproduction and fertility, and examples are evident across Europe. A study carried out in Scotland found multiple instances of decreased fertility in men. This was diagnosed by the poor semen quality caused by consumption of only half the daily requirement of selenium [15, 48]. The relationship between selenium and increased male fertility is due to increased selenium concentration in testicular tissue which mainly takes the form of glutathione peroxidase 4 (GPx4). The GPx4 enzyme reduces hydrogen peroxide (H_2O_2), organic hydroperoxides and lipid peroxides. Additionally, it also plays an essential role in constructing the spermatozoan-midpiece thus connecting the importance of sperm quality and fertility with selenium [49]. Selenium is a crucial part of glutathione peroxidase (GPx) enzymes as each molecule incorporates four selenium atoms [50]. Selenium is also essential in male fertility due to its involvement in testosterone synthesis [50].

1.2.2 Selenium toxicity

Selenium, while essential, can be toxic in circumstances of high level chronic ingestion. This causes an accumulation of selenium and can lead to selenosis. Selenosis is accompanied by a number of symptoms such as loss of hair or nails, a garlic breath odour, itchy rashes, skin conditions and some gastrointestinal issues [35, 51, 52]. However, it must be noted that, while too much selenium can be toxic, it largely depends on the type of selenium present i.e. the selenium species. Relevant research has shown diets rich in selenium (Brazil nuts, chicken, meat and fish) have not demonstrated evidence of selenosis such as the population of the Brazilian Amazon [53]. Although, this could be due to confounding such as not measuring heavy metals as mentioned by Vinceti *et al.*, who also noted the specific selenocompounds in the diet were not determined [54]. However, studies administering as much as 800 µg Se/day (almost 15 times the RDA) over a period of years in the form of selenised yeast also showed no selenium toxicity [55]. Despite this, it must be noted that other studies show that selenosis is prevalent in areas such as China where the high selenium levels in the soil is absorbed by the dominant food source – rice. Ingestion of this selenium source leads to selenosis [16, 56]. This suggests that determining the concentration of selenium present in these foods, while important, is not sufficient information to determine toxicity or deficiency. To get a more detailed perspective of selenium content, analysis must be carried out to determine the chemical composition of the selenium present, i.e. selenium speciation. Pharmacokinetic studies suggest that after a single dose of 200 µg selenomethionine (SeMet), adults had an average SeMet turnover of 0.01-1.2 days in the plasma, 1.6-3.1 days in the liver and 61-86 days in the peripheral tissues [52, 57]. Similar plasma turnovers were reported by Wastney *et al.* although the study reported faster SeMet turnover in the liver of a few hours [57]. If selenium intake outweighs this turnover (which can vary person to person), then excess accumulation could occur thus leading to selenosis. Therefore, numerous factors such as individual metabolic rates, individual tolerance, size, gender, selenium species, quantity and frequency of selenium consumption, all play important roles in selenium deficiency or toxicity.

1.2.3 Selenium as a trace element

Selenium has established itself as an essential trace element in human health. Numerous studies have identified selenium's function in antioxidant enzymes [31, 58]. A

selenoprotein contains at least one selenocysteine (SeCys) residue in its amino acid sequence. A selenium-containing protein contains non-specifically incorporated selenium in the structure [4, 59]. This significance has led to selenocysteine gaining the title of the 21st amino acid [4].

As previously mentioned, it is also possible to have selenium non-specifically incorporated into proteins and other compounds. This inclusion of selenium is in place of the sulfur atom and in the case of methionine, for example, results in the formation of selenomethionine. These processes result in selenium-enrichment of plants and thus foods. As previously shown in Figure 1.1, Section 1.1, selenocompounds also follow the sulfur metabolic pathway. By taking advantage of specific selenium incorporation through SeCys or non-specifically through SeMet, nutritional supplements such as selenised yeast are produced.

1.2.4 Glutathione peroxidases

Glutathione (GSH) is a tripeptide that consists of glutamate, cysteine and glycine [14]. Glutathione selenocompounds such as selenodiglutathione were discovered in aqueous yeast extracts thus suggesting that it is an important intermediate/end-product in the selenium metabolic pathway [60]. Earlier research on glutathione had established its ability to maintain the redox balance within cells before its function as a selenoprotein peroxidase [61]. Glutathione peroxidase (GPx) was first proven to contain stoichiometric amounts of selenium in 1973 by Flohé and co-workers thus identifying GPx as a selenoenzyme [62]. These selenoenzymes, such as glutathione peroxidase and thioredoxin reductase (TRx), have antioxidant capabilities, thus reducing oxidative damage [49, 63]. The primary activity of these enzymes is to prevent cell and tissue damage by reducing hydrogen peroxide and organic hydroperoxides. This in turn, decreases the level of free radicals, such as reactive oxygen species (ROS) [64], present in the cells leading to less cell damage [49, 63]. Other selenoproteins such as iodothyronine deiodinase (DIO) enzymes are essential for thyroid function and hormone release [65].

The first six members of the glutathione peroxidase family all have prominent antioxidant characteristics. GPx1-4 are found in mammals and contain a selenocysteine catalytic centre, GPx5 has been linked to male fertility and GPx6 is specifically a human selenoprotein [66, 67]. The GPx features are spread through the body but mainly

occur in the cell cytosol and mitochondria (GPx1), the gastrointestinal tract, specifically the intestinal epithelium (GPx2), the extracellular space and plasma (GPx3) and in the cell membrane and sperm (GPx4) [66]. The first glutathione peroxidase discovered was GPx1 which predominantly reacts with hydrogen peroxide and soluble low molecular mass hydroperoxides (t-butyl hydroperoxide, cumene hydroperoxide, hydroperoxy fatty acids, hydroperoxy lysophosphatides) [66]. GPx2 functions by protecting mammals from ingested lipid hydroperoxides. GPx4 is often referred to as phospholipid hydroperoxide GPx because of its detoxification of lipid peroxides, while GPx5 has restricted expression in the epithelium and is known as the epididymal GPx. Being epididymis-specific, GPx5 has been linked to fertility by protecting mammalian sperm membranes from lipid peroxidation [67]. GPx6 is located in the olfactory epithelium and embryonic tissues, and its reported function is antioxidant protection [68]. GPx7 and GPx8 are cysteine containing GPx's (CysGPx) and have reduced GPx activity [66]. In nature, it is reported that there are more than 700 CysGPx homologous sequences identified; of these, only a minority contains selenium [66].

1.2.5 Animal health - Selenium deficiency and toxicity

In animals and livestock, selenium deficiency diseases were recognised in the mid-twentieth century and were a result of inadequate soil selenium levels in numerous countries [69]. This deficiency of selenium was linked to White Muscle Disease (WMD), reproductive impairment and a myopathy of heart and skeletal muscle mainly affecting lambs and calves [15, 70, 71]. The condition of WMD also manifests itself in adult animals as a walking impediment and through irregular postural positions. In addition, it is widely known that selenium supplementation can reduce, among other things, the incidence of cancer in animals [72]. These diseases caused by selenium deficiency can affect all animal species, but ruminants seem to be more susceptible, with a higher severity in small ruminants like goats and sheep [73]. It has also been observed that some abortions in ruminants are caused by a selenium deficiency, a trait which can be reversed by the supplementation of selenium [74].

Ruminants have a higher vulnerability to selenium deficiency because of the reticulo-rumen environment. Certain microorganisms and bacteria in the rumen will also consume selenium. This competition coupled with the formation of insoluble forms of selenium due to conditions within the reticulo-rumen such as pH result in significant

reduction of selenium available to the animal for absorption [75]. Decreased absorption caused by a deficiency of the element or inappropriate chemical form has a knock-on effect to the health of animals (and humans). Relevant research has shown selenium deficiency causes a decrease in blood levels of immunoglobulins M (IgM), G (IgG) and A (IgA) in rats and a reduction in IgG and IgM levels in humans [12]. Immunoglobulin A is found mainly in mucous membranes, saliva and tears. Immunoglobulin G is found in all body fluids and IgM is mostly found in the blood and lymph fluid and constitutes the first antibody to be made by the body to fight a new infection [76].

Dietary and soil selenium deficiencies can be reversed by selenium fertilisation of pasture, thus increasing the selenium concentration of hay [77]. This approach was found to be an effective method of selenium supplementation to meet dietary requirements of certain animals [77]. Unless their diet contains sufficient selenium for their needs, then selenium supplementation is necessary to ensure animals remain healthy. However, research suggests that the effects of this supplementation are dependent on the composition or chemical form of the selenium supplied which will be expanded on in more detail later.

1.2.6 Plants

Plants, like animals and humans, need essential nutrients to grow and prevent bacterial, fungal and viral infections [41]. A deficiency of nutrients can lead to poor plant health. Humans are also affected by nutrient deficiencies and since the majority of our elemental dietary demand is obtained either directly or indirectly from plants (fruits and vegetables) or animals (who eat plants), respectively, plants are closely correlated to human and animal health.

Inorganic selenium was previously mentioned in relation to production (Section 1.1.1) and geology (Section 1.1.2). However, organic selenium is obtained from the conversion of inorganic selenium to organic selenium in plant metabolic pathways [41]. This process also occurs in yeast and is applied as a method of selenium enrichment [78]. Plants are the primary source of selenium in the human diet, so it is essential to understand plant selenium metabolism for human health and nutrition [79].

1.2.6.1 Selenium and sulfur metabolism

As noted previously, selenium is very similar in chemical characteristics to sulfur (Table 1.4), which is why selenium and sulfur compete in sulfur metabolic pathways

[28]. A carbon-sulfur bond is 1.8 Å (1 Å = 0.1 nm), but the carbon-selenium bond is only slightly longer at ~1.95 Å [80, 81]. While sulfur is available to plants as a sulfate or sulfite, selenium is present as selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}). Selenate transportation across the plasma membrane of the root epidermal cells is caused by electrochemical gradients [82]. The uptake process for each selenate ion is driven by co-transportation of three protons [82]. The pH of the majority of aqueous and aerobic sedimentary soils allows for the existence of selenium as an oxyanion in selenate, selenite and also as a biselenite ($H_3SeO_3^-$) [83]. Elemental selenium may be found in more reduced sediments. Chemical differences exist between these elements, therefore not all biochemical processes utilising selenium will also incorporate sulfur and vice versa [82]. Selenium is a larger atom compared to sulfur, with radii of 0.5 and 0.37 Å, respectively. This difference translates to longer and weaker bonds between two selenium atoms than between 2 sulfur atoms [4, 82].

Table 1.4: Comparison of sulfur and selenium atomic and elemental properties.

	Sulfur	**Selenium**
Atomic Weight	32.07	78.96
Atomic Number	16	34
Electronic Configuration	(Ne) $3_s^2\ 3_p^4$	(Ar) $3_d^{10}4_s^24_p^4$
Covalent Radius Å	1.05	1.16
Atomic Radius Å	1.00	1.4
Ionic Radius Å	1.84	1.98
Common oxidative states	-2, 0, +2, +4, +6	-2, 0, +4, +6
Ionisation Potential, eV	10.36	9.75
Electronegativity	2.58	2.55

Adapted from Ullrey *et al*. [84].

Nevertheless, certain plants can metabolise and accumulate up to a few thousand parts per million of selenium as dry mass. An example is the genus *Astragalus* which can accumulate 0.6 % selenium in their shoot tissues [85]. These hyperaccumulating plants mainly belong to the *allium*, *leguminosae*, *cruciferae* and *compositae* families [86].

Selenium is incorporated as multiple selenocompounds and in various quantities in different plants as a result of diverse metabolic pathways. In selenium-accumulating plants and the root system of garlic and onions, the most abundant selenium compound

is Se-methylselenocysteine [15, 87]. The incorporation of selenium into garlic is thought to greatly enhance garlic's cancer prevention and inhibition capabilities [87, 88]. Selenocysteine, selenomethionine and Se-methylselenocysteine were discovered in onions and garlic [88, 89].

Relevant research suggests that selenium uptake by plants is largely dependent on the soluble selenium levels in the soil rather than the total selenium figures that are mainly reported [90]. Further evidence supporting the importance of selenium speciation development can be seen in numerous countries. In Hawaii, the soil total selenium concentration varies between 6 and 15 mg/kg [91, 92]. While these levels are considered high, they do not create any selenium toxic vegetation or plants. The same cannot be said for South Dakota or Kansas in the United States which have less than 1 mg/kg of total selenium in their soils but do produce seleniferous *Astragalus* vegetation [91]. Therefore, total selenium analysis of the soil is only part of the speciation information needed since the form of the selenium species and the type of plant will determine accumulation. In addition, soluble selenium in the soil is fundamentally reliant on physiochemical properties and biological conditions of the soil, some of which may increase or decrease the soluble selenium fraction available for bioaccumulation in plants [91]. These include, but are not limited to; soil pH; soil type; organic matter content - iron hydroxides can bind selenium and minimise bioavailability to plants; presence of sulfur species from fertilisation which can compete with selenium for absorption; leaching of selenium from soil due to rainfall and efficiency of soil microbes that can convert insoluble selenium to soluble forms for absorption by plants [55, 91]. Furthermore, high soil pH favours selenium anion adsorption on to clays and metal oxides thus selenite is adsorbed more strongly than selenate, leaving selenate the dominant form for plant uptake [93]. However, inorganic selenides and elemental selenium are typically insoluble except when present in low pH, wet, reducing conditions [93]. This justifies the need for the development of selenium speciation techniques to calculate the composition of the soluble selenium and total selenium present in soils. Additionally, these techniques are fundamental to determine the selenium content in hyper-accumulating plants but also food supplements too.

As mentioned, plants assimilate selenium non-specifically into compounds that usually contain sulfur, leading to selenium-enriched vegetables, such as onions, broccoli and garlic. The main selenium species in these vegetables are Se-methylselenocysteine

and γ-glutamyl-Se-methylselenocysteine (Figure 1.1). Research suggests that these methylated forms of selenium are more efficient tumour inhibitors compared to other organic (SeMet) or inorganic species (Se (IV)) [94-96]. These examples highlight why selenium speciation is beneficial. If some plants accumulate tumour-inhibiting selenocompounds, detecting and then quantifying them would be advantageous.

1.2.7 Yeast and selenised yeast

Yeasts are ubiquitous, eukaryotic microorganisms classified in the kingdom Fungi. They have a rigid cell wall which is extremely robust. The cell wall composition is not identical for each strain. Therefore, the extraction procedure to liberate intracellular components from selenised yeast must take into account these differences in the cell wall structure. Yeast is predominantly used for baking, brewing and selenium supplementation. The yeast strain of choice for brewing and baking is *Saccharomyces cerevisiae*. This strain is also widely used for selenium enrichment [17, 97]. *Saccharomyces cerevisiae* yeast cells are round and oval in shape with a diameter of ~5-6 µm but other types of yeast can grow to ~40 µm in diameter [98, 99]. Reproduction takes place by budding where another yeast cell grows out from the parent yeast and splits off forming a new identical yeast cell. The *S. cerevisiae* cell wall can represent between 20-30 % of cellular dry mass, which consists mainly of mannoproteins and β-glucans as well as lipids and chitins [100]. The cell wall contains two layers, the inner and the outer layer. The outer layer of the yeast cell wall (YCW) is dominated by glycosylated mannoproteins which limit intracellular access by foreign enzymes. Chitin and β-glucans primarily make up the inner layer. β-1,3-glucan gives the cell wall its strength due to its triple helical structure and accounts for 85 % of glucan in *S. cerevisiae* [100].

The production of yeast is carried out by fermentation. This technique is highly dependent on the rate that substrate feed is added, the quality of the desired product, time and the formation of ethanol which has an inhibitory effect on the biomass productivity thus reducing yield and the specific growth rate of biomass [101].

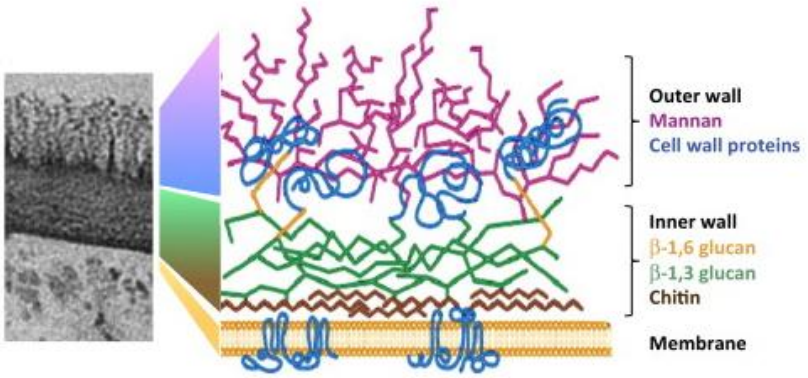


Figure 1.5: Yeast cell wall structure.
Taken from Brown *et al.* [102].

1.2.7.1 Production of selenised yeast

A fed-batch fermentation method for the production of selenised yeast was reported and patented in 1985 [103]. The selenised yeast itself is produced by aerobic fermentation of baker's yeast [55] that incorporates water-soluble selenium salt as a part of the culture medium. This is typically in the form of sodium selenite (Na_2SeO_3) and results in a high uptake of selenium into the yeast [17, 104]. The inorganic selenite is converted to organic selenium species with improved nutritional properties [105, 106]. The critical parameters for selenium incorporation are nutrient addition rates, dissolved oxygen in the culture, temperature, pH value and strain specific consumption [104]. The base medium and nutrient feed for selenised yeast fermentation can vary but commonly consist of molasses, urea, $(NH_4)_2HPO_4$, KH_2PO_4, H_3PO_4, $MgCl_2$, NaCl, $CaCl_2$ and NH_4Cl [107, 108]. The base medium also contains a selection of amino acids, vitamins and trace elements as seen in Table 1.5.

Table 1.5: Concentrations of vitamins, amino acids and trace elements utilised in the base medium.

Vitamin	mg/L	Trace element	mg/L	Amino acid	mg/L
Biotin	0.002	H_3BO_3	0.50	Adenine	10
Pantothenate, Ca	0.4	$CuCl_2$	0.04	L-arginine, HCl	50
Folic acid	0.002	KI	0.10	L-aspartic acid	80
Inositol	2.0	$FeCl_3$	0.20	L-histidine	20
Niacin	0.4	$MnCl_2$	0.40	L-isoleucine	50
PABA	0.2	Na_2MoO_4	0.20	L-leucine	100
Pyridoxine, HCl	0.4	$ZnCl_2$	0.40	L-lysine, HCl	50
Riboflavin	0.2			L-phenylalanine	50
Thiamin, HCl	0.4			L-threonine	100
				L-tryptophan	50
				L-tyrosine	50
				Uracil	20
				L-valine	140

Taken from Ouerdane *et al.* [107].

Selenium is readily assimilated into proteins by yeast due to its similar chemical properties to sulfur (Table 1.4). The amount of selenium that a yeast cell can accumulate depends on the sulfur and methionine content but can be as high as 6,000 mg/kg [109]. However, the majority of selenised yeast products have concentrations of ~3,000 mg/kg selenium with commercially-available yeasts usually between 500-2,500 mg/kg of selenium [109]. The International Union of Pure and Applied Chemistry (IUPAC) defines dry food yeast as "the whole organism of one individual yeast, or a mixture of several yeasts belonging to the family *Saccharomycetaceae*, obtained either as a by-product of fermentation processes or by special culture" [109]. Yeasts are chemoorganotrophs, they obtain their energy and carbon source by oxidation of organic compounds. Their primary energy source during the selenium-enriched yeast production process are carbohydrates which are in the form of beet/cane molasses. The culture medium also contains vitamins and nutritional salts to maximise biomass. Once the fermentation is finished, the yeast can be spray-dried into a fine powder for encapsulation or packaging. The image in Figure 1.6 was captured by a scanning electron microscope (SEM) to show the finished product of commercial spray-dried yeast. Selenium speciation and total selenium analysis of the yeast product can be used to determine the success of the fermentation. Measurement of selenomethionine by an enzymatic or chemical extraction is the preferred method and is discussed in more detail in Section 1.4. The yeast is also screened for heavy metals like As, Cd, Pb and Hg since

yeast is very efficient at accumulating metals [110], and this ensures product safety prior to consumption. Baker's yeast is classified as generally regarded as safe (GRAS), therefore it can be used for production of a selenium supplement for consumption by humans and animals [111]. As such, selenised yeast is used for selenium supplementation in ruminants to increase milk selenium content which subsequently has beneficial use for human consumption [112].

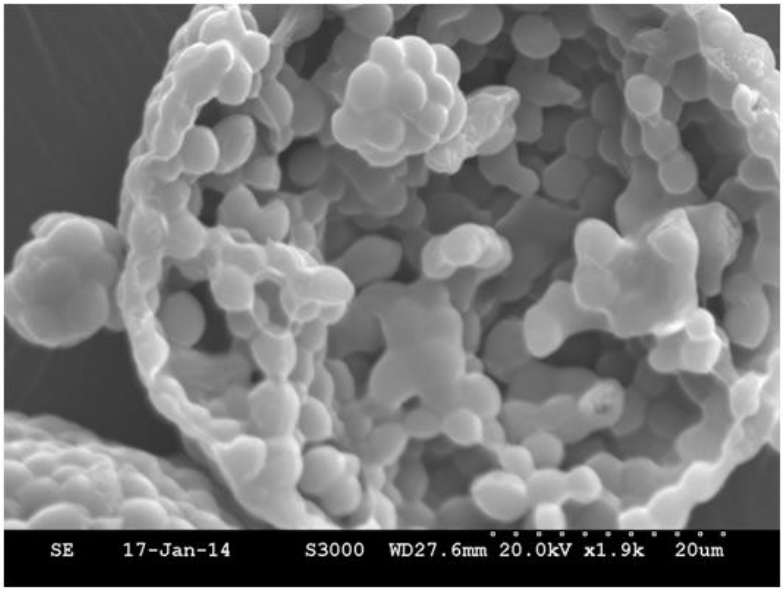


Figure 1.6: Hitachi S3000 Scanning electron microscope (SEM) image of spray dried Saccharomyces cerevisiae (magnification x1.9k).

1.3 Selenium speciation analysis

1.3.1 The importance of selenium speciation

The IUPAC defines speciation as "the process yielding evidence of the atomic and molecular form of an analyte" [113] and over the last two or three decades, selenium speciation has developed considerably in its precision, accuracy and necessity. It is no longer sufficient to solely determine the total selenium content of soil, tissue or food; instead, it is essential to identify what forms of selenium are present [114, 115]. Is the selenium present an inorganic or organic species? Is the selenium bioavailable and/or bioaccessible? Bioavailability is defined as 'the total amount of a chemical present in a specific environment', that is available or can be made available to plants [116]. Similarly, bioaccessibility, is defined as the total amount of a chemical present, in ingested food, for example, that can release during digestion [116].

Selenium can exist in many different chemical forms. Accurate speciation capabilities are necessary as different species can ultimately have varying toxicities depending on their concentrations [117]. The dominant inorganic species in soil are selenate (Se VI) and selenite (Se IV), which are soluble in water, unlike insoluble elemental selenium (Se 0) and metal selenides (Se -II) [118, 119]. In biological systems, and in particular the human body, organic selenium is the dominant form, with selenocysteine (SeCys), selenomethionine (SeMet) and methylated selenium compounds present in selenoproteins and selenoenzymes [119, 120]. Speciation analysis of selenium species is crucial, especially since the bioavailability of each selenium species will differ. Research has proven that organic selenium is less toxic and more bioavailable than inorganic selenium to animals and humans [115, 121]. Selenium supplementation trials have been carried out on cattle [121, 122]. Analysed samples included milk, urine, blood, plasma, tissue and sometimes rumen contents. These samples were analysed for selenium species content and results were compared back to the original supplementation dose. This allowed for in-depth knowledge of the selenium distribution and accumulation in the animal [121, 122]. Selenium accumulation in humans has also been documented for whole blood, plasma, serum, hair and nails [123].

Speciation analysis is also used for product determination and verification. Methods were developed for total selenium analysis of feed and supplements [124, 125]. However, this provides us with only a piece of the speciation information available. It is possible for two commercial samples to contain the same concentration of total selenium but due to the presence of different selenium species, each may have varying efficacy. One sample may be formulated with inorganic selenium while the other might contain an organic form of selenium, such as SeMet. Once there is sufficient digestion, the sample which contains the majority of its total selenium as an organic species (SeMet, SeCys) will be better absorbed in the small intestine [126]. This means more selenium speciation information must be reported along with total selenium analysis [9, 11, 127] unlike previous research that only looked at the total selenium content [124, 125, 128]. Furthermore, yeast strains may not assimilate inorganic selenium during fermentation at the same inclusion rates. This leads to variation in SeMet levels, resulting in two entirely different products. Research has also shown that supplemental selenomethionine has twice the bioavailability over selenite [129].

1.3.2 Selenomethionine

Selenomethionine is the primary selenoamino acid in cereal grains, grassland legumes and soybeans [87] and is the preferred source of selenium supplementation due to its increased bioavailability and reduced toxicity compared to selenite [130]. Selenomethionine (2-amino-4-(methylseleno)-butanoic acid) is the selenium analogue of methionine and has both D- and L- enantiomers. In water, selenomethionine is less soluble than methionine due to the greater hydrophobicity of the CH_3-Se group compared to the CH_3-S group [130].

The substitution of selenomethionine for methionine does not alter protein structure but may alter enzyme activity if incorporated near the active site [131]. Absorption of selenium into the yeast and subsequent production of selenomethionine can account for almost 74 % of total selenium [55]. Selenomethionine is metabolised by the same enzymes as methionine and at similar rates. In relation to health, *in vitro* and *in vivo* studies on selenomethionine have shown anticarcinogenic properties for prostate and colon cancer [132]. Selenomethionine is an important pharmacological agent against methyl mercury induced neurotoxicity [133, 134] and cisplatin-induced nephrotoxicity [135, 136]. Selenomethionine is absorbed into the body via the small intestine and becomes incorporated into the body's reserves [131, 137, 138]. Absorption of SeMet occurs through the methionine transporter system whereas inorganic selenium, such as selenite/selenate, is absorbed by passive diffusion which is less efficient [139]. Furthermore, the storage of SeMet allows it to be retained in the body longer than other selenium species [140]. Fermentation of selenised yeast is one of the most economical ways to produce SeMet which makes it one of the most studied and preferred selenium food sources [137]. All selenium sources must undergo metabolic transformation to selenide before incorporation into SeCys and then into selenoproteins. This is not necessary for SeMet which allows for quicker and more efficient protein assimilation [141].

1.3.3 Oxidation of selenomethionine

Methionine residues in proteins can undergo oxidation to form methionine sulfoxide and sulfone. Selenomethionine also undergoes oxidation creating methionine selenoxide and selenone [142]. The oxidation of methionine has both a biochemical and

physiological role [143]. However, oxidation of SeMet to methionine selenoxide is reversible as demonstrated in the following equation (Figure 1.7);

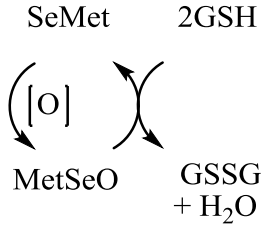


Figure 1.7: Reduction of methionine selenoxide to selenomethionine by glutathione.
Glutathione (GSH). Adapted from Assman *et al.* [142].

Although Met and SeMet oxidise similarly, they both undergo different reduction pathways. While methionine selenoxide reduces by reaction with glutathione (GSH), methionine sulfoxide requires the enzyme sulfoxide reductase [143].

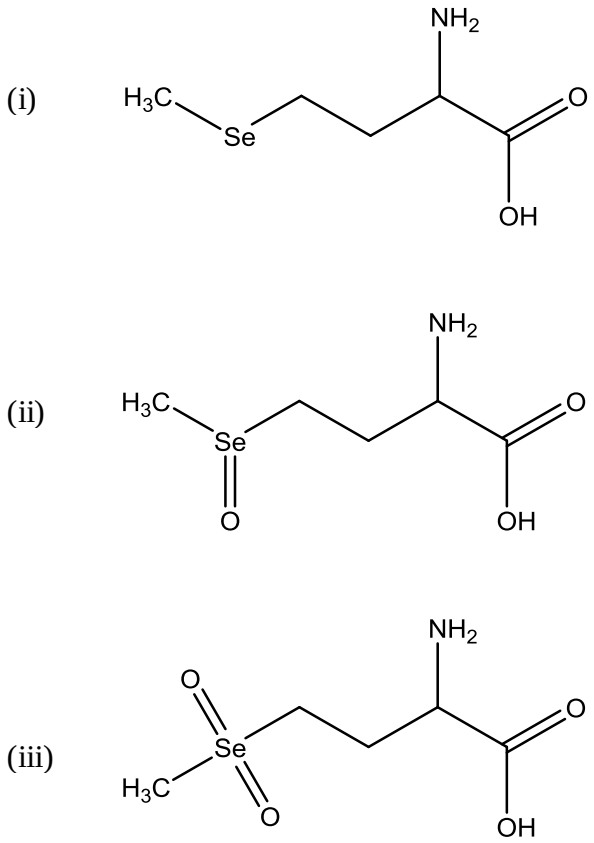


Figure 1.8: Structures of selenomethionine oxidation products.
(i) selenomethionine, (ii) methionine selenoxide and (iii) methionine selenone.
Taken from Read *et al.* [144].

1.3.4 Chiral speciation

The chirality of an enantiomerically pure sample may be determined by the way it rotates plane polarised light. Chiral compounds with no plane of symmetry or point of symmetry are thus described as being left or right handed. Left-handedness is denoted by L for laevorotatory (left) while right-handedness is denoted by R for dextrorotatory (right). Chiral speciation describes the analysis and resolution of chiral compounds and is an important technique due to the substantial physiological similarities and differences between such compounds. The existence of chiral compounds and their biological importance was identified by Pasteur in the 1800's [145]. Resolution, in relation to stereochemistry, was detailed by IUPAC as the separation of racemic compounds into their enantiomers [146].

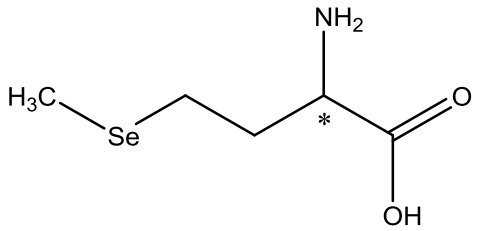


Figure 1.9: Structure of D, L-selenomethionine.
An asterisk [*] represents the chiral centre. Adapted from Nagar *et al.* [147].

The structure of SeMet allows it to be physically present as both the D- and L- conformations due to its single chiral carbon centre (Figure 1.9). While both conformations are possible, the L- form of the selenoamino acid is biologically favoured [148]. The pharmacological differences between these enantiomers to moderate toxic agents have not been examined thoroughly, but studies have shown both D- and L- conformations can offset methylmercury toxicity in *Chlorella sorokiniama* [149]. Moreno *et al*. [149] suggested that it was primarily the L-enantiomer of SeMet that detoxified methylmercury and that the D-SeMet enantiomer had to be converted to L-SeMet to be effective. Furthermore, studies carried out on humans and rats have shown that the difference in bioavailability between D- and L-SeMet is negligible but that D-SeMet is converted to L-SeMet *in vivo* [148, 150].

One of the main metabolites present in the urine of people with diets containing excess selenium is the trimethyl selenonium ion (TMSe). This metabolite is absent in normal or low level selenium diets [151] which may explain the absence of TMSe in studies by Kuehnelt and co-workers [152]. After supplementation with L-SeMet and D,L-SeMet, analysis of human urine showed that the SeMet enantiomers were

metabolised differently resulting in varying excreted concentrations, the major metabolites being selenosugars [152, 153]. Other relevant research examined ways to determine the conversion of D-SeMet into L-SeMet for pharmacokinetic purposes [154]. While some research was carried out on chiral enantiomers of SeMet, these reported differences demonstrated the necessity for the resolution of chiral species, especially due to the large variations in enantiomer activity. Chiral resolution is defined by IUPAC as the separation of a racemate (an equimolar mixture of a pair of enantiomers) into the component enantiomers (in this instance; D- and L-SeMet) [146]. However, enantio-resolution of non-racemic mixtures were also of importance since not all biological enantiomer mixtures are equimolar, such as SeMet in selenised yeast.

1.3.4.1 HPLC for chiral speciation

The most recent and detailed chiral speciation review of selenoamino acids in biological samples was in 2014 [155]. This comprehensive publication documented the analytical techniques used and stated there were only about 30 papers specific to the chiral speciation of selenoamino acids.

Speciation of certain compounds may need more complex sample preparation such as the inclusion of derivatisation steps. Chiral speciation by HPLC can be divided into two sections; indirect or direct enantioseparation [156]. Indirect methods employ derivatisation steps to produce diastereoisomers which can then be separated by GC or HPLC [157, 158]. Derivatisation was employed with GC speciation before detection of selenoamino acids. This action is necessary to enhance the volatility of the compounds for accurate MS detection [159]. The most widely used reagent for indirect derivatisation is Marfey's reagent, a chiral variant of Sanger's reagent, 2,4-dinitrofluorobenzene (2,4-DNFB) [160, 161].

Direct enantioseparation is carried out with a chiral chromatographic column. There were only a few commercially-available columns referred to in the literature up to 2014 detailing chiral separation of selenoamino acids. The columns of choice were the Cyclobond 1 β-CD, Daicel Crownpak CR(±) and the Chirobiotic T [155, 162].

As described by Claus *et al.* the Chirobiotic T column was used to resolve D- and L-methionine enantiomers (Figure 1.10). Based on the chemical similarities between methionine and selenomethionine, similar resolution should also be achieved for D- and L-selenomethionine. Another positive for using this method is the possible transfer across to LC-MS for identification of selenocompounds if necessary.

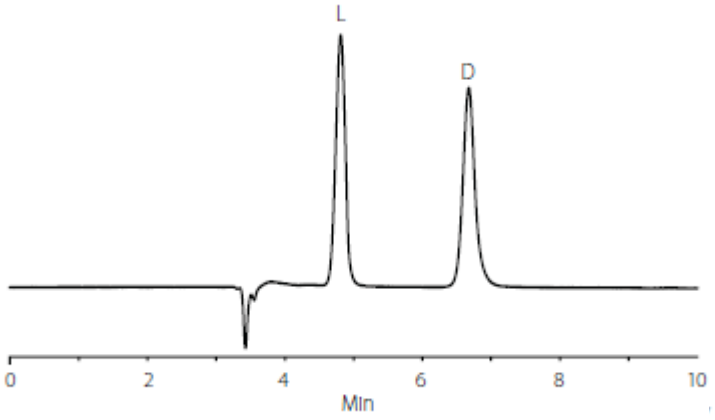


Figure 1.10: Chromatogram of D, L-methionine on Astec Chirobiotic T.
Flow Rate; 1.0 mL/min
Temperature; 25 °C
Injection volume; 10 µL
Mobile phase; water: methanol: formic acid (30:70:0.02)
Taken from Claus *et al*. [163].

When examining yeast, Egressy-Molnár *et al*. determined the D-SeMet concentration in SELM-1 to be 0.5 ± 0.03 % and 2.2 ± 0.5 % of total selenium content after different enzymatic and acidic extraction methods, respectively [164]. Enzymatic extractions were favoured over chemically harsh methanesulfonic acid (MSFA) extractions since some L-SeMet will convert to D-SeMet due to the MSFA [164]. Therefore, a water extract or enzymatic extraction is best for accurate D- and L-selenomethionine resolution.

The least concentrated SeMet enantiomer in selenised yeast, from current literature, was D-SeMet [155, 164], with ratios of 18:82 for D- and L-SeMet [155]. However, Chen *et al*. noted that not all studies detected D-SeMet, perhaps due to the insufficient sensitivity of their analytical methods [162]. Free SeMet is vulnerable to oxidation, degradation and isomer inversion [165]. Recent research from 2015 reported that, in rats, approximately 60 % of intravenously injected free D-SeMet was converted to L-SeMet [148]. This study also proposed a possible metabolic pathway for the chiral conversion of D-SeMet into its L-form, Figure 1.11.

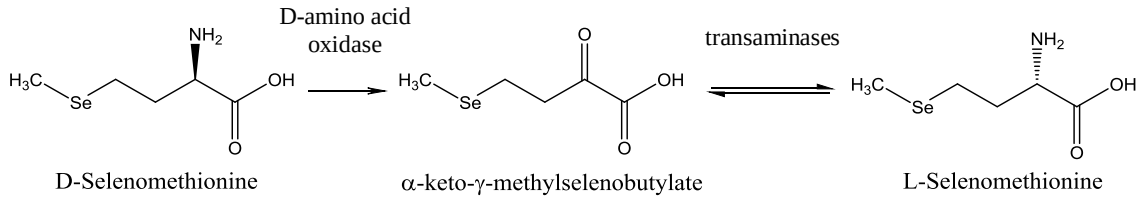


Figure 1.11: Proposed conversion of D- to L-selenomethionine.
Taken from Matsukawa *et al.* [148].

Direct enantioseparation of chiral compounds by HPLC is possible (Figure 1.10). When ICP-MS is coupled to an HPLC system, it is a very sensitive speciation analytical instrument capable of parts per trillion levels of detection. A hydrogen collision reaction cell (which is discussed later in greater detail, Section 1.5.3) is ideal for selenium analysis and helps reduce background interference caused by the Ar-Ar dimer which has the same mass as ^{80}Se. Certain ICP-MS models can only tolerate a certain percentage of solvent passing into its argon plasma, this was ~30 % for the 7700× ICP-MS model. To combat higher levels of solvent, such as the ~50-70 % MeOH that would be needed for chiral SeMet separation [163], the ICP-MS needs to be altered to withstand these high organic solvent levels. This is discussed in more detail in Chapter 4.

1.4 Sample preparation

Extraction methods for SeMet determination vary from water [166], accelerated solvent extraction [167], chemical (acidic and alkaline) [78, 168] and enzymatic extractions, [169-171] to more recent microwave-assisted extractions [117, 172, 173]. Food samples and in particular, milk and cheese samples need defatting and protein precipitation steps for optimum species determination [174]. However, some samples require little or no extra preparation for analysis such as cerebrospinal fluid (CSF) which can be analysed for total selenium by direct infusion into an ICP-MS. Water extracted samples can also be analysed directly for selenium species by HPLC-ICP-MS thus providing information about water-soluble compounds like unbound free selenomethionine [175]. More complex samples need more vigorous extraction methods such as a chemical approach with strong acids (HCl) or enzymatic extractions with a cocktail of enzymes [176]. There are issues with both extraction methods as unfortunately, use of a strong acid may liberate the intracellular components but can also degrade the selenium compounds and

enzymatic extractions depend on the consistency of enzyme activity in different batches of product [78, 176]. Enzymatic hydrolysis is often favoured over chemical hydrolysis for its mild temperature (~37 °C) and pH (5-9) conditions [177]. Temperate conditions like these can also minimise analyte loss caused by chemical volatilisation. Enzymes are highly selective, allowing for the precise targeting of only certain bonds [178]. Numerous studies use protease XIV to cleave peptide bonds in proteins to determine low molecular weight selenocompounds [179, 180]. One problem with conventional enzymatic extractions for selenium speciation analysis has been the long extraction times. These enzymatic extractions were 24 hours long and were repeated up to three times (72 hours) which involved changing the enzyme solution every 24 hours [181, 182]. Not only is this approach time consuming, but it also increases the chances of sample loss due to excess steps and degradation losses to oxidation. Recently, considerable time and cost savings were made possible by incorporating microwave digesters to extract intracellular components from biological samples, thus reducing extraction times. Microwave digesters were largely used for total elemental determination from as far back as 1975 [183, 184] and they have continued to be utilised throughout the years up to the present day, where they are also applied to speciation assays [185-188].

1.4.1 Enzymatic extraction

Selenomethionine analysis carried out by contract and research laboratories worldwide initially used enzymatic extraction methods. These methods examined enzymes such as protease, lipase and driselase to break down the glucan and mannan-based yeast cell wall (Figure 1.5) [185]. One of the most popular methods, which typically recovered 95-100 % of SeMet, was a three-day proteolytic enzymatic extraction described by Mester *et al.* [78]. This proteolytic extraction was part of an overall study to certify a selenised yeast reference material, SELM-1, and was employed by the Pau research group [169]. The method used a 0.2 g subsample of the selenised yeast which was extracted with 5 mL of 30 mM Tris-HCl (pH 7.5) solution that contained 20 mg of Protease XIV and 10 mg of lipase. The sample was incubated for 17 hours at a temperature of 37 °C and centrifuged to obtain a supernatant. This first step was repeated twice more. To reduce oxidation of the extracted SeMet, 5 µL of β-mercaptoethanol (0.1 %) was added to the supernatant. The three supernatants were

pooled for total selenium and SeMet analysis. However, some of the major drawbacks obviously include time constraints, cost of enzymes and variable activity of enzymes. Such a long extraction process, including the analysis time, means SeMet results are not available until at least 5 days after receiving the sample. Therefore, there was a need to increase sample throughput and thus develop a much faster extraction procedure for SeMet determination. To reduce the cost of enzymes, other laboratories extracted SeMet with methanesulfonic acid (MSFA). Switching to MSFA also had the benefit of avoiding enzyme activity issues (e.g. variability) which were documented by other researchers who worked with Protease XIV [128].

1.4.2 Chemical extraction

Chemical extraction of selenium compounds from selenised yeast provides a cheaper alternative to the proteolytic extraction discussed in Section 1.4.1 and typically, it is a lot quicker too. Instead of three 24 hour extractions, it can be reduced to one 16 hour reflux extraction [189]. Three of the participants in the certification of SELM-1 applied some variation of the MSFA reflux extraction protocol and 4 of the remaining 7 participants employed the proteolytic extraction procedure [78]. The typical chemical extraction involved boiling ~0.2 g of selenised yeast under reflux in 10 mL of 4 M MSFA [190]. After the reflux was completed, samples were centrifuged and 1 mL aliquots of the supernatant were taken either for derivatisation and/or dilution in deionised water [190]. Other research has also investigated chemicals to extract selenocompounds including hot water [32, 191, 192], HCl [193-195], TMAH [191, 195] and MeOH [191] but more recently, investigators have started to investigate the use of microwave-assisted extractions for both chemical and enzymatic extractions.

1.4.3 Ultrasonication

Ultrasonication refers to frequencies greater than 20 kHz [196]. Ultrasonication was incorporated in numerous experiments over the years primarily for the purpose of accelerated cell lysis [197] or leaching of analytes from samples [198]. The two main types of instrumentation are ultrasonic baths and ultrasonic probes. Both sonication techniques have distinct benefits. Ultrasonication baths allow the ultrasonication of a sample in a container, such as a beaker or test tube, either partly or entirely submerged in water. Moreover, the sample can be contained in a sealed vessel, so there is no risk of

sample loss or contamination. In addition, it is possible to ultrasonicate multiple samples at one time compared to just one sample with the ultrasonication probe. It should be noted that multiple pronged ultrasonication probes are also available on the market but are significantly more expensive than a single probe. The ultrasonic energy imparted to the sample by an ultrasonic bath nevertheless tends to be much lower than that of an ultrasonication probe. However, sample volume size can be much bigger depending on the size of the ultrasonication bath. The ultrasonication probe can provide a higher energy as its ultrasonication energy is imparted over a smaller surface area. Furthermore, because the surface area of the probe head is extremely small, it allows for the ultrasonication of very small sample volumes and it is not uncommon to sonicate sub 200 µL volumes. Another benefit of an ultrasonication probe is the ability to change the titanium coated probe tip in the event of degradation or when treating even smaller sample volumes. In addition, probe ultrasonication ruptures yeast cells and liberates intracellular components such as SeMet [127, 187]. Sonication of selenised yeast is also responsible for reducing the time of the enzymatic extraction process discussed in Section 1.4.1 [187, 199]. While decreasing extraction times is always welcomed from a productivity perspective, the technique has its faults especially if the right precautions are not taken. Some of the negatives regarding probe ultrasonication include;

- increased risk of sample loss through splashing due to the sample vessel being open.
- Another concern with ultrasonic probes is increased sample temperature. The high energy delivered to such a small volume can cause the sample to overheat, potentially causing boiling, bubbling/splashing and subsequent evaporation and loss of volatile compounds [200].
- Cross contamination is a possibility due to the use of only one probe.

All these issues have the ability to reduce the sample volume thus increasing the margin of error by altering the dilution factor. Steps can be taken to mitigate these errors such as utilisation of larger volumes, keeping the sample chilled with ice through the sonication process and the use sonication pulses instead of prolonged sonication times [178].

1.5 Analytical techniques

1.5.1 Hydride generation

Before the recent accessibility of ICP-MS technology, hydride generation (HG) was one of the most useful techniques for selenium determination on account of its low interference potential, excellent sensitivity and efficient analyte transfer [201]. Successful coupling to HG includes HG-atomic absorption spectrometry (HG-AAS), HG-atomic fluorescence spectrometry (HG-AFS), HG-atomic emission spectrometry (HG-AES), HG-ICP-MS and HG-microwave induced plasma-mass spectrometry (HG-MIP-MS). Utilisation of HG-AAS is possible for total selenium analysis and also for inorganic selenium speciation. Research carried out in 2001 also illustrated the possibility of coupling HPLC to HG-AAS for SeMet determination [201]. The direct technique for selenium analysis operates by reducing selenium with sodium borohydride ($NaBH_4$) thus creating selenium hydride (H_2Se). The volatile selenium hydride is then brought via a carrier gas (nitrogen or argon) to the quartz tube that is suspended in the flame for atomisation and ultimately atomic absorption spectrometry, and subsequent determination. Sample preparation depends on the analyte but typically for quantitative analysis, the sample matrix needs to be completely broken down. This is assisted with some form of chemical hydrolysis. A popular digestion solution for this hydrolysis is a nitric/perchloric acid mix ($HNO_3/HClO_4$) [202]. This digestion liberates total selenium in the form of Se (IV) and Se (VI). Conversion of the Se (VI) to Se (IV) requires a reduction step. Hydrochloric acid (HCl) is added to the sample solution and refluxed for roughly between 20 minutes to an hour. This step ensures that all of the selenium in the sample is present as Se (IV) and is ready to create a gaseous hydride for detection by HG [203].

1.5.2 GC, GC-ICP-MS and GC-MS separation and detection methods

Prior to the popularity of inductively coupled plasma-mass spectrometer (ICP-MS), one method for SeMet determination was analysis by gas chromatography-flame ionisation detection (GC-FID). The extraction method was similar to that by Wolf *et al.* [204]. The SeMet analyte was extracted from selenised yeast and derivatised by a cyanogen bromide (CNBr) reaction [204]. Gas chromatography (GC) is an analytical technique that is mainly employed for the analysis of volatile compounds. The hyphenated GC

approach was applied to numerous volatile selenocompounds such as methylselenol (CH_3SeH), dimethylselenide $(CH_3)_2Se$ and dimethyldiselenide ($CH_3SeSeCH_3$) [205, 206]. Non-volatile selenoamino acids were also analysed by GC, but these analytes needed derivatisation for this to occur. Selenomethionine, for example, was reacted with cyanogen bromide (CNBr) and from the subsequent reaction (Figure 1.12) created the volatile methylselenocyanate, CH_3SeCN, for detection by GC [195]. However, there can be disadvantages to some CNBr derivatising assays with ~35 % lower SeMet recoveries reported [195]. Another issues involving the CNBr reaction with SeMet was the mechanism created the volatile methylselenocyanate compound but does not differentiate between SeMet and selenomethylselenocysteine. Thus, the CNBr will react with any compound that contains a CH_3-Se functional group which could lead to incorrect identification and quantification [207].

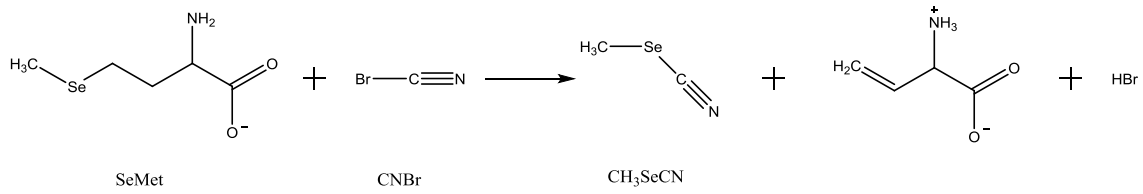


Figure 1.12: Reaction of CNBr with selenomethionine.
Adapted Wolf et al. [204].

The most reliable GC method for selenium speciation involves coupling with ICP-MS or MS [205]. GC provides a perfect sample introduction pathway for mass spectrometry due to the presence of a gaseous mobile phase. The absence of any solution allows the plasma to operate at a higher temperature which reduces plasma oxide ratios and polyatomic interferences. While this will result in a more stable plasma and provide better atomisation, the sensitivity will also be improved due to less matrix interference. Plasma oxides are measured based on a CeO:Ce ratio and are usually monitored to ensure they remain at ~1 %. The advantage of ICP-MS coupling will be highlighted in more detail in Section 1.5.3. Nevertheless, since ICP-MS is solely an elemental detector, it allows for increased sensitivity by only monitoring the element and its individual isotopes of interest regardless of compound structure.

1.5.3 ICP-MS

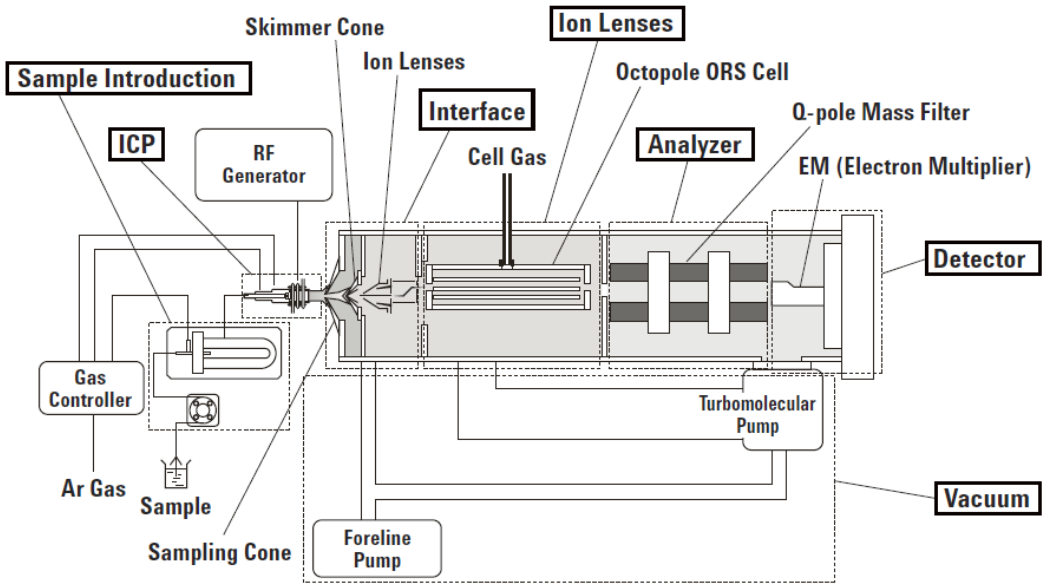


Figure 1.13: ICP-MS system.
Taken from Agilent Technologies 7700 ICP-MS literature [208].

Almost 30 years ago the Houk, Date and Gray research teams developed ICP-MS [209-213]. Since then ICP-MS has grown to be one of the go-to instruments for elemental quantification and remains one of the best detection techniques for trace element speciation [214, 215]. While researchers have coupled GC to ICP-MS [216], the coupling of HPLC and ion chromatography (IC, see Table 1.6) to ICP-MS are more popular techniques with literature searches generally showing more results for HPLC-ICP-MS and IC-ICP-MS than GC-ICP-MS. The introduction of the aqueous solution to the HPLC-ICP-MS spray chamber is carried out via a pneumatic nebuliser. The Micromist nebuliser creates a fine spray or aerosol which is transported through a cooled spray chamber. The spray chamber, made of quartz or perfluoroalkoxy alkanes (PFA), is mounted in an aluminium block and is cooled by a Peltier thermoelectric module. This cooling allows for aqueous samples to be maintained at 2 °C while organic solvents can be cooled to -5 °C, further demonstrating the range of samples that may be analysed by ICP-MS. The inert carrier gas, typically argon, carries the aerosol through the cooled spray chamber to the inductively coupled plasma ion source. Argon is the preferred gas for the plasma since it is a good source of singly charged positive ions. This allows a broad range of elements to be ionised to more than 90 % efficiency according to the manufacturers [208]. Furthermore, other gases can be added on-line to the argon gas to increase the ICP-MS sensitivity [217]. A radio-frequency (RF) at

approximately 27.1 MHz coupled through a load coil creates an oscillating magnetic field that is seeded with electrons thus igniting the plasma. With high energies generated by the plasma, gaseous atoms become ionised thus sustaining the plasma through electrons colliding with more gaseous atoms and initiating further ionisation [218]. The plasma (~8,000 to 10,000 K) atomises, excites and ionises all material in the aerosol. A quartz torch consisting of three concentric tubes in a high-frequency electric field creates the plasma [219]. The auxiliary gas flows around the centre concentric tube which modifies the horizontal position of the axial plasma relative to the torch. The inner walls of the torch are cooled by this Argon coolant gas flowing tangentially through the outer tubes, which function as the primary plasma gas but also serves to centre and stabilise the plasma. Plasma ionisation is independent of the molecular structure of the original sample since the entire sample is completely dissociated. While this dissociation and atomisation removes all structural information, it allows for element-specific analysis regardless of the sample matrix, thus becoming one of the main reasons for the adoption of ICP-MS.

Early argon plasma ICP-MS instruments for selenium speciation had selectivity and sensitivity issues caused by spectral and non-spectral interference [220]. Non-spectral interference is matrix dependent and relates to the signal suppression or enhancement. Matrix effects are predominantly caused by high salt concentrations in the sample or due to the reagents included throughout sample extraction [220]. To combat matrix effects, the inclusion of an internal standard with similar ionisation energies, such as germanium for selenium analysis, is monitored to account for any signal variation [221]. Additionally, the limit of detection in early ICP-MS was not sensitive enough and needed to be lowered for adequate analysis (ppm-ppb) [222]. This problem was created by comparatively high first ionisation potential causing lower ionisation efficiency in the plasma and also by the interferences of the argon/argon dimers and polyatomics [223]. Another spectral interference issue pertaining to ICP-MS analysis is the formation of oxides and doubly charged analytes in the plasma [222]. The majority of ions produced in the plasma are singly charged (M^+), although formation of mono-oxides (MO^+) and doubly charged ions (M^{2+}) also occur [224]. Analyte oxides may interfere with other analyte masses resulting in signal enhancement and possible incorrect elemental determination. Douglas and Houk perceived cerium oxide (CeO^+) as an example of the worst ions for oxide formation [225] which explained why the

monitoring of this oxide prior to ICP-MS analysis was necessary. Studies observed that a reduction of the interfering signal caused by doubly charged ions and oxides by an order of magnitude can be attributed to spray chamber cooling and desolvation [226].

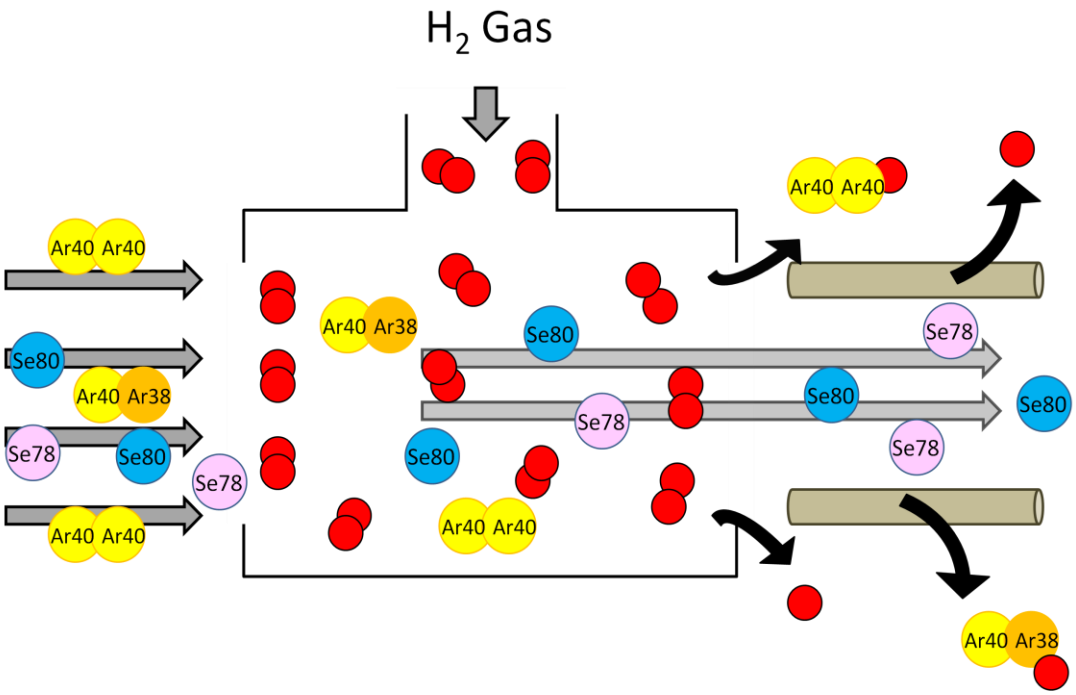


Figure 1.14: Schematic of octopole reaction system.

Other benefits of the ICP-MS used in this study include an octopole reaction system (ORS) which allows for better ion focusing of the analyte and improved collision efficiencies (Figure 1.14). These conditions can employ either hydrogen (H_2) or helium (He) collision gas (Figure 1.15 and 1.16, respectively) and enable multi-element analysis of complex sample matrices. The goal of these collision gases is to reduce the molecular interference to below that of the blank solution. To keep interference at a minimum, the ORS is encased in stainless steel. This steel vessel is then pressurised with H_2 or He. H_2 in the ORS removes plasma based polyatomic ions such as ArO and the Ar_2 dimer [227]. The H_2 gas in the ORS is essential for selenium speciation as it removes the argon dimer of molecular weight 80, which interferes with the most abundant isotope (^{80}Se) see Figure 1.15.

$$Ar_2^+ + H_2 \rightarrow Ar_2H^+ + H \qquad k = 1x10^{-9} cm^3 s^{-1}$$
$$Se^+ + H_2 \rightarrow Se^+ + H_2 \qquad no\ reaction$$

Figure 1.15: Reaction of Hydrogen with Argon in octopole reaction system.

Similarly, the helium gas suppresses hypochlorite ions (ClO^-, MW ~ 51), NaAr (MW ~ 63) and ArCl (MW ~ 75) sample matrix species which interfere with ^{51}V, ^{63}Cu and ^{75}As respectively, Figure 1.16.

$$NaAr^+ + He \rightarrow Na^+ + Ar + He \qquad dissociation$$
$$Cu^+ + He \rightarrow Cu^+ + He \qquad no\ reaction$$

Figure 1.16: No reaction of Helium with Copper in octopole reaction system.

All these performance enhancements allow for increased detection sensitivity of the target analyte and improve tolerance towards matrix effects, thus making ICP-MS the preferred detector for quantitative analysis. Once through the ORS, the quadrupole mass analyser separates the ions based on their mass-to-charge ratio. The quadrupole consists of four hyperbolic rods with each rod capable of receiving both radio frequency (RF) and direct current (DC) voltage. The voltages are ramped rapidly to scan the entire mass range (2-260 amu) in 100 milliseconds. This allows the operator to control what ions are investigated by controlling the voltages applied to the quadrupole to filter all other ions and only let through the isotopes of interest. In addition to these instrumental controls for increased sensitivity, constructive or destructive matrix interference may be accounted for by monitoring the isotopic ratio of the element in question thus ruling out any matrix effects that may cause inaccurate results. Furthermore, isotopically enriched standards can be included for isotope dilution to determine analysis specificity and to achieve accurate data.

1.5.3.1 HPLC-ICP-MS

Numerous selenium speciation publications have utilised ICP-MS, whether it is coupled to GC [216, 228], CE [229, 230], SEC [231-233] or HPLC [182, 187, 234-237]. HPLC-ICP-MS is one of the most popular hyphenated approaches for speciation analysis. The separation is carried out by HPLC and ICP-MS is used as an element/isotope specific detector. Coupling an ICP-MS to an HPLC allows detection to focus solely on the elemental species in question. Selenium speciation, for example, only monitors the selenium products and ignores any other background interference that is not a selenium isotope e.g. sulfur based compounds. Furthermore, due to the optimisation and the vast amount of research carried out on HPLC as an analytical

technique, numerous types of HPLC formats may be coupled to ICP-MS (see Table 1.6) [205].

Table 1.6: HPLC selenium speciation techniques.

Column	Species	Matrix	Detector	Ref
Enantiomeric separation				
Cyclobond I (250×4.6 mm)	D and L-SeMet	Yeast	ICP-MS	[238]
Chirobiotic T (250×4.6 mm)	D and L-SeMet	Yeast	ICP-MS	[238]
Lichrospher C18, (250×4.6mm, 5 µm)	D and L-SeMet	Standard mixture	UV	[239]
Chirobiotic T (250×4.6 mm)	D and L-SeMet	Microalgae	ICP-MS	[149]
Ion Exchange				
Hamilton PRP-X200 (250×4.1mm, 10µm)	$SeCys_2$, TMSe, SeMet, Se(IV), Se(VI)	Fish, oysters	ICP-MS	[59, 240]
Hamilton PRP-X100 (250×4.1mm, 10µm)	Soluble compounds	Yeast	ICP-MS/ESI-MS	[241]
Hamilton PRP-X100 (250×4mm)	SeMet, SeCys, SeMeSeCys, Se(IV), Se(VI)	Flour and bread	ICP-MS	[242]
Hamilton PRP-X100 (250×4mm)	Se(IV), Se(VI), SeMet	Malt, wort, beer made with Se-enriched barley	ICP-MS	[243]
Hamilton PRP-X100 (250×4mm, 10µm)	SeCyst, SeMetSeCys, Se(IV), SeMet, Se(VI)	Strawberries	AFS	[244]
Supelco SCX	Soluble compounds	Yeast	ICP-MS	[241]
Supelcosil LC-SCX (250×4.6 mm)	SeMet, Se(IV), Se(VI),$SeCys_2$	Se nutritional supplements	ICP-MS	[245]
Merck Polysphere ICAN 2 (120× 4.6 mm, 10 µm)	Se(IV), Se(VI), SeMet, $SeCys_2$	Plants (white clover)	FAAS/ICP-MS	[246]
Mono Q 5/5 FPLC (50×5mm)	Selenoproteins	Human serum	ICP-MS	[247]
Ionosphere-C (100 mm ×3 mm)	Se(IV),Se(VI), SeMet and $SeCys_2$	Plants (white clover)	FAAS/ICP-MS	[246]
Dionex IonPac AG11 HC (250×2mm)	Se(IV) and Se(VI)	Urine	ICP-MS	[248]
Dionex Ionpac CS5 (250×4mm, 9µm)	TMSe, SeMet and species unidentified	Urine	ICP-MS	[248]
Ionosphere-C (100×3mm)	Se(IV), Se(VI), $SeCys_2$, SeMet, MetSeCys, PrSeCys, SeCys, $SeHoCys_2$, MeSeMet, SeEt and TMSe	Standard mixture, yeast and algal extracts	ICP-MS	[249]
PRP-X100 (250×4.1mm)	SeMet, MeSeCys, SeHLan and species unidentified	Australian wheat cultivars (Mace, Janz, Emu Rock and Magenta)	ICP-MS	[250]
Dionex AS-7 (250×4.1mm)	Se(IV), Se(VI)	River water	ICP-MS	[251]
Hamilton PRP-X100 (250×4.1 mm, 10 µm)	Se(IV), Se(VI), SeCys, SeMet	Green peas (*Pisum sativum L.*)	ICP-MS	[252]
PRP-X100 column (250×4.6 mm, 10 µm)	SeMet, $SeCys_2$, Se(IV), Se(VI)	Red leaf lettuce (*Lactuca sativa L.)*	ICP-MS	[253]

Table continued

Table 1.6 continued

Column	Species	Matrix	Detector	Ref
PRP-X100 (250×4.6 mm, 10 µm)	SeMeSeCys, SeMet, γ-glu-SeMeSeCys, Se(IV), SeHLan, SeOMet, deaminohydroxy-selenohomolanthionine, NAC-SeMet, methyl-seleno-Se-pentose-hexose, Se-methyl-Se-glutathione, 2,3-DHP-propionyl-selenocysteine-cysteine, methyltio-selenoglutathioneand 2,3-DHP-selenolanthionine and other unknown compounds	Sunflower, radish, garlic,SELM-1	ICP-MS/ESI-MS	[254]
Size exclusion				
Superdex200 10/30 (600-10 kDa)	Soluble compounds	Chicken, turkey, oyster, duck, lamb, pig and cow	ICP-MS	[255]
BioSep SEC 2000 (300-1 kDa)	Soluble proteins	Chicken liver	UV/ICP-MS	[256]
Superdex G-75 HR 10/30 (70-3 kDa)	SeMet, selenised haemoglobin selenoprotein P and other soluble compounds	Cow serum	ICP-MS	[257]
Superdex peptide 10/30 (14-0.18 kDa)	Soluble proteins	Onion leaves	UV/ICP-MS	[258]
Superdex 75 HR 10/30 (70-3 kDa)	Soluble proteins	Plants (Indian mustard)	ICP-MS	[259]
Size exclusion/affinity/ion exchange/				
5 mL HiTrap®Desalting Columns/ HEP-HP/BLU-HP affinity columns/ PRP-X100 column (250×4.6 mm, 5 µm)	Selenoprotein P, extracellular glutathione peroxidase, selenoalbumin (SeAlb), Se(IV), Se(VI)	Human serum	ICP-MS	[260]
5 mL HiTrap®Desalting Column/ HEP-HP/BLU-HP affinity columns	Selenoprotein P, extracellular glutathione peroxidase, selenoalbumin (SeAlb),	Blood samples, cord blood	ICP-MS	[261]
Size exclusion/ion exchange				
Shodex Asahipak GS 220 HQ (300×7.6mm)	MetSeCys, SeMet	Plants (Indian mustard and garlic)	ICP-MS	[262]
Shodex GS220 HQ (>3 kDa) (300×7.6mm)	SeMet	Mushrooms (*Lentinula edodes*)	ICP-MS/ESI-MS/UV	[263]
Shodex GS 520 7G (>300 kDa) (300×7.6mm)	SeMet and soluble compounds	Mushrooms (*Lentinula edodes*)	ICP-MS/UV	[263]
Ion pair reverse phase				
Symmetry Shield RP8 Waters (150×3.9mm, 5µm)	Se(IV), Se(VI), SeMet, $SeCys_2$, Se-lanthionine, methaneseleninic acid, Se-cystathionine, MetSeCys, Se-2-propynilselenocysteine, GMetSeCys, Se-allylselenocysteine, cis-Se1-propynilselenocysteine, Trans-Se-1-propenylselenocy steine, Se-1-propylselenocysteine, SeEt, SeHoCys, Se-1-methyl-2-propenylselecysteine	Standard mixture, selenised yeast and garlic	ICP-MS/ESI-MS	[264]
AG 11 (precolumn 50×4 mm) + AS 11 (analytical column 250×2 mm)	Se(IV), Se(VI), HSA-Se,Selenoprotein P, SeMet, SeCys, SeGpX, SeTRxR	Human serum	ICP-RDC-MS	[265]
Zorbax RX-C8 (250×4.6mm, 5µm)	SeMet, MetSeCys, GMetSeCys	Selenised yeast	ICP-MS/ES I-MS/MS	[167]
Zorbax SB-C18 (150×2.1 mm, 5 µm)	SeMet, SeCys	Rice (*Oryza sativa*)	AAS-HFS	[266]

Table continued

Table 1.6 continued

Column	Species	Matrix	Detector	Ref
Zorbax SB-C8 (150 × 4.6 mm)	Se(IV), Se(VI), SeMet, SeMeSeCys,	Se-enriched garlic, beer, wine, yeast	HG-AFS	[267]
Zorbax RX-C8 column (150×4.6 mm, 5 µm)	SeMet	Se-enriched peanut	ICP-MS	[268]
Zorbax StableBond (SB-Aq) (250×4.6 mm, 5µm)	MeSeMet, MeSeOCys, SeOMet, MeSeCys, SeMet, MSeA, Se(IV), Se(VI)	Se-dietart supplements, SELM-1	ICP-MS /ESI-MS	[269]
Discovery Bio Wide Pore C8 (150×2.1mm, 3µm)	Soluble fractions	Simulated gastric extract of selenised yeast	ICP-MS	[137]
Alltima C8 (250×2mm, 5µm)	MetSeCys, SeMet	Plants (Indian mustard and garlic)	ICP-MS	[262]
XTerra MS C18 column (250×2.1 mm, 5µm/ Biosil C18 (250×4.6mm)	Selenomethyl-N-acetylhexosamine	Human urine	ICP-MS /ESI-MS	[270]
Aeris PEPTIDE XB-C18, (100×2.1 mm,3.6µm) with ULTRA C18 Security Guard	TMSe, SeMet, Se-MeSeCys, SeGalac, Se(IV), Se(VI)	HeLa cells	ICP-MS	[271]
LiChorsorb RP 18 (250×4.6mm, 5µm)	Se(IV), Se(VI), SeCys, SeEt, SeMet, SeCM, SeUr, TMSe	Standard mixture and nutritional supplements	ICP-MS	[272]
Alltech Alltima C18 (250×4.6 mm, 5 µm)	Se(IV), Se(VI), SeMet, SeCys, MeSeCys	SELM-1, Urine	PB/MS	[273]
Nucleosil (250×4.0mm C18, 5µm)	Se(IV), Se(VI), SeMet and other soluble compounds	Nutritional commercial supplements	ICP-MS	[192]
Nucleosil 120 Å (C18, 5 µm)	SeCys2, selenocystamine	Seagull eggs	ICP-MS	[274]
Nucleosil (250×4.0mm C18, 5µm)	TMSe	Human urine after Se supplementation	ICP-MS	[275]
C18 shim-pack CLC (150×4.6mm, 5µm)	Se(IV), Se(VI), $SeCys_2$, SeMet, MeSeCys, SeEt	Se enriched yeast and clover	ICP-MS	[276]
ODS-3 C18 (250×4.0mm C18, 5µm)	Se(IV), Se(VI)	Water, urine, plasma	UV	[277]
YMC-Triart PFP (250×3.0mm, 3 µm)	SeO_3^{2-}, MeSeCys, SeMet, $TMSe^+$, selenosugar 1	UROtsa, CCF-STTG1 and HepG2 cells	ICP-QQQ-MS	[278]
YMC-Triart PFP (250×3.0mm, 3 µm)	$TMSe^+$, SeMet, MeSeCys, methyl-2-acetamido-2-deoxy-1-seleno-beta- D-galactopyranoside (SeSugar 1), γ-glutamyl-Se-MeSeCys and Se-Me-Se-glutathione	Human hepatoma cells, (HepG2)	ICP-MS, ESI-MS	[279]
Affinity chromatography				
Hitrap Heparin-Sepharose, Hitrap blue-Sepharose	SelP, Se-Albumin, p-GSH-Px and soluble compounds	Human serum	ICP-MS	[247]
MARC (Multi-affinity removal column)	Se-albumin and selenoproteins	Human serum	UV	[232]
WatersSpherisorb ODS2 (4.6×250mm, 5µm)	Se(IV), Se(VI), SeCys, SeMet, Methyl-SeCys	Lentil (*Lens culinaris Medik*)	ICP-MS	[280]
Hitrap Heparin–Sepharose and Hitrap blue-Sepharose	Selenoproteins	Human serum	ICP-MS	[247]

Adapted table from Szpunar *et al.* 2000 [281] and Pedrero *et al.* [205] 2009, updated to 2017.

Table 1.6 highlights the popularity of liquid chromatography for selenium speciation, whether it is size exclusion, affinity, ion exchange, or enantiomeric separations. With the benefits of being able to analyse non-volatile samples directly unlike gas chromatography, HPLC can also be easily interfaced to ICP-MS [282]. The different column chemistries enables a broad range of samples to be analysed from

human urine and serum, to onion leaves, oysters and selenised yeast. There are disadvantages though, some chromatographic approaches are limited in that they can only analyse inorganic selenium forms or only organic forms of selenium. Furthermore, some columns might be better suited to analysing selenoproteins such as affinity chromatography. However, having the ability to analyse a range of samples by numerous different approaches allows flexibility to analytical facilities and research groups who may not have sufficient instrumentation. Therefore, if HPLC is not available, other options such as SEC could be employed, likewise, if ICP-MS is not available, other detectors could be utilised like UV or AFS.

Selenium compounds that occur in food or biological samples tend to be non-volatile which is why HPLC is suitable for selenium speciation of yeast [205]. The multiple HPLC columns available for selenium speciation allowed a wide range of samples to be analysed. These samples varied from selenised yeast to human serum to fruit, vegetables and meat. The success of HPLC as an analyte separation technique and ICP-MS as a detector is clearly evident from this range of samples analysed for selenocompounds. Mass spectrometry, either by ICP or ESI, were by far the most popular techniques employed. These analytical instruments both offer sub ppt detection capabilities with ESI tandem mass spectrometry offering structural information about the selenocompound detected, which will be discussed in more detail in Section 1.5.5.

The ability to identify the form of the selenium species present is necessary for toxicity and deficiency but also for selenium bioavailability as discussed in Section 1.3.1. This was a primary reason for the selection of HPLC-ICP-MS for development of accelerated selenium speciation methods. The large number of potential chromatographic columns further enhances selenium speciation capabilities.

1.5.3.2 Compound independent calibration

Compound independent calibration (CIC) is a valuable tool in the ICP-MS arsenal and provides quantitative applications for speciation analysis [283]. The increasing instrumental sensitivity of mass spectrometers enables scientists to quantify species at lower and lower concentrations (parts per trillion). These improvements in sensitivities have both advantages and disadvantages. Increased sensitivity allows for detection of species which until now, we have not been able to see. While this is a huge plus towards identification of new compounds, unless tandem MS is available, identification by hyphenated ICP-MS techniques relies heavily on retention time matching. However, if

analyte standards are not available for ICP-MS analysis and identification is not possible, CIC can estimate the concentration of the species in question [284]. Unknown species can be quantified based on their elemental concentrations when compared to the elemental concentration of a known standard [285]. ICP-MS is ideally suited for analysis by CIC due to the extremely high temperatures in the ICP plasma. This complete atomisation in the ICP ion source guarantees that the elemental signal from the target element is independent of the compound in which the element is present. Therefore, the response of any selenium-containing compound or species is determined by the selenium signal. Hence, it is possible to calibrate based on selenium concentrations irrespective of the species. The CIC becomes important for novel research since some standards for new species may not be commercially-available or may not be available in sufficient purity. Situations like these rule out quantification by calibration standards but CIC allows researchers to at least estimate the quantity of selenium associated with each species detected.

1.5.4 Quantification of total selenium

Total selenium analysis has evolved from flame atomic absorption spectrometry (FAAS) [125] to inductively coupled plasma-optical emission spectrometry (ICP-OES) [286] and ICP-MS analysis [287]. FAAS aspirates a liquid sample and creates an aerosol that is mixed with combustible gases, such as acetylene and air. The mixture is then atomised in the ignited flame and using absorption spectrometry, the concentration of analyte present may then be calculated. This analytical technique requires the comparison of the sample signal produced to the signal of known standards of varying concentrations. Total selenium analysis today is mainly carried out by ICP-MS [269, 288]. For ICP-MS analysis, the sample solution is introduced into the nebuliser creating a fine aerosol. The aerosol is then transferred into high-temperature argon plasma which atomises and ionises the sample to produce a cloud of positively charged ions. The sample ions are extracted from the plasma into a vacuum system containing a quadrupole analyser. This acts as a mass filter which can be optimised by the operator from day to day. A multi-elemental scan can be performed on the sample simultaneously, allowing for detailed analysis of samples [289]. Ions are then focused into the analyser and separated based on their mass/charge ratio (*m/z*) [208]. The count

rate obtained for a particular ion is compared with a calibration curve to give the concentration of that element in the sample.

1.5.5 LC-MS/MS

1.5.5.1 Peptide mass fingerprinting

The identification of selenoamino acids, selenopeptides and selenoproteins has become more and more important in modern selenium speciation research and owes a lot of these discoveries to the invention of soft ionisation techniques [290, 291]. Advanced instrumentation and thus increased sensitivity has satisfied the thirst for precise identification. Firstly, peptide mass fingerprinting (PMF), sometimes referred to as peptide mass mapping (PMM), involves separation of the proteins of interest usually by HPLC, UPLC or gel electrophoresis [292]. Once separation is complete, the proteins need to be broken down into their subsequent peptide chains. Enzymatic digestions, with enzymes such as endoprotease-trypsin, will cleave the protein predominantly at lysine and arginine sites, creating smaller peptide fragments. These fragments are then analysed for their molecular mass and are compared to theoretical fragmentation masses that are stored in databases such as the Dayhoff database [292, 293]. Accurate mass determination of these peptides is carried out by mass spectrometry including MALDI-TOF and ESI.

1.5.5.2 Electrospray ionisation for selenium speciation

There are numerous mass spectrometry techniques employed throughout the analytical community e.g. the pharmaceutical, environmental and life science industries. Soft techniques such as electrospray ionisation (ESI) are well documented for the identification of selenium biomolecules [294-297]. Since the earliest reports of coupling electrospray ionisation and mass spectrometry by Yamashita and Fenn [298, 299], the technique has become popular for the analysis of polar and ionic compounds. A primary benefit of ESI is that solutions of the sample analytes can be exploited to create ions in the mobile phase which are easily transported into the MS. Electrospray ionisation proved to be helpful in identifying selenium peaks detected by HPLC-ICP-MS [300, 301] and since then it was coupled to numerous mass analysers for selenium compound identification such as quadrupole, ion trap, double focusing and time-of-flight to name a few [302, 303]. In brief, single or multiple protons can be attached to the molecule of

interest thus creating a singly or multiply charged ion thereby allowing for precise measurement of the molecular mass of the species [304]. The protonated molecule ions can be represented by $(M + nH^+)^{n+}$ and can be broken down by increasing the voltage on the orifice i.e. ionisation energy [304]. This popularity was due to the fact that multiply charged ions of high molecular mass proteins could be analysed by quadrupole MS [305]. The identity of such high molecular mass proteins can then be confirmed by comparing the mass spectra of the peptides against theoretical peptide mass databases. Additionally, the "bottom-up" approach (Section 1.5.5.3) exploits two methods for protein identification; peptide mass fingerprinting and tandem MS (MS/MS). Figure 1.17, describes the ESI process.

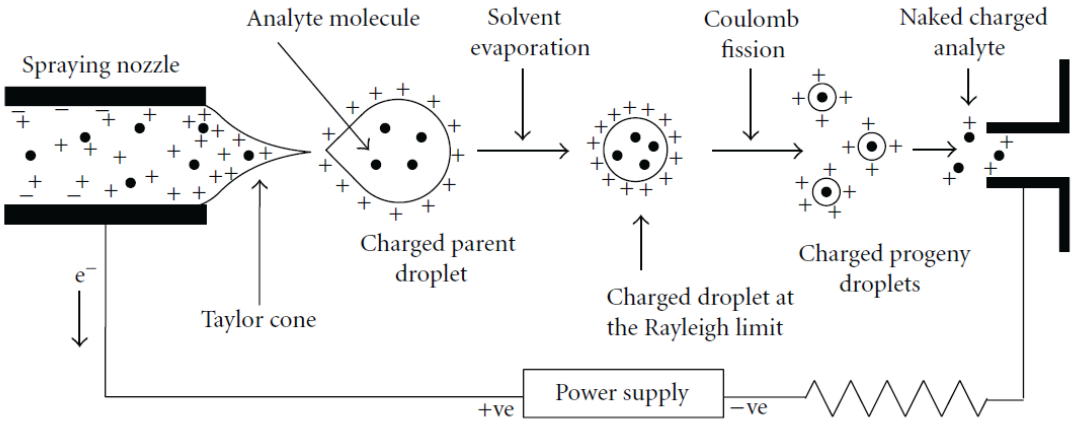


Figure 1.17: Schematic of electrospray ionisation.
Taken from Banerjee *et al.* [306].

The electrospray ionisation process described in Figure 1.17 demonstrates the three distinct stages. Stage one; once the analyte solution is injected, droplets become charged due to the high-voltage capillary tip. Stage two; the charged droplet undergoes solvent evaporation and disintegration, making the charged droplet smaller and smaller. Finally, the third stage; generation of the gas phase ion for the mass analyser [306]. The ESI technique was successfully employed for the identification of novel selenocompounds by Preud'homme *et al*. and also by Arnaudguilhem *et al*. [8, 9]. Nonetheless, these ionisation techniques have their drawbacks. Zwitterions with their overall neutral charge, as observed with some selenium species, can result in decreased sensitivity and thus poor limits of detection (LOD) [307, 308].

When ESI is compared to other soft ionisation techniques such as atmospheric pressure chemical ionisation (APCI), there are significant differences. The APCI

process begins with electrons from the corona ionizing nitrogen in the source which in turn reacts with the solvent molecules resulting in deprotonated or protonated analytes. Differences between ESI and APCI include the ranges of analyte polarity and molecular mass. The majority of organometallic species of interest are highly polar and cover a wide range of molecular masses. Therefore, this range of tolerance makes ESI a widely accepted technique for selenocompound analysis. In relation to the literature and relevant research, ESI is a far more popular technique for selenocompound identification than APCI. Additionally, the principle of ESI allows it to preserve the selenospecies when compared to APCI [305]. However, ESI has its disadvantages compared to APCI such as increased vulnerability to matrix interferences and mobile phase changes [305].

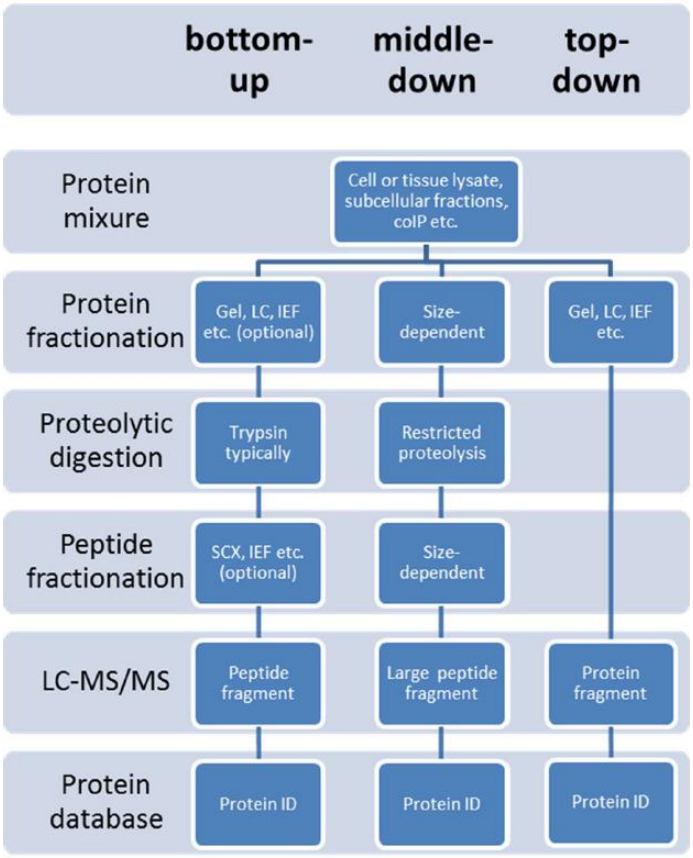


Figure 1.18: Schematic breakdown of protein identification by different techniques.
Taken from Zhang *et al.* [309].

1.5.5.3 Bottom-up mass spectrometry for selenium speciation

The term 'bottom-up' is a reference to the approach taken for large peptide or protein analysis. The method utilises proteolytic cleavage to liberate peptides thus determining the composition and characterisation of the protein in question [309, 310]. McSheehy *et al*. utilised such an approach to account for SeMet present in selenoproteins [311]. The term shotgun proteomics is often used in conjunction with bottom-up protein analysis. Shotgun proteomics was coined by the Yates lab [312] and refers to the practice of carrying out 'Bottom-up' analysis on a mixture of proteins [309]. This 'bottom-up' approach was successfully applied to selenised yeast to identify 16 novel selenium-containing proteins by Tastet *et al.* [313]. More recently, this popular approach was also applied to selenised yeast by Bierla *et al.* (2013) to account for Se/S substitution ratios in methionine and cysteine [314]. Similar to other analytical techniques, there are advantages and disadvantages and the 'bottom-up' strategy is no exception. 'Bottom-up' mass spectrometry identifies a portion of the total peptide population thus only accounting for a fraction of the protein. Therefore, it is possible to obtain numerous protein isoforms due to a range of post-translational modifications (PTMs). Such PTMs include methylation, amidation, acetylation, phosphorylation and glycosylation. The latter two PTMs are significant for not only cell metabolism but also the regulation of protein function. Furthermore, by only sequencing a fraction of the protein via 'bottom-up' spectrometry, a large quantity of PTM data is lost. Additionally, collision-induced dissociation (CID), which will be discussed next, alters PTMs requiring neutral loss scanning to identify them.

1.5.5.4 Top-down mass spectrometry for selenium speciation

Top-down mass spectrometry examines the completely intact protein or molecule as opposed to the proteolytically digested fractions analysed by bottom-up mass spectrometry [254, 315]. Once the protein is injected into the mass spectrometer, the protein molecular ions generated by ESI are fragmented by a gas phase. The main impediment to this particular approach is the formation of multiply charged product ions. This can create uncertainty in the interpretation of MS/MS spectra. Reduction of this ambiguity can be achieved by charge state manipulation via gas phase ion-ion interactions and by exploiting increased instrumentation sensitivity with high mass measurement accuracy (MMA). Ion charge state manipulation utilises gas-phase anions

to remove protons from the multiply charged product. Small molecule/peptide analysis after a water extraction of selenised yeast can be classed as top-down mass spectrometry. Once extracted, the sample matrix is separated by LC and undergoes tandem mass spectrometry fragmentation via MS^2 (or MS^3/MS^4) to identify the molecule, peptide or protein [316]. Selenocompounds have previously been identified by this approach [8, 9]. More recently, over 100 selenocompounds were detected in selenised yeast extracts by Gilbert-López *et al.* [317], aided by the increased sensitivity provided by top-down mass spectrometry approaches such as ESI-Time-of-Flight MS. This will be discussed in greater detail in Chapter 4.

1.5.5.5 Tandem mass spectrometry for selenium speciation

Tandem mass spectrometry, sometimes referred to as MS/MS or MS^2, is the step after the ionisation of the peptide or molecule of interest and helps provide information about structural properties [306]. This method is useful for peptide and protein identification and works on the fundamental principle that the ion is isolated in the first mass analyser and undergoes dissociation for further fragmentation in the second. Selenium speciation of selenised yeast has benefited from tandem MS and as a result numerous research groups have identified novel selenocompounds [8, 9, 318]. Apart from novel identification of selenocompounds, tandem MS can also be used to verify the synthesis of selenocompound standards as performed by Egressy-Molnár *et al*. [319]. This valuable confirmatory approach allows research groups to create and validate their own standards which are not commercially-available. Each selenocompound will have its own unique fragmentation. The dissociation that takes place is typically achieved by CID. Collision-induced dissociation exploits neutral atoms or gas molecules for collisional activation [320]. A similar technique was employed by ICP-MS which collided gaseous H_2 or He with the analyte, thus changing the mass of any interfering matrices that might be reducing the signal of the analyte in question. In tandem MS/MS, the masses of the dissociated fragment ions can be tallied to infer the amino acid sequence of the precursor ion. While this method of identification is not fast and quite arduous, alternative methods involve identifying an amino acid sequence and then searching, via a database, for this ‘sequence tag’ instead. However, the shotgun approach pioneered by Eng *et al.* [321], previously mentioned in Section 1.5.5.3, which compared product ion spectra to numerous databases by cross-correlation analysis has become one of the more favoured techniques. This method utilises uninterpreted

fragmentation data, i.e. ignoring any enzyme specificity that created the peptide [321]. Tandem MS will continue to be an analytical tool that will advance selenium speciation and the identification of novel selenocompounds.

1.6 Conceptual framework of this study

Based on the information presented in this introduction, it is clear that speciation analysis of selenium supplements is beneficial. Without selenium speciation information, selenium supplementation for humans and animals could be ineffective, especially since organic selenium has a longer half life in the body than selenite. Furthermore, excess selenium intake could be toxic. Excess accumulation can lead to selenosis, while a deficiency can cause a multitude of problems ranging from fertility issues to cardiovascular diseases. Methods to determine the form and concentration of these selenium species help inform selenium supplemented diets. The development of new selenium speciation techniques that can reduce extraction times and provide reliable information is critical to supplementation strategies adopted by agricultural and nutraceutical industries. The EFSA states that, "a cause and effect relationship has been established between the dietary intake of selenium and protection of DNA, proteins and lipids from oxidative damage, normal function of the immune system, normal thyroid function and normal spermatogenesis" [322]. Since selenium dietary intake is essential, the understanding of its composition has never been more important.

The core pillars of this research will examine and investigate the analytical techniques of liquid chromatography and mass spectrometry essential for selenium speciation. The marriage of industrial efficiency with novel academic approaches will combine to try to increase the effectiveness of current analytical methods. Industrial research does not always lend itself to the liberated approach of academia and as such, relevance to that industry is essential. However, unification of both codes provides the foundation for crucial research. New approaches for selenium speciation of selenised yeast will be developed and if successful, will proceed towards validation.

Therefore, the primary goal of this study was to investigate the methods of selenium speciation of selenised yeast supplements and to improve on these methods using various extractions techniques. Furthermore, if method development was successful, the selenised yeast will be examined further for potential novel selenium species. The specific objectives of this doctoral thesis were broken down per chapter:

Chapter 2;

- development of an accelerated enzymatic extraction of selenomethionine from selenised yeast.
- development of an accelerated chemical extraction of selenomethionine from selenised yeast.
- validation of any new accelerated selenomethionine extraction methods.
- validation of any analytical methods utilised for selenomethionine quantitation.

Chapter 3;

- determined if newly developed enzymatic and chemical extraction methods for selenomethionine analysis could differentiate between commercially-available selenium-enriched yeast products.
- investigated if water extraction methods extracted other selenoamino acids, selenoproteins or organoselenium metabolites and examined if there were any differences between commercial selenised yeast products.
- investigated and measured the chiral composition of selenomethionine in the water extracts of selenium-enriched yeast.

Chapter 4

- identification of any other selenoamino acids, selenoproteins or organoselenium metabolites that were present in water extracts of selenium-enriched yeast and investigated the presence of novel, previously unreported compounds.

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Chapter 2: Determination of selenomethionine in selenised yeast using accelerated enzymatic and chemical extraction methods with High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry analysis

Abstract

The objective of this study was to reduce the extraction time, to hours instead of days, for quantification of the SeMet content of selenised yeast. Two approaches were investigated, one enzymatic, the other by chemical means. Firstly, sonication coupled with microwave energy and various enzymes were investigated to design a faster extraction assay for SeMet from selenised yeast. Once optimised, the method was applied to a selenised yeast certified reference material (SELM-1; 2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet). Separation and determination of SeMet in yeast extracts were performed by high performance liquid chromatography with inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). A hydrogen (H_2) collision cell was utilised with the Agilent 7700× ICP-MS. The limits of detection and quantitation of the analytical sample were 5 and 15 µg/kg SeMet, respectively, and the signal response was linear up to 1,500 µg/kg SeMet. The average recovery of spiked SeMet from the selenised yeast matrix was 97.7 %. Analysis of SeMet in SELM-1 using this newly optimised method resulted in average recovery yields of 100.9 % of the certified value. This extraction and analytical method are suitable for fast, reliable determination of SeMet in selenised yeast. Development of an accelerated chemical method was also investigated. This study was carried out to explore the validity of chemical extraction, as an alternative to enzymatic extraction, for selenomethionine (SeMet) determination in multiple strains of selenised yeast using HPLC-ICP-MS. Microwave assisted chemical extraction (MACE) was exploited to accelerate the chemical extraction of SeMet from the selenised yeast. A 10 minute extraction took place in 4 M MSFA at 200 °C. An acid tolerant column, the YMC Triart column (pH 1-12), was selected for separation of the extracted selenium species. Again, SELM-1 was extracted and analysed throughout the validation process. The limit of detection and quantification were 1 and 5 µg/kg SeMet, respectively, and the signal response was found to be linear from 5 to 750 µg/kg SeMet. The certified reference material, SELM-1, was spiked with 50, 100 and 150 % of the analyte concentration (SeMet) in the sample and yielded an average recovery of 96.7 ± 2.1 % (n=9). Analysis of SELM-1 with this method yielded a SeMet recovery of 100.2 ± 0.1 % of the certified value. Compared with other selenium speciation techniques like gas chromatography (GC), the proposed chemical extraction method simplified the analysis by decreasing both sample preparation time and potential experimental error caused by derivatisation. This MACE HPLC-ICP-MS method was

successfully applied to commercially-available selenised yeast products. When compared to the enzymatically validated method, it resulted in an average SeMet recovery of 99.97 %.

2.1 Introduction

The main objectives of this study was to develop a fast, efficient and accurate enzymatic and chemical extraction method to reliably and repeatedly extract ≥95 % of SeMet from selenised yeast. To reach this objective, sonication and microwave technologies were investigated in conjunction with enzymes and chemicals. With both techniques capable of supplying high quantities of energy, investigations were carried out to reduce the extraction times.

The first objective of this study was the investigation of the enzymatic extraction of SeMet from selenised yeast. The current three day extraction method involved three sequential proteolytic extractions [1, 2]. The goal was to reduce this three day extraction of SeMet to a maximum extraction time of one day or less.

Sonication and the use of microwaves were investigated to speed up extraction times. The main benefit of using the ultrasonic probe was to break up the robust yeast cell wall by employing high-frequency (above 16 kHz) to release intracellular components such as SeMet [3]. The mechanism of disruption by shear force is due to the phenomena of cavitation [4]. The high-frequency sound wave energy creates gas and vapour bubbles. When these bubbles collapse, sonic energy is converted to mechanical energy resulting in a release of intense shock waves where pressure can be thousands of atmospheres at that very point [5, 6]. To prevent pitting damage by cavitation from the surrounding liquid, the probe tip was constructed of titanium [5].

A microwave digester was the second instrument to be investigated to speed up the extraction while still maintaining ≥95 % recovery of SeMet. Microwave digestion works on the premise that in a closed vessel under pressure, boiling points can be raised thus increasing digestion speed and efficiency. An example would be digestion of organic material in nitric acid, like the digestion of yeast samples before analysis of its total selenium content. Such an approach would be beneficial for determining the mass balance, described in detail later (Section 2.2.10). The boiling point of nitric acid is approximately 120 °C, but at 5 atm this increases to 176 °C [7]. If the digestion took place on a hot plate, biological organic matrices would remain intact. Therefore, perchloric acid would have to be added to destroy the biological matter. If perchloric acid is added to sample digestions an exhaust air scrubber system is necessary in the fume hood to emit a fine water spray thus diluting the perchloric acid fumes and

vapours. This reduces the formation of perchlorate salts which are highly explosive and are sensitive to small vibrations even from the normal vibrations created by a working fume hood. However, the ability to increase the boiling point of nitric acid to 176 °C by use of microwave digestion is sufficient to break down carbohydrates, proteins and lipids which decompose at temperatures of 140 °C, 150 °C and 160 °C, respectively [7, 8]. Complete digestion of biological samples was not always the goal and fortunately, the energy provided by the microwaves has previously worked well with enzymes for the liberation of amino acids and proteins [9, 10]. Accelerated methods were previously described [10] to reduce multiple proteolytic extractions that usually are ~17-24 hours per extraction step [1, 2, 11, 12]. Therefore, as previously mentioned the goal of this study was to reduce current methods further with the aid of sonication and enzymatic microwave digestion and subsequently validate the method for use in other laboratories. Temperature, time and power parameters were all investigated to develop, optimise and validate an accelerated enzymatic extraction of SeMet. The aim of selenium speciation was to quantitatively extract selenium-containing compounds from various samples while still maintaining the integrity and composition of the original compound. Any modification of the analyte would result in flawed analysis and thus incorrect results [13]. To reduce degradation and any stability issues, the analysis should be performed within 12 hours of extraction. Once extracted, some compounds like SeMet degrade easily through oxidation (SeMet oxidation will be examined closer in Chapter 3). Steps can be taken to counteract this degradation such as the application of antioxidants like β-mercaptoethanol (C_2H_6OS) or dithiothreitol (DTT) ($C_4H_{10}O_2S_2$) [14, 15]. Introducing a reducing step may also reverse oxidation of the analyte in question leading to more efficient and accurate yields [15].

Once spectral interference levels (oxides and doubly charged, see Chapter 1, Section 1.5.3) and tuning solution conditions (sufficient signal counts) were optimised, the ICP-MS could be coupled to an analytical separation instrument that was suitable for analysis of the selenium samples. Currently, selenium speciation analysis can be carried out by HPLC-ICP-MS, ultra pressure liquid chromatography (UPLC), GFAAS, GC-MS, GC-FID and capillary electrophoresis [16-19]. Derivatisation is also required for certain chiral analytical methods for the determination of chiral amino acids or selenocompounds, e.g. SeMet [20-22].

While numerous analytical detectors are available for selenium speciation, ICP-MS is one of the most widely utilised [23-26]. Furthermore, numerous separation techniques have been reported. In this present study HPLC was the selected separation technique, primarily due to its capability to couple to ICP-MS and also since selenium speciation separation techniques by HPLC are well documented [27-30]. A collision cell with H_2 gas, as previously described, provided highly sensitive and selective analysis of selenium compounds in selenised yeast extracts. Therefore, the coupling of these two analytical instruments was employed for investigations of an accelerated enzymatic extraction of SeMet from selenised yeast.

The second objective of this study was investigation of the chemical extraction of SeMet from selenised yeast. One of the challenges associated with selenised yeast speciation is the recovery of selenocompounds from complex matrices without modifying their native forms. Yeast strains differ in cellular structure, organoselenium composition [31] and total selenium concentration [32] so extraction methods must accommodate such variations in order to be universally applicable. However, validated enzymatic methods are dependent on consistent enzymatic activity between different batches and earlier studies have demonstrated that batch-to-batch variability is an issue in some commercial enzyme products [33]. Essentially this could lead to fluctuations in SeMet recovery due to different extraction efficiencies. Subsequent extraction of SELM-1 with different batches of enzyme (protease XIV) demonstrated significantly different SeMet recoveries ($p \leq 0.05$), see Table 2.12. This highlights the importance of employing enzymes with consistently reliable activity. Fortunately, this was the case for the enzymatic method development in this study but this issue emphasises the need for screening of enzyme batches before any studies are undertaken. Therefore, another objective of this study was to develop, optimise and validate a chemical extraction of SeMet from selenised yeast that would remove the uncertainty associated with batch-dependent enzyme activity.

To date, there have been two detailed selenium speciation studies carried out on the certified reference material SELM-1 [9] and selenised yeast supplements [1]. The former was an inter-laboratory study that involved both chemical and enzymatic extraction conditions as well as different methods for selenomethionine determination. Some of these approaches also incorporated methyl chloroformate or cyanogen bromide (CNBr) derivatisation. The instrumentation also varied between liquid chromatography-

UV-atomic fluorescence spectroscopy (LC-UV-AFS), amino acid analysers, high performance liquid chromatography-inductively coupled plasma-tandem mass spectrometry (HPLC-ICP-MS/MS), gas chromatography-mass spectrometry (GC-MS) and LC-MS/MS. The second study, which took place 2 years later, used many of the same extraction conditions and analytical techniques and most of the methods applied in these studies are still widely used for selenium speciation [10, 34-41]. More recent research investigated accelerated microwave assisted chemical extractions (MACE) for the determination of SeMet in SELM-1 [42]. However, the method of analysis employed isotope dilution GC-MS and needed a derivatising step. The benefit of the MACE HPLC method over GC methods is the removal of the derivatisation steps. Derivatisation creates more experimental steps in the method protocol thus increasing extraction time and inconvenience. Furthermore, derivatisation could result in an incomplete reaction with the analyte (SeMet) leading to inaccurate recovery of SeMet while also increasing the complexity of the analysis. The MACE approach was adopted to avoid time consuming reflux conditions thus completing SeMet extractions from selenised yeast at an accelerated rate [43].

Reflux conditions examined for the acid extraction of selenised yeast predominantly employed MSFA [1, 12, 44]. The method involved heating a mixture of the sample and MSFA under reflux to liberate intracellular SeMet. Extraction times were still lengthy (8-16 hours) [1, 45-47]. Selenomethionine recoveries from the SELM-1 certified reference material, ranged from 95-110% in previous studies [1, 9, 43, 47-53]. While recoveries of SeMet match the certified reference values reasonably well, the duration of the reflux extractions were still excessively long to be considered an accelerated method. Furthermore, none of these investigations carried out a complete validation protocol in accordance with the ICH tripartite guidelines [54] or any other protocol, with most choosing to analyse the SeMet content of SELM-1 for confirmation instead.

Refluxing the yeast samples in aqueous MSFA acid can be 8-16 hours long, thus requiring the need to prevent SeMet oxidation [1]. While reverting oxidised SeMet back to SeMet is possible, as well as the inclusion of antioxidants as a preventative measure, both approaches could be minimised or even avoided if there was a faster extraction method. Microwave-assisted chemical extraction methods can significantly decrease digestion times due to the benefit of a closed vessel system [55]. Since the microwave

vessel remains sealed, the sample suspension experiences increased pressure. The resulting pressure increases the boiling point of the acid/sample suspension, allowing higher temperatures to be reached than possible at ambient pressures. A higher boiling point translates to higher reaction rates and thus faster extraction.

Recent MACE methods have utilised HCl as the acid for the extraction step [56], while research carried out at the same time as the present study also used MSFA [42, 57]. The time and cost were influential factors when researching alternatives to enzymatic extractions for SeMet determination. So much so, that other researchers examined ways to reduce the volume of enzyme used for SeMet determination [58]. Development of a chemical extraction method would be more cost effective than that of a protease XIV extraction. The chemical reagents employed for SeMet extraction, such as 6 M HCl or MSFA [44], are easier to standardise and much cheaper than enzymatic reagents. Development of a chemical extraction method for SeMet also provides an alternative method of analysis to confirm any enzymatic extraction results.

The focus of this study was to develop, optimise and validate accelerated enzymatic and chemical extraction methods that could be applied to the determination of SeMet in selenium-enriched yeast. In summary, there were four significant reasons for the development of these extraction methods:

- to benefit from having more than one set of conditions for extracting SeMet from selenised yeast.
- to reduce extraction times and,
- to decrease the cost of routine SeMet determination.
- also a chemical method would remove potential issues with batch-to-batch variation when carrying out the enzymatic extraction method.

2.2 Experimental

2.2.1 Instrumentation

Moisture analysis for the certified reference material (CRM) SELM-1 was carried out on a Denver Instrument, IR 35. A Techne Hybridiser HB-1D was used to maintain a constant temperature for enzymatic sample extractions. The instrument also allowed gentle mixing of the sample solution as the test tubes were constantly turned

end-over-end inside the oven. An ultrasonic probe was used to homogenise and disrupt the cells causing cell lysis. The chosen ultrasonic probe was a Heinemann 130 W ultrasonic homogeniser (HTU SONI 130, USA) equipped with a 3 mm double-step titanium probe. The analytical separation and detection utilised the Agilent Technologies 1260 infinity series HPLC system coupled to an Agilent Technologies 7700× series ICP-MS, respectively. Solutions for total selenium or SeMet analysis were introduced to an Agilent Technologies 7700× series inductively coupled plasma-mass spectrometer, equipped with a gas cell that contained either helium (99.99 % purity) or hydrogen (99.99 % purity) when operating under collision or reaction mode, respectively. The samples were introduced using a Micromist nebuliser and a Peltier-cooled quartz spray chamber. The entire system was controlled using Mass Hunter software, full version A.01.02. Instrumental parameters are listed in Table 2.1.
Even though the 1260 HPLC system has an inbuilt degasser, an Ultrawave QS-12 ultrasonic bath was utilised to degas all liquids and solutions before any applications such as the mobile phase or dilution solutions. The microwave digester used for the experiments was a CEM Discover equipped with an Explorer SP-D Plus 24/48 autosampler.

The reflux apparatus is shown in Figure 2.1. This included a 6 position Electrothermal EM6 mantle (capable of incremental heating to nominal maximum of 450 °C) for refluxing samples in round bottom flasks equipped with vertically attached Liebig condensers.

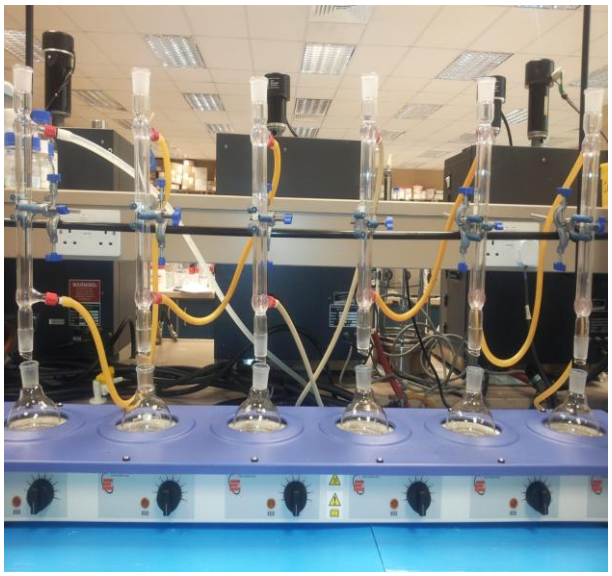

Figure 2.1: Reflux apparatus set-up with mantle and condensers for the extraction of selenomethionine from selenised yeast.

2.2.2 Chemicals and reagents

An ELGA Purelab Flex S7 system was used to produce >18 MΩ cm water. L-SeMet standard powder (>98 % by TLC) was obtained from Sigma (UK). This was used to prepare a 100 mg/L stock solution in 0.1 M HCl. Aliquots (0.1 mL) of this solution were frozen (-20 °C) and used fresh each day. Protease XIV (*Streptomyces griseus*), lipase, driselase and trizma base primary standard were obtained from Sigma (UK). Hydrogen peroxide (30 % H_2O_2 w/w in H_2O), methanesulfonic acid (MSFA; 70 wt. % in deionised water), methanol (HPLC grade) and TFA (Sigma ReagentPlus® grade (99 %)) were also supplied by Sigma (UK). The certified reference material, SELM-1 (National Research Council (NRC), Canada; 2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet or 1390 ± 59 mg/kg Se as SeMet), was used throughout the optimisation process and was the test material for the validation study. Selenised yeast product A (YPA) was obtained for application of the enzymatic extraction to compare a different strain of selenium-enriched yeast to SELM-1. Non-selenised *Saccharomyces cerevisiae* sample of yeast product A (YPA) was provided by Alltech. Two non-consecutive batches of 3 different selenised yeast products (YPB, YPC and YPD) were acquired for application purposes. Daily ICP-MS instrument performance was checked using a multi-element standard (Agilent Technologies) containing Ce, Co, Li, Mg, TI and Y at concentrations of 1 µg/kg.

2.2.3 HPLC set-up

2.2.3.1 Enzymatic method

Sample vials were cooled and kept constant at 4 °C in the HPLC autosampler with the aid of a chiller module. All HPLC mobile phases were filtered before analysis with glass microfibre filters (0.6-0.8 µm, GF/F, Whatman, UK). The entire HPLC system (column, pumps and lines) was purged with high concentrations (100 %) of methanol (2-3 hours). The HPLC was then primed with mobile phase (97.9 % H_2O, 2 % MeOH and 0.1 % TFA). The mobile phase flow rate was slowly adjusted by 0.1 mL/min from 0 to 0.9 mL/min through the column over a one hour period. The column compartment of the Agilent 1260 infinity series was set to 25 °C and when the ICP-MS was fully tuned analysis was initiated. The columns used for the HPLC separation were the RX-C8, 4.6 × 250 mm (5 µm) and the Poroshell 120 EC-C8 3.0 × 100 mm (2.7 µm) column. The

column was changed to the shorter Poroshell 120 EC-C8 which still maintained similar chromatography chemistry but provided faster analyte elution. The injection volume was 45 µL for the RX-C8, 4.6 × 250 mm I.D (5 µm) column and 5 µL for the 120 Poroshell EC-C8 3.0 ×100 mm (2.7 µm) column.

2.2.3.2 Chemical method

The column oven was set to 25 °C with the aid of a handheld pilot remote and analysis was initiated when the ICP-MS was fully calibrated and tuned. Sample vials were cooled and kept at a constant 4 °C in the HPLC autosampler with the aid of a chiller module. The columns used for the HPLC-ICP-MS analysis were the Zorbax RX-C8, 4.6 × 250 mm (5 µm) (Agilent Technologies) and the YMC Triart C8, 4.6 × 250 mm (5µm) (Apex Scientific). The injection volume was 45 µL for the RX-C8 column and 20 µL for the YMC Triart C8 column. The isocratic mobile phase consisted of deionised water: MeOH: TFA (97.9: 2.0: 0.1). The mobile phase flow rate was 0.9 mL/min and the analysis time was 30 minutes per sample with SeMet eluting after ~13-14 minutes.

2.2.4 ICP-MS

The ICP-MS was calibrated using its built-in parameter assessment based on concentrations of elements in a specified tuning solution provided by Agilent Technologies (1 µg/kg Li, Ce, Co, Tl and Y). Once the torch was clean, fully aligned and warmed up, a series of further auto tunes and checks were manually carried out to ensure the ICP was working at its optimum performance. Isotopes ^{76}Se, ^{77}Se and ^{78}Se were monitored to confirm the selenium peaks based on natural isotopic abundance.

2.2.5 HPLC-ICP-MS calibration standards

SeMet contains 40.3 % selenium by weight, thus SeMet results can be reported as selenium as SeMet, or as SeMet. A calibration curve (20.15-100.75 µg/kg Se as SeMet) was used to quantify the analyte in the selenised yeast sample extracts. Each SeMet standard was analysed in triplicate. The analysis would only continue once the correlation coefficient of the calibration standards was ≥0.995. A frozen aliquot (100 µL) of previously made SeMet stock standard solution (100 mg/kg) was used to ensure consistency throughout the development and validation process. A 20 µL volume of this stock solution was added to 1.98 mL of deionised water to give a 1 mg/kg solution of

SeMet. Aliquots of 50, 100, 150, 200 and 250 µL of this 1 mg/kg solution were made up to 1 mL respectively, to give 20.15, 40.3, 60.45, 80.6 and 100.75 ng/mL Se as SeMet.

2.2.6 Post HPLC-ICP-MS analysis

Once samples were analysed, they were corrected for any constructive or destructive drift. Adjustment for drift was carried out by analysis of a 120 µg/kg independent SeMet check standard that was made separately from the calibration standards and tested every 6-8 samples. The samples were then corrected constructively or destructively based on the drift that this test sample underwent. The column was flushed, after every analysis, for approximately 3 hours with a methanol gradient up to 100 %.

2.2.7 Sample extraction methods

2.2.7.1 Three day enzymatic extraction

The three day proteolytic extraction was carried out as described by Polatajko *et al.* [2]. Approximately 0.2 g of selenised yeast powders were weighed out and placed into 15 mL plastic test tubes. A 30 mM Tris HCl 7.5 pH buffer was made for the protease XIV/lipase enzyme (0.364 g Tris in 100 mL H_2O). The pH was adjusted using 6M HCl. Protease XIV and lipase were added in a ratio to ensure there was ~20 mg protease XIV and ~10 mg lipase enzyme in every 5 mL of enzyme solution. This enzyme solution (5 mL) was then added to the yeast samples and placed into the hybridisation oven for 17-24 hours at 37 °C, with end on end rotation at the second slowest rotation speed (7.5 rpm). Once incubation was completed, tubes were centrifuged for 30 minutes at 6,000 rpm. The supernatant was poured into another 15 mL test tube and 10 µL of β-mercaptoethanol was added. An aliquot of each supernatant from the three extractions was stored separately to determine the efficiency of the extraction. A 5 mL aliquot of fresh protease XIV/ lipase enzyme solution was then added to the remaining pellet for a second extraction. The tubes were vortexed to break up the pellet and placed back into the hybridisation oven for another 17-24 hours. This process was repeated once more and at the end there was ~15 mL (minus any aliquots (~60 µL per 5 mL) taken for further analysis) of supernatant for selenium speciation and total selenium analysis. For selenium speciation analysis, the supernatant was filtered using Chromafil Xtra

RC-20/25 0.2 µm RC filters (Macherey-Nagel, Germany) and diluted 1 in 50 (20 µL + 980 µL deionised water). Fresh mobile phase of 2 % MeOH and 0.1 % TFA was made for every analysis. SeMet concentration was then quantified by external calibration.

2.2.7.2 Microwave assisted enzymatic extraction

Microwave extractions were carried out to determine SeMet content of selenised yeast. The enzymes used were protease XIV and driselase. Protease XIV and driselase concentrations were 40 mg each per 250 µL Tris buffer for each sample. The protease XIV solution was added to 40 mg selenised yeast and vortex mixed until homogenous. The extraction was carried out at times from 15-120 minutes with fixed microwave power outputs between 5 and 80 W. The contents of the 1.5 mL eppendorfs were then centrifuged for 3 minutes at 14,000 rpm and the supernatant was poured into a 15 mL test tube half-filled with deionised water containing 15 µL β-mercaptoethanol. The pellet was washed with 900 µL deionised water, centrifuged and the wash solution was added to the test tube. The pellet was re-extracted and digested with 120 mg /750 µL driselase solution and extracted in the MW (15 minutes at 37 °C). Once centrifuged again and added to the test tube, the wash step was repeated. The test tube was made up to a final volume of 15 mL with deionised water and was filtered and diluted before analysis by HPLC-ICP-MS.

2.2.7.3 Sonication

Sonication was examined with a Heinemann 130 W ultrasonic homogeniser. The sonicated sample volume was 500 µL based on the titanium probe's double-step 3 mm diameter. Additionally, sample volume size was kept to 500 µL to suspend the yeast sample (~40 mg) in enzyme solution. Sonication parameters of time and amplitude were investigated. The probe was rinsed with enzyme solution (250 µL) and deionised water after each sonication with washings transferred to 15 mL test tubes. A 500 µL solution of protease XIV (40 mg/250 µL) was added to ~40 mg selenised yeast, vortex mixed until homogenous and sonicated for 30, 60 and 90 seconds at varying amplitude (40-100 %). A further 250 µL of protease XIV solution was used to rinse down the probe and the sample was vortexed.

2.2.7.4 Reflux chemical extractions

Extraction by chemical reflux was carried out by the method developed by Wrobel *et al*. [44]. In brief, 100 mg of selenised yeast was placed in a round bottom flask with 10 mL of 4 M MSFA. A volume of 200 µL of β-mercaptoethanol antioxidant was added and the mixture was refluxed for 16 hours.

2.2.7.5 Microwave-assisted chemical extraction

SELM-1 was chosen as a reference material since its SeMet content was previously certified [9]. To summarise the approach; 0.045 ± 0.05 g of sample was accurately weighed into 10 mL microwave vessels. A solution of 4 mL of MSFA (4 M) was added and vortex-mixed for 2 minutes. The extraction program had a ramp time of 2 minutes and a 10 minute hold at 200 °C with varying microwave power. Once cooled to <40 °C the chemical extract was then transferred into 50 mL centrifuge tubes. The microwave vessel was rinsed 3 times with water and all rinsings were transferred to the 50 mL centrifuge tube. The volume was made up to 50 mL using deionised water and the tube was inverted 20-30 times to ensure homogeneity before removing an aliquot (2 mL) for filtration (0.2 µm regenerated cellulose filter) and dilution (1 in 25; 40 µL sample + 960 µL H_2O) with deionised water into glass HPLC vials before analysis.

2.2.8 Sample analysis

The 2 mL aliquots of the enzyme extracted solution were filtered using 0.2 µm regenerated cellulose filters. Further dilutions (1 in 50; 20 µL sample + 980 µL H_2O) were made with deionised water into HPLC glass vials. This ensured adequate concentration of the SeMet analyte for the calibration curve. The isotopes monitored were ^{76}Se, ^{77}Se and ^{78}Se.

All calculations were based on ^{78}Se

$$C = \frac{D \times V \times A}{m \times 1000}$$

C = SeMet concentration in the sample (mg/kg)

D = Dilution factor (usually 50 but may be adjusted as appropriate)

V = Volume after sample extraction (mL)

A = SeMet content of analysed samples (µg/L) based on peak area from the chromatogram

m = Sample mass (g)

1000 = Conversion of µg/kg to mg/kg

2.2.8.1 Moisture correction

It was documented by the National Research Council of Canada that the selenium speciation results of the selenium-enriched yeast CRM, SELM-1, were based on the weight of a dried yeast product. Therefore, the moisture content of the CRM was measured to ensure accuracy of results. SELM-1 moisture content was calculated with a moisture analyser and gave a value of 6.42 ± 0.25 % (n=3). This figure agreed with the moisture content of spray dried selenium-enriched yeast which normally has between 5-10 % moisture content [1]. All results presented in this study were moisture corrected unless stated otherwise.

2.2.9 Method validation

For validation of the newly developed accelerated method, SeMet determination using HPLC-ICP-MS investigated the precision, accuracy, repeatability, reproducibility, linearity, range, limit of detection (LOD), limit of quantification (LOQ), specificity and selectivity of the method as described by the ICH guidelines [54]. The final chromatography conditions that were utilised for the validated enzymatic and chemical methods are outlined in Table 2.1.

Table 2.1: Liquid chromatography and ICP-MS conditions.

Liquid chromatography conditions for enzymatic extraction	
Column A	Agilent 120 Poroshell EC-C8 3.0 × 100 mm (2.7 μm)
Flow rate	0.9 mL/min
Injection volume	5 μL
Mobile phase	97.9 % H_2O: 2 % MeOH: 0.1 % TFA
Column B	Agilent Zorbax RX-C8, 4.6 × 250 mm (5 μm)
Flow rate	1 mL/min
Injection volume	45 μL
Mobile phase	97.9 % H_2O: 2 % MeOH: 0.1 % TFA
Liquid chromatography conditions for chemical extraction	
Column	YMC Triart C8, 4.6 × 250 mm (5 μm)
Flow rate	1 mL/min
Injection volume	20 μL
Mobile phase	97.9 % H_2O: 2 % MeOH: 0.1 % TFA
Column Oven	25 ℃
ICP-MS parameters	
RF Power (W)	1550
RF Matching (V)	1.4
Carrier Gas (Ar) (L/min)	0.91
Makeup Gas (Ar) (L/min)	0.20
Nebuliser Pump (rps)	0.4
Collision cell gas flow (H_2)	6.6
Monitored Species	^{76}Se, ^{77}Se, ^{78}Se [59]

2.2.10 Mass balance

To ensure the extraction process of the assays were efficient, a mass balance was completed. The total selenium content of the soluble fraction was calculated by chemical digestion in concentrated nitric acid followed by analysis on the ICP-MS. The total selenium content of the enzymatic and chemical extracts were calculated by the ICP-MS CIC tool in conjunction with the SeMet calibration standards. The selenium content of other selenocompounds or selenoproteins that appeared as unidentified peaks in the HPLC-ICP-MS were estimated by CIC.

2.2.11 Statistical analysis

Statistical analyses of results were performed using Minitab statistical software version 17 (Coventry, UK). Two-sample t-test and one way analysis of variance (ANOVA)

were carried out to test significant differences between means, where the confidence level was set to 95 %. Significant levels were defined using $p \leq 0.05$.

2.2.11.1 Horwitz function

The Horwitz function was applied to determine the acceptable relative standard deviation of the various measured analytes throughout this thesis. The equation was;

$$H(\%) = 2^{(1 - 0.5 \times \log C)}$$

Where C = relative concentration.

2.3 Results and discussion

2.3.1 Accelerated enzymatic method

The standard published three day enzymatic extraction of SeMet from selenised yeasts was extremely time-consuming for routine industrial analysis [2]. This justified the need to investigate and try to develop a faster extraction method. However, such a long extraction increased the risk of oxidation of selenomethionine. β-mercaptoethanol was added as an antioxidant to prevent this oxidation. Reduction of any methionine selenoxide is chemically possible using glutathione (GSH) (see Chapter 1, Section 1.3.3) although studies in this field stick to the prevention of oxidation with β-mercaptoethanol.

A sample of selenised yeast (YPA) was chosen for comparison purposes as it had similar concentrations of total selenium (determined by AA, ICP-OES and ICP-MS) as the CRM but also because it had comparable levels of SeMet (~1,400 mg/kg) (determined by the aforementioned three day enzymatic extraction [1]).

2.3.1.1 Selenomethionine stock solution stability

Standards were made as described in Section 2.2.5. To investigate the reliability of these standards, the slope of each standard curve was recorded and monitored over the course of a few weeks to examine the stability of frozen SeMet over time (Figure 2.2). These results confirmed that SeMet was stable when frozen at concentrations of 100 mg/kg for at least 25 days. The average slope was 1035.42 ± 38.11 (n=9).

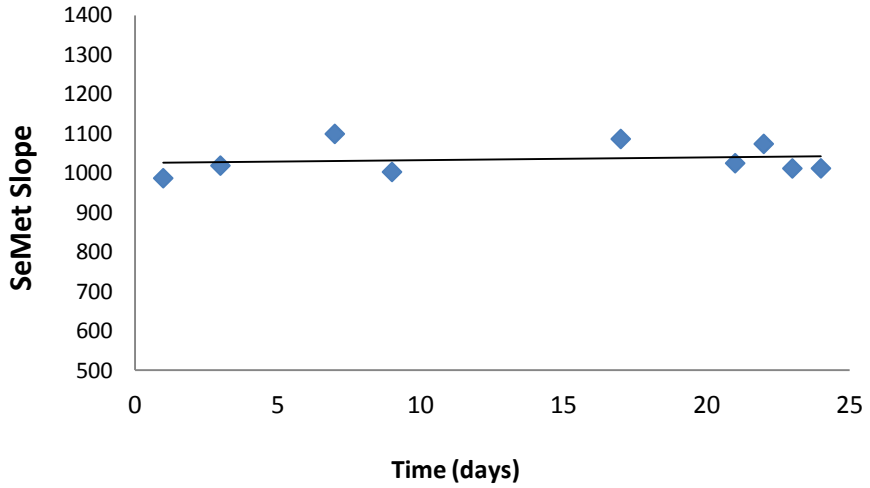

Figure 2.2: Selenomethionine standard stability.
SeMet standards were measured over the course of 25 days by HPLC-ICP-MS.

2.3.1.2 Sample extraction

2.3.1.2.1 Enzymatic extraction of selenomethionine from selenised yeast

The 72 hour enzymatic extraction, as mentioned in Section 2.2.7.1, was carried out on the two yeast products, YPA and SELM-1 (Table 2.2). The extracted supernatants were not pooled together but separated from the insoluble pellets for SeMet determination and analysed by HPLC-ICP-MS. When all the extracts of the two yeasts were analysed, it was clear that no SeMet was recovered in the last incubation extraction (Table 2.2). Results suggested that the final incubation extraction served little purpose regarding SeMet recovery. Therefore, the extractions could be reduced from three to two. Furthermore, the recovery of SeMet from the second incubation extraction was at most 1-4 % of the total selenium. The majority of SeMet was liberated from the selenised yeast in the first incubation extraction.

Table 2.2: Recovery of selenomethionine from each 17 hour enzymatic extraction of selenised yeast.

Samples	Total Se (mg/kg)	1st Extraction Se as SeMet (mg/kg)	2nd Extraction Se as SeMet (mg/kg)	3rd Extraction Se as SeMet (mg/kg)	Total Se as SeMet (mg/kg)	Recovery of CRM SELM-1 (%)
YPA	2004.5 ± 21	1313.4 ± 8	56.8 ± 2	0.0 ± 0	1369.8	
SELM-1	1949.4 ± 45	1337.9 ± 50	60.0 ± 32	0.0 ± 0	1378.0	99.1

SELM-1 certified values; 2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet or 1390 ± 59 mg/kg Se as SeMet. YPA = yeast product A. Results are based on triplicate readings (n=3).

2.3.1.2.2 Reduction of enzymatic extractions by utilising microwave energy

As part of the development and optimisation process, different methods were investigated. One of these methods involved microwaves (described in Section 2.2.7.2) and specifically looked at the development of a microwave assisted enzymatic extraction. An experiment was designed to investigate microwave extraction time and microwave power to increase the efficiency of the enzymatic extraction. The hypothesis was to investigate microwave extractions as a means to accelerate the extraction of SeMet from selenised yeast. SELM-1 was the test sample for this study. The experimental design included the investigation of microwave power from 5 to 80 watts across different times, varying from 15 to 120 minutes with triplicate analysis for every data point (Figure 2.3). A combination of protease XIV and driselase enzymes were investigated due to previously reported success with this enzyme mixture for preparing yeast extracts for selenium speciation analysis [32]. The highest SeMet recovery in the shortest microwave extraction time was observed at a fixed power output of 30 W for 15 minutes. These parameters were chosen for further investigation.

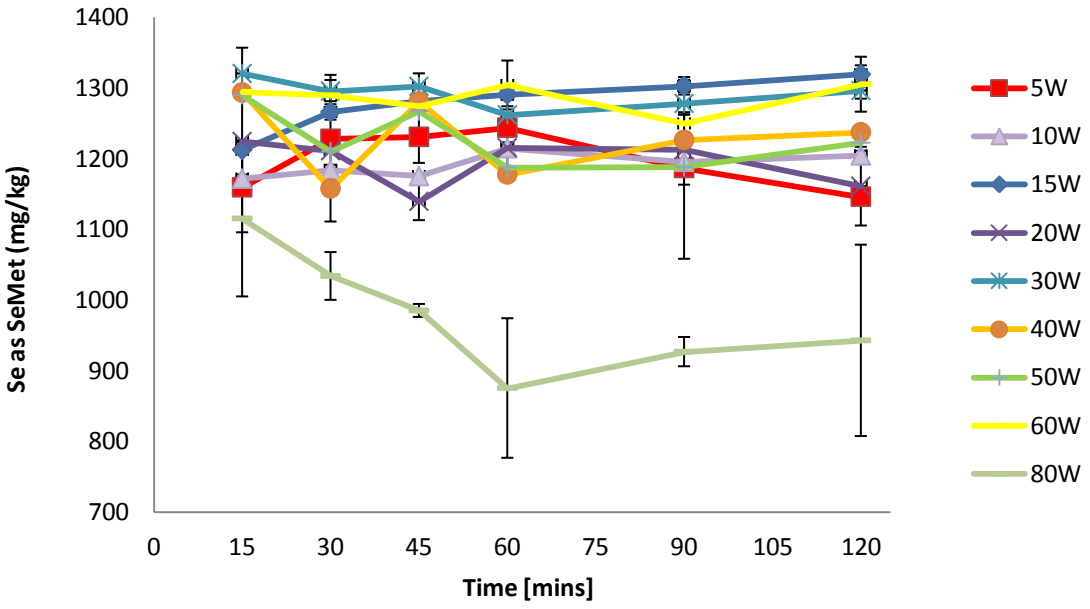

Figure 2.3: Selenomethionine extraction from SELM-1 after microwave extractions with varying power (5-80 W) and time (15-120 minutes).
Results are based on triplicate readings (n=3).

It was also noted that at microwave power 80 W (Figure 2.3) the SeMet recoveries decreased significantly which can be attributed to the enzyme/yeast solution slightly curdling and solidifying. The 80 W extractions reached a temperature of 100 °C for the

majority of the microwave extraction (see Table 2.3). With increased power came an increase in temperature of the sample during extraction. This temperature increase resulted in a poor recovery for the 80 W extraction. These findings were confirmed by the supplier (Sigma, UK) who specified that protease XIV is "completely inactivated by heating above 80 °C for 15-20 minutes" [60].

Table 2.3: Temperature (°C) during various microwave extraction power outputs.

Power (W)	Temperature Reached (°C)
5	~30
10	~40
15	~45
20	~45
30	~65
40	~65
50	~75
60	~90
80	~96-100

The information recovered from the microwave assays allowed for the comparison of the microwave parameters. These were compared to each other to determine which conditions achieved the highest recoveries of SeMet. The microwave work aimed to replace the enzymatic extractions in the incubator thus two microwave extractions were carried out (the third proteolytic extraction yielding little or no SeMet, Table 2.2). However, while these microwave extractions were much quicker than the three day method, it still involved tedious, time-consuming steps such as washing the pellet and centrifuging. These steps took a long time to complete as the pellet was created using the 14,000 rpm conditions, thus taking a long time to re-suspend the pellet. While two enzymatic extractions (protease XIV and driselase) were investigated, ideally a single microwave extraction would be preferential. This would reduce not only the time taken for the overall extraction but also decrease the number of steps needed to complete the extraction.

The amount of SeMet liberated in a second microwave extraction, under the same conditions, was investigated. These parameters were 15 minutes at 30 W of fixed power. The results are shown in Figure 2.4.

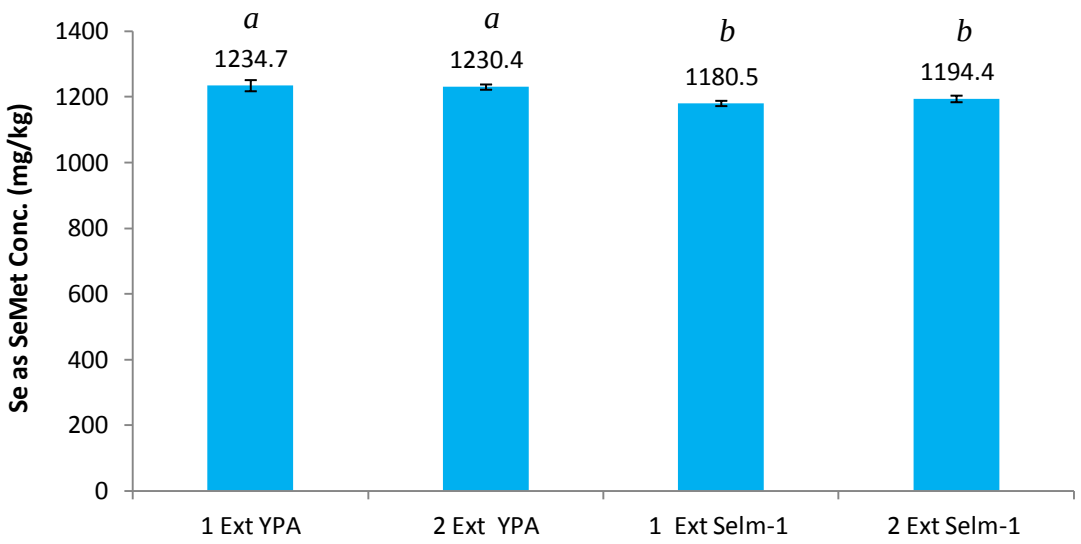

Figure 2.4: Selenomethionine extraction from one protease XIV extraction compared to one protease XIV extraction followed by a driselase extraction.
[a,b] Values that have a common superscript letter do not differ in significance ($p \geq 0.05$). YPA = yeast product A. Results are based on triplicate readings (n=3).

The extractions were performed on YPA and the CRM SELM-1. The results revealed that there were no differences between YPA samples or between SELM-1 samples when carrying out one protease or sequential protease and driselase extractions ($p \geq 0.05$). The results in Figure 2.4 were almost identical for the respective samples regardless of whether there were one or two 15 minute extractions. This evidence suggested that it was possible to reduce the number of extractions from two to one and also utilise protease XIV on its own in the extraction assay without affecting SeMet recovery. The extraction time was effectively halved as there was no significant benefit to carrying out the second extraction. The single protease XIV extraction of the CRM, SELM-1 resulted in SeMet recovery of 84.9 % (1180.5 ± 9 mg/kg) when compared to the three day extraction method (Section 2.2.7.1). These results suggested that the extraction was not entirely efficient with respect to SeMet determination.

Before protease XIV was used on its own in the microwave extractions, the possibility of using a cocktail of enzymes was examined i.e. a protease XIV/driselase mix. The enzyme solutions were added to ~40 mg selenised YPA and SELM-1. The samples were vortex mixed until homogenous and extracted in the microwave for 15 minutes at 30 W.

Table 2.4: Determination of selenomethionine in SELM-1 by single microwave extractions with protease XIV and driselase.

Samples	Mean Se as SeMet (mg/kg)	S.D.	R.S.D.	Recovery of CRM SELM-1 (%)
YPA 40 mg protease XIV	1234.9	15	1.2	
YPA 40 mg driselase	139.7	4	2.9	
YPA 20 mg protease XIV + 20 mg driselase	1040.0	40	3.9	
YPA 40 mg protease XIV + 20 mg driselase	1248.0	22	1.8	
SELM-1 40 mg protease XIV	1160.9	34	2.9	83.5
SELM-1 40 mg driselase	100.5	5	4.9	7.2
SELM-1 20 mg protease XIV + 20 mg driselase	1093.1	14	1.3	78.6
SELM-1 40 mg protease XIV + 20 mg driselase	1151.9	23	2.1	82.8

Enzyme amounts were per 250 µL. Final volume before microwave extraction was 750 µL. YPA = yeast product A. SELM-1 certified values; 2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet or 1390 ± 59 mg/kg Se as SeMet. Results are based on triplicate readings (n=3).

The results in Table 2.4 showed that protease XIV as an enzyme solution on its own was more efficient at extracting SeMet from YPA and SELM-1 than the 50:50 cocktail of protease XIV and driselase ($p \leq 0.05$ respectively). The single protease XIV extraction method was repeatable at concentrations of 40 mg of protease XIV (Figure 2.4) with a consistent recovery of approximately mid-1200 mg/kg each time for YPA and mid-1100 mg/kg for SELM-1. This gave a SeMet recovery of 83.5 % of the CRM. Driselase on its own resulted in a low SeMet recovery of 7.8 ± 0.4 % of the CRM, SELM-1. Similarly, low recovery was experienced for YPA. Such a low SeMet recovery from a driselase extraction was not expected, however a cocktail of driselase and protease gave only slightly more total selenium recoveries when compared to just protease in other relevant research [32]. These results were similar to what was recorded in this study with driselase responsible for only a small overall recovery. When 20 mg of driselase was added on top of the 40 mg of protease XIV enzyme, although slightly higher in recovery for YPA (1248.0 compared to 1234.9 mg/kg for YPA; 1160.9 compared to 1151.9 mg/kg for SELM-1, respectively), there was no significant difference to that of the extractions with just 40 mg of protease XIV (both $p \geq 0.05$). Therefore, an enzymatic solution of 40 mg protease XIV was selected for the sonication extraction analysis.

2.3.1.3 Sonication

The microwave enzymatic extraction procedure (Table 2.4) for SeMet determination of YPA yielded 1234.9 ± 15 mg/kg of selenium as SeMet. The same method was applied to the CRM SELM-1. The expected certified value of 1390 ± 64 mg/kg selenium as SeMet was not reached. Instead the best SeMet recovery from SELM-1 was 83.5 % (1160.9 ± 34 mg/kg). To enhance SeMet recovery sonication was investigated. The benefit of a probe sonicator was that it imparted higher frequency energy than that of an ultrasonic bath [61]. A further benefit of a sonication probe was the ability to impart this higher energy to a much smaller sample volume size.

2.3.1.3.1 Sonication with protease XIV

Sonication was carried out as described in Section 2.2.7.3, followed by microwave extraction for 15 minutes at 30 W. These microwave conditions were applied since they gave the best recovery of SeMet in this study (Figure 2.5). Rinsing the probe ensured no sample was lost and also reduced possible cross contamination to the next sample. Microwave extraction was carried out as described in Section 2.2.7.2

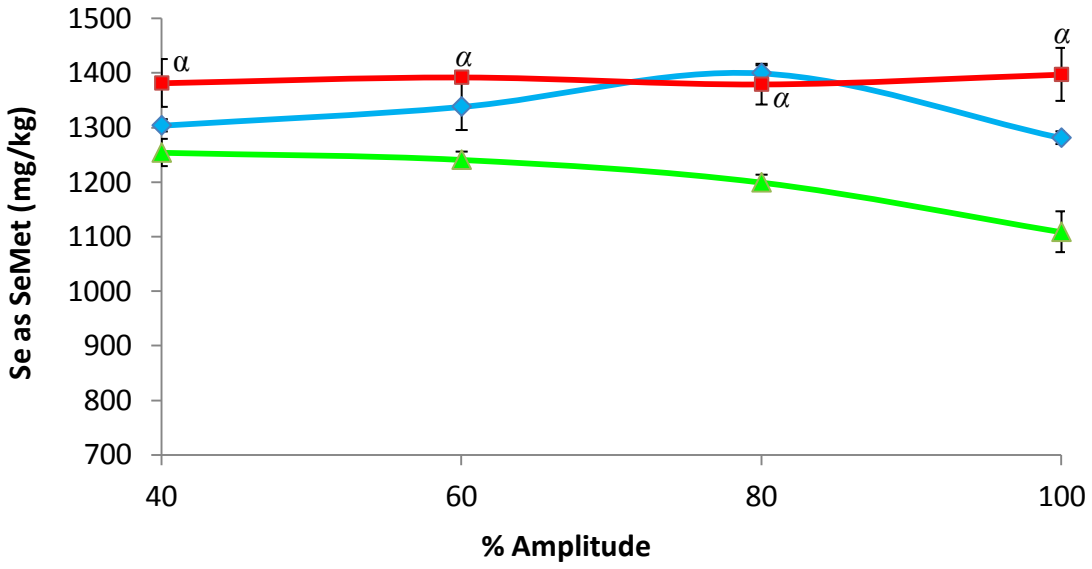

Figure 2.5: Extraction of selenomethionine from SELM-1 by sonication followed by microwave digestion at 30 W for 15 minutes.
Sonication was carried out for 30, 60 and 90 seconds at 40, 60, 80 and 100 % amplitude. ♦ = 30 second sonication; ■ = 60 second sonication; ▲ = 90 second sonication. Results are based on triplicate readings (n=3). $^{\alpha}$ Values that have a common superscript letter do not differ in significance ($p \geq 0.05$).

Sonication times of 30 to 90 seconds, along with the incorporation of enzymes, were investigated. The literature suggested 30 seconds would be sufficient for extracting

SeMet from selenised yeast [62]. Another variable sonication parameter was amplitude. Amplitude is a measure of the height of the sine wave over single period or cycle. The higher the amplitude, the more energy the sonicator probe imparted. The amplitude of 40 to 100 % was investigated at each time point. The aim of this experiment was to investigate if the combination of sonication followed by microwave extraction increased the recovery of SeMet when compared to only a microwave extraction. The CRM, SELM-1, had a recovery of ~85 % of the certified value after a microwave extraction. The recoveries in Figure 2.5 varied from 1108.4 ± 37 mg/kg (90 seconds sonication at 100 % amplitude) to 1399.6 ± 14 mg/kg (30 seconds sonication at 80 % amplitude).

Figure 2.5 demonstrated that longer sonication times could possibly be responsible for reduced SeMet recovery from the CRM with all 90 second sonications performing the worst. This could be caused by splashing and thus loss of sample. Sonication for 60 seconds was the most consistent time for SeMet recovery. There was no significant difference between any of the SELM-1 samples sonicated at varying amplitudes for 60 seconds ($p \geq 0.05$). While there was no significant difference between 30 and 60 seconds sonication at 80 % amplitude ($p \geq 0.05$), a 30 second sonication at 80 % amplitude was favoured as it gave the highest recovery of SeMet in the shortest sonication time. Sonication in conjunction with a protease XIV microwave extraction gave higher selenium as SeMet recovery (1399.6 ± 14 mg/kg) compared to just a single protease XIV microwave extraction (1180.5 ± 9 mg/kg Se as SeMet). In industry, and in this analysis, the bottleneck was sample throughput. Therefore the sonication time remained at 30 seconds and 80 % amplitude. This recovery meant that the accelerated enzymatic extraction was 100.6 ± 1.0 % (1399.6 ± 14 mg/kg) efficient by comparison to its expected SeMet value. The objective of the study was achieved since the assay was reduced from three days to ~40 minutes and provided ≥95 % recovery of SeMet in the CRM.

2.3.1.3.2 Final sonication and microwave parameters

The temperature reached by the end of the 30 W microwave extraction for 15 minutes was approximately 65 °C. To reduce the temperature, ice was added to the extraction vessel carousel. High temperatures during the microwave extractions were documented in Table 2.3. Addition of ice-water instead of room temperature tap water reduced the temperature of the 30 W extraction for 15 minutes, to approximately 40 °C. This

resulted in slightly higher recoveries of SeMet from SELM-1 (Table 2.5). The final change was a reduction of the sonication time from 30 seconds to 25 seconds. This change was also implemented to ensure the sample remained cooler with 5 seconds less of sonication energy imparted into the sample.

Table 2.5: Recovery of selenomethionine from SELM-1 under optimised sonication and microwave extraction conditions.

Sample	Mean Se as SeMet (mg/kg)	S.D.	R.S.D.	Recovered of CRM SELM-1 (%)
SELM-1	1411.4	50	3.5	101.5

Optimised conditions were sonication for 25 seconds at 80 % amplitude followed by microwave extraction with fixed power (30 W) for 15 minutes and inclusion of ice-water in the microwave vessel. Results are based on six readings (n=6).

Therefore, with good recoveries of the certified reference material (101.5 %), and with R.S.D's (3.5 %) below the expected Horwitz function R.S.D's of 5.4 % these microwave and sonication operation parameters were employed for the validation process.

2.3.1.4 Optimised HPLC conditions for selenomethionine separation

The goal of HPLC optimisation was to reduce the analysis time of SeMet. Selenomethionine determination was made using isocratic elution with H_2O: MeOH: TFA (97.9: 2.0: 0.1), TFA was included as an ion pairing agent [11]. The mobile phase was ultrasonicated to reduce oxidation of the SeMet that could occur due to dissolved oxygen in the deionised water [63]. Sample vials were cooled to 4 °C in the HPLC autosampler with the aid of a chiller module to prevent unnecessary degradation that can be caused by heating [64]. The injection volume was investigated, with 45 µL providing the best peak shape and area for the RX-C8, 4.6 × 250 mm (5 µm) column. Regular blank injections were also included to check on the cleanliness and performance of the system [65]. Sample analysis time per injection was 30 minutes. The analyte of interest, SeMet, eluted around 14-15 minutes. However, to allow for complete sample elution and sufficient column clean-up, the run time was set for 30 minutes. The first time the analysis was carried out, the run time was set to 60 minutes to verify no peaks were eluting after 30 minutes. This confirmed there were no late eluting peaks thus ensuring there was no carry over to the next injection, possibly causing interference.

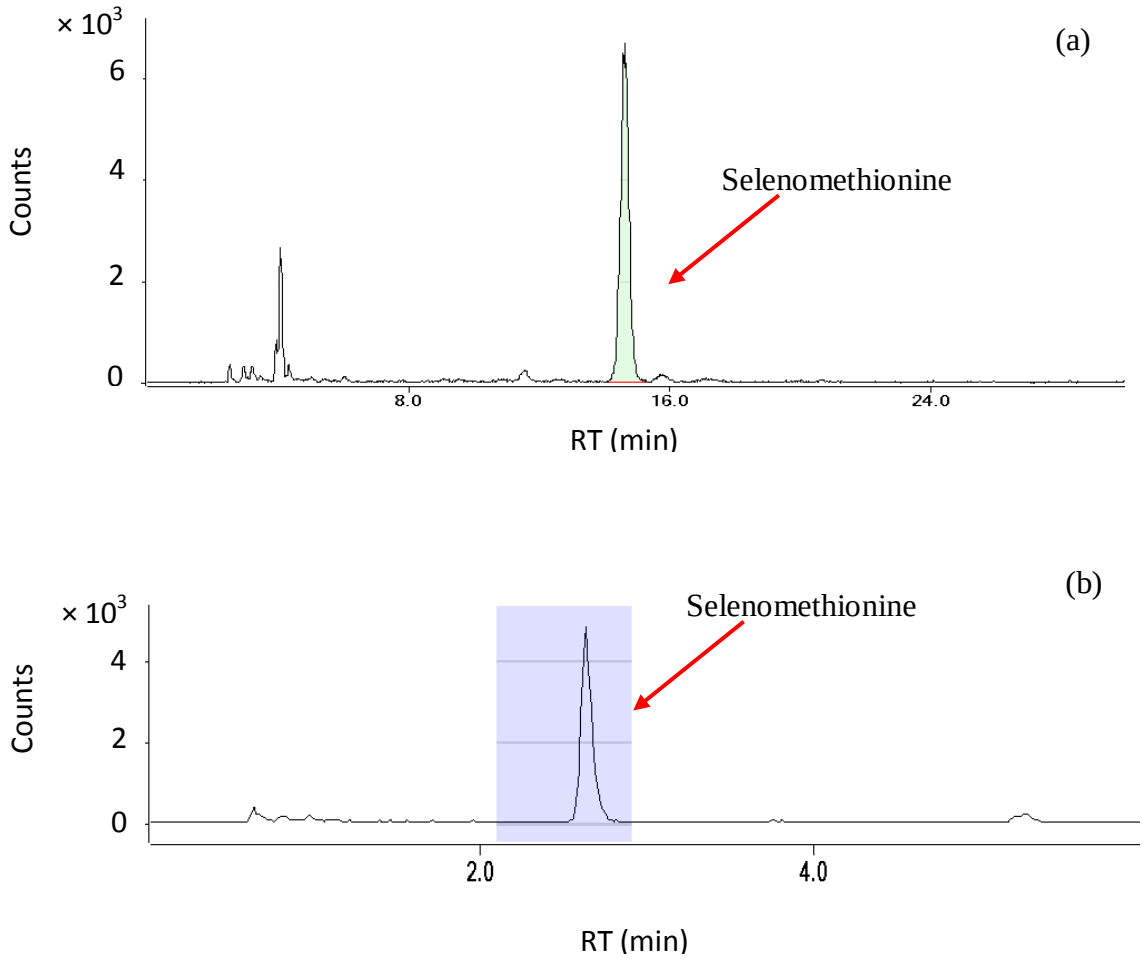

Figure 2.6 (a) and (b): Elution of selenomethionine extracted from SELM-1 by (a) the RX-C8 column and (b) the Poroshell 120 EC-C8 column.

To reduce analysis time, the reversed-phase HPLC column was investigated. The original column, 4.6 × 250 mm RX-C8 with a 45 µL injection volume, was selected for isocratic separations due to previously published SeMet analysis results [1]. The column was switched to the 120 Poroshell 120 EC-C8 3.0 × 100 mm, (2.7 µm) column with an injection volume of 5 µL. Both injection volumes on each column were selected due to good peak shape and resolution, see Figure 2.6 (a) and (b), respectively. The Porshell column was chosen to increase sample throughput, decrease oxidation of the analyte and allowed for the removal of antioxidants. The column was shorter, had a smaller diameter than the previous column and smaller particle size (2.7 µm) and resulted in higher pressure (~400 bar at 0.9 mL/min flow rate), better resolution and faster elution times. The theoretical plate number for the 4.6 × 250 mm RX-C8 column was calculated to be N = 9635, while the shorter 3.0 × 100 mm Poroshell EC-C8 was N = 8155. This equated to only a decrease of ~18 % efficiency but at a much fast elution time for the SeMet analyte. Combining all these factors gave a more rapid SeMet

elution. The previous column had an elution time around 14-15 minutes with the same mobile phase. However, the new Poroshell column reduced this retention time by almost 12 minutes to ~2.5 minutes. Figure 2.6 (a) and (b) illustrates the chromatography optimisation. Overall, sample extraction times were reduced to ~40 minutes and analysis times were reduced from ~30 minutes to ~6 minutes. A deionised water blank sample was injected onto the column before analysis to check the column baseline was free from contamination. Relevant research suggest storage conditions of SeMet are of significance, therefore the HPLC autosampler was maintained at 4 °C to prevent oxidation [66]. Since analysis time could vary from one hour to >12 hours, it was imperative to keep the samples refrigerated while they were waiting to be analysed. Once samples were analysed, the column was cleaned with 100 % MeOH, this removed any sample matrix that remained on the column throughout the analysis. Samples were corrected for any constructive or destructive drift that occurred throughout the analysis as described in Section 2.2.6. Signal drift can overestimate or underestimate the concentration of the analyte in question by anything the region of approximately ± greater than 10 %.

2.3.1.5 Mass balance of selenised yeast fractions

A mass balance was utilised to identify if there was 100 % recovery of selenium. This method was used to account for the selenium in the soluble fraction by two methods. The first method measured the total selenium of the solution by taking an aliquot of the solution and digesting it completely in nitric acid before direct infusion into the ICP-MS. This total selenium value was then compared to the sum of the concentration of all the selenium peaks detected using the HPLC-ICP-MS. The mass balance of the soluble fraction, shown in Figure 2.7, demonstrated good agreement between the two types of analysis.

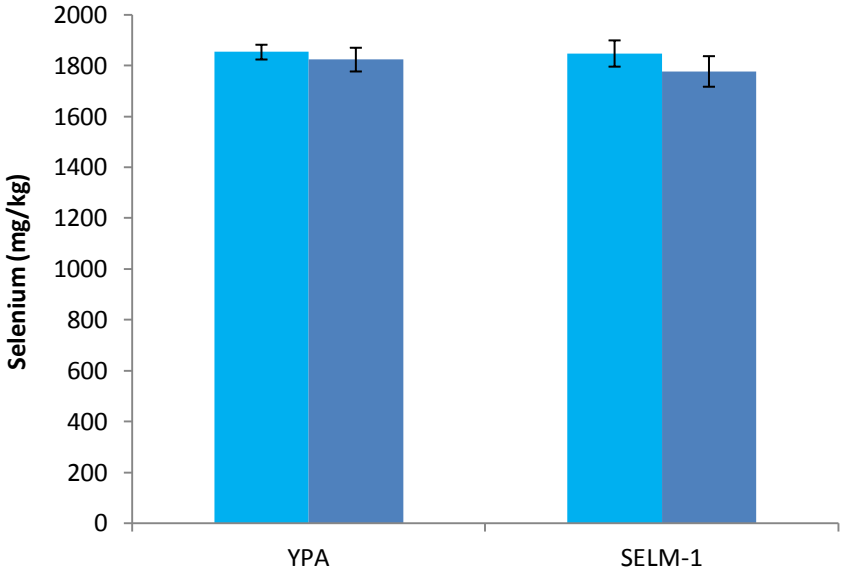

Figure 2.7: Total soluble selenium mass balance of yeast product A and SELM-1.
After extraction by the optimised conditions described in Section 2.2.7.2, total selenium of the soluble yeast fraction was analysed by ICP-MS and compared to the total selenium in selenocompounds detected by HPLC-ICP-MS. ■ = selenium concentration from LC; ■ = total selenium concentration from ICP-MS. YPA = yeast product A. Results are based on triplicate readings (n=3).

The sum of the total selenium from other organoselenium compounds in the supernatant, after the three day extraction, was estimated by compound-independent calibration (CIC). The results were then compared to the total selenium concentration of the sample. This calculated the percentage of selenium as organic selenium. Quantifying the selenium from the HPLC-ICP-MS analysis was essential to see if it matched the total selenium of the overall enzymatic supernatant. Once both of the total selenium results agreed with each other, there was confidence in the HPLC-ICP-MS results. To strengthen this confidence, a mass balance was repeated with the inclusion of the total selenium content of the remaining pellet. This also included a total selenium mass balance based on the total selenium digestion of the supernatant. A good mass balance recovery confirmed the absence of significant matrix effects [67] and gave validity to the method employed [68]. This created three fractions, the total selenium content of the yeast before any enzymatic or chemical digestions, the enzymatic supernatant extract and the pellet which remained after an enzymatic extraction. The sum of the pellet and its corresponding enzymatic extract should equal the total selenium content of the yeast sample to complete a mass balance, see Figure 2.8.

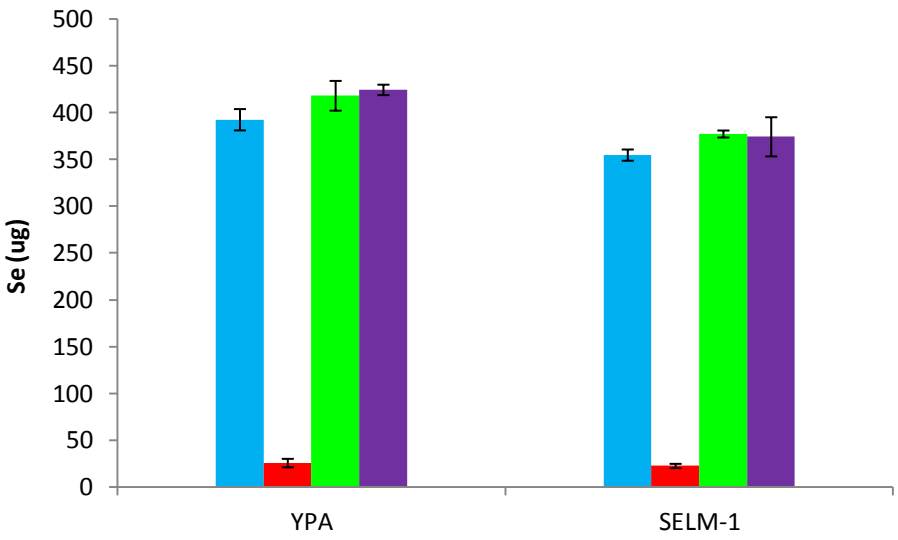

Figure 2.8: Complete mass balance of selenised yeast by total selenium analysis of the soluble and insoluble fractions after a protease XIV extraction.
■ = total selenium from LC speciation; ■ = total selenium from pellet; ■ = total selenium from pellet + supernatant; ■ = total independent selenium from selenised yeast. YPA = yeast product A. Results are based on triplicate readings (n=3).

The mass balance, in Figure 2.8, showed good agreement and presented sufficient information to suggest that the enzymatic extraction was robust and reliable.

2.3.1.6 Optimised microwave assisted enzymatic extraction

Samples of CRM SELM-1 (0.04 g) were weighed into 1.5 mL push-capped micro-centrifuge tubes. Protease XIV enzyme solution (500 µL; 40 mg protease XIV/250 µL Tris buffer) was added to each sample and vortexed (2 minutes). The yeast/enzyme suspension was sonicated using an ultrasonic probe (25 seconds at 80 % amplitude). The probe was rinsed with 250 µL enzyme solution into the 1.5 mL eppendorf and further rinsed with deionised water (8-10 mL) into a 15 mL test tube. Each eppendorf was vortexed again (2 minutes). The 1.5 mL eppendorfs were placed into the CEM discovery microwave carousel, which had ice and water in the bottom to cool the samples during the microwave extraction. The microwave programme was set up to run for 15 minutes at a fixed power output of 30 W and afterwards the eppendorfs were centrifuged (3 minutes at 14,000 rpm). The supernatant was poured into the 15 mL test tube that contained the deionised water washings from the sonicator probe. The pellet was washed with deionised water (900 µL) and vortexed until homogenous. The eppendorf was centrifuged (6 minutes at 14,000 rpm) and the supernatant was poured

off again into the 15 mL test tube. The 15 mL test tube was filled with deionised water up to its graduation mark. The test tube was inverted 20-30 times to ensure homogeneity. A 2 mL aliquot of this 15 mL solution was then filtered through 0.2 µm regenerated cellulose filters into a push-capped micro-centrifuge tube.

2.3.1.7 Method validation protocol

Assay precision relates to the closeness in agreement between measurements made on different replicates of the same sample. Accuracy is the application of an analytical procedure to an analyte of known purity (e.g. reference material). However, it may be inferred once precision, linearity and specificity have been established [54]. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability in this study was determined by a minimum of 6 determinations at 100 % of the test concentration, 9 determinations were analysed in this study [54]. The limit of detection (LOD) was established based on the standard deviation of the blank response and the slope of the calibration curve.

This was expressed as follows:

$$LOD = \frac{3.3\delta}{S}$$

δ = standard deviation of the blank response

S = slope of the calibration curve

The limit of quantitation (LOQ) was expressed as:

$$LOQ = \frac{10\delta}{S}$$

δ = standard deviation of the blank response

S = slope of the calibration curve

Instrumental LOD and LOQ was carried out for ten replicate injections of the blank samples, followed by standard solutions of increasing SeMet concentration until the analyte could be accurately and precisely detected. According to the International Conference on Harmonisation (ICH) guidelines for validation of analytical procedures, 'specificity is the ability to assess the analyte unequivocally in the presence of components which may be expected to be present' [54].

The LOD is defined by IUPAC as the lowest concentration level that can be determined to be statistically different from an analytical blank and LOQ as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy [69]. Linearity was evaluated by linear analysis on the linearity and range data and also by visual inspection of a plot of signals as a function of analyte concentration or content which should contain a minimum of 5 data points. A linear relationship was evaluated across the range of the analytical procedure [54]. Method linearity is a measure of a method's ability (within a given range) to produce test results that are directly proportional to the amount of analyte in the sample. Linearity and range were assessed by analysing replicate samples of standard solutions under normal assay conditions. Solutions containing up to 100 times, the LOQ for SeMet were used [54]. A range of 20.15 to 604.5 µg/kg selenium as SeMet was investigated and the results are shown in Figure 2.9 and Table 2.9.

2.3.1.7.1 Precision and repeatability

Assay precision relates to the closeness in agreement between measurements made on different replicates of the same sample. Results from the analysis of 9 separate extracts of SELM-1 are presented in Table 2.6. The 3.6 % R.S.D was also below that of the expected Horwitz function R.S.D of 4.7 %.

Table 2.6: Method precision data.

Replicate Number	SeMet (mg/kg)
1	3280
2	3439
3	3275
4	3293
5	3273
6	3258
7	3194
8	3115
9	3030
Mean ± S.D. (% R.S.D) = 3240 ± 117 mg/kg (3.6 %)	

Repeatability was assessed by making 9 measurements of the SeMet content of the same test material (SELM-1) within a short time frame (2 days) in the same

laboratory by the same operator using the same equipment. Results shown in Tables 2.7 were almost identical with less than 11 mg/kg SeMet in the difference between the averages over two separate days of analysis. Furthermore, the experimental R.S.D's on both days were lower than the expected Horwitz function R.S.D's of 4.7 %.

Table 2.7: Method repeatability for day 1 and day 2.

Replicate Number	Day 1 SeMet (mg/kg)	Day 2 SeMet (mg/kg)
1	3244	3212
2	3274	3145
3	3223	3232
4	3327	3280
5	3330	3255
6	3270	3293
7	3269	3326
8	3288	3330
9	3223	3280
Mean ± S.D. (% R.S.D)	3272 ± 39 (1.2 %)	3262 ± 59 (1.8 %)

2.3.1.7.2 Limits of detection and quantitation

Table 2.8: LOD/LOQ results (blank readings).

Replicate Number	SeMet (µg/kg)
1	5.7
2	8.7
3	7.7
4	4.7
5	7.2
6	7.2
7	7.2
8	8.7
9	7.7
10	8.2
Mean ± S.D. = 7.3 ± 1.3 µg/kg	

Ten consecutive blank samples were analysed, followed by standard solutions of decreasing SeMet concentration until the analyte could not be accurately and precisely detected. These results were used to verify the LOD and LOQ calculations. The measured LOD was 5 µg/kg SeMet (2.015 µg/kg Se as SeMet), see Table 2.9.

Table 2.9: LOD/LOQ results (standard readings).

Actual SeMet (µg/kg)	Measured SeMet (µg/kg)	Recovery (%)
5	6.00 ± 0.84	120.1
10	10.60 ± 0.25	105.9
15	15.53 ± 0.17	103.6
20	20.35 ± 0.25	101.7
25	24.99 ± 0.79	100.0

Results are based on triplicate readings (n=3).

The measured LOQ was 15 µg/kg SeMet (6.045 µg/kg Se as SeMet). The 15 µg/kg SeMet standard was selected as the LOQ since it was the lowest standard quantified within ±5 % of the actual value.

Calculated LOD = 4.3 µg/kg SeMet (1.7 µg/kg Se as SeMet)

Calculated LOQ = 12.9 µg/kg SeMet (5.2 µg/kg Se as SeMet)

When S = 1.0078 (from calibration curve)

2.3.1.7.3 Linearity and range

Method linearity is a measure of a method's ability (within a given range) to produce test results that are directly proportional to the amount of analyte in the sample [54]. Linearity and Range were assessed by analysing replicate samples of standard solutions under normal assay conditions. Solutions containing up to 100 times the LOQ for Se as SeMet were used and the results are shown in Figure 2.9. Linear regression analysis was also carried out on the linearity and range data. When analysed by the fitted line plot for linear regression through Minitab, the standard error of the regression (S) was very low (S=3.52). Since S represents the average distance the observed value falls from the regression line, the precision of the data was acceptable.

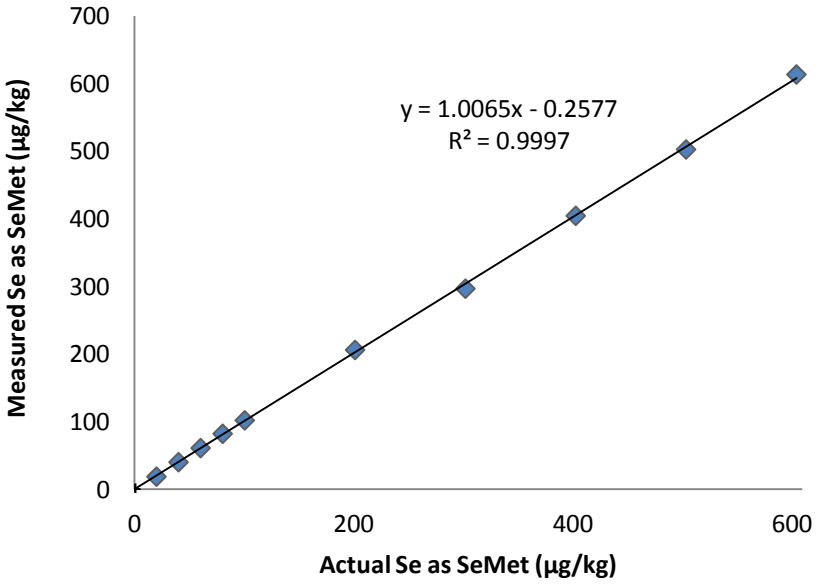

Figure 2.9: Linearity and range data plot of 5 times the calibration curve used for selenomethionine determination.
Results are based on triplicate readings (n=3).

2.3.1.7.4 Specificity and selectivity

Specificity and selectivity relate to the ability of a method to differentiate the analyte from other components in the sample matrix [54]. This discriminating power may be determined by spiking test samples with known amounts of the analyte and measuring its recovery in the presence of the sample matrix. Standard SeMet solutions were used to spike a sample at levels of 50, 100 and 150 % of the analyte concentration in the sample. The results shown in Table 2.10 highlight that spiked amounts of SeMet were successfully recovered from the selenised yeast sample SELM-1. All recoveries of spiked SeMet were ≥95 %.

Table 2.10: Specificity and selectivity data.

SeMet spike (µg)	SeMet Spike recovery (% Mean ± S.D.)
75.5	96.7 ± 1.4
151.0	100.0 ± 2.3
226.5	96.4 ± 1.1

Results are based on triplicate readings (n=3).

2.3.1.7.5 Accuracy

Accuracy defines how close a result is to the true value and may be inferred once precision, linearity and specificity have been established [70]. However, it is considered

good practice to check method accuracy with appropriate certified reference materials when available. In this case, the certified reference material was the test material also.

Three replicates of two different sample bottles of SELM-1 were extracted and analysed, the results are presented in Table 2.11. Reliability in the results from this extraction and analysis is strengthened due to a successful validation procedure. The SeMet recoveries from the newly validated extraction method agreed well with the previously certified reference values for SeMet in SELM-1. The relative standard deviation was also acceptable for SELM-1 (a) and (b) when compared against the Horwitz function R.S.D of 4.7 %.

Table 2.11: Method accuracy data.

Sample	SeMet (mg/kg)	Certified SeMet (mg/kg)	Recovery (%)
SELM-1 (a)	3461 ± 125	3448 ± 146	100.4
SELM-1 (b)	3496 ± 43		101.4

Results are based on triplicate readings (n=3).

2.3.2 Accelerated chemical method

Although carried out around the same time, the present study differed to the accelerated MACE method developed by Yang *et al*. [42] by producing sample extracts that were suitable for HPLC-ICP-MS analysis with minimal workup. This study also investigated a range of MSFA concentrations and was applied to a variety of commercially-available selenium-enriched yeast products. In comparison to reflux extractions, this investigation resulted in shorter extraction times without affecting SeMet recoveries. SELM-1 was chosen as a reference material as its SeMet content has been previously certified [9] and the HPLC conditions were the same as before (98:2, H_2O: MeOH in 0.1 % TFA) [71]. The optimised method was validated in accordance with specific guidelines [54].

Variability in enzyme activity between batches was identified as a potential concern so the certified reference material (SELM-1) was extracted with two different batches of Protease XIV. In one case, the recovery of SeMet was 100.5 % while for the other it was just 90.6 % under the exact same analytical conditions (Table 2.12).

Table 2.12: Selenomethionine recovery from SELM-1 by microwave assisted enzymatic extraction.

Sample	Se as SeMet (mg/kg)	S.D	R.S.D	Recovery of CRM SELM-1 (%)
SELM-1 (Protease XIV Batch A)	1397	35	2.5	100.5^{a}
SELM-1 (Protease XIV Batch B)	1259	13	1.0	90.6^{b}

Different batches of protease XIV were utilised on the same day and under the same analytical conditions. Values that lack a common superscript letter differ in significance ($p \leq 0.05$) and are denoted by ^{a,b}. Results are based on triplicate readings (n=3).

These significantly different results ($p \leq 0.05$) indicated that an alternative approach to enzymatic sample extraction, and its associated variability, would be beneficial. It was decided to investigate the extraction of SeMet from selenised yeast by chemical means. Chemical reagents would have better consistency and less variation than proteolytic enzymes from batch-to-batch.

2.3.2.1 Chemical extraction

2.3.2.1.1 Reflux chemical extraction

One disadvantage of refluxing selenised yeast in acid, especially when employed for SeMet determination, was the need to include antioxidants such as β-mercaptoethanol [9]. The duration of the reflux (8-16 hours), increased the risk of SeMet oxidation [44].

The 16 hour reflux extraction method, Section 2.2.7.4, was applied to the certified reference material SELM-1 and also to selenised YPA. Analysis by HPLC-ICP-MS gave recoveries of 1201 ± 62 and 1206 ± 92 mg/kg Se as SeMet (n=6), respectively. This represented a SeMet recovery of 86.4 % of the certified value for SELM-1 which was below the acceptable limit of 95 %. Furthermore, the goal of this study was to develop an accelerated chemical extraction (<1 hour long), thus a 16 hour reflux for the digestion of selenised yeast samples was too long. While the extraction time constraints were the primary concern of this study, reflux chemical extractions were not convenient for instrumentation footprint either. Each replicate for each sample needed a mantle position, a round bottom flask and a condenser, this equated to a throughput of 2 samples per day for four days (with analysis carried out the following day) by the Electrothermal EM6 mantle (Figure 2.1). Modern microwave digesters, like the CEM discover, are automated with autosamplers and do not need individual mantle

positions like chemical reflux extraction. This makes microwave extractions beneficial for sample throughput while also reducing the instrument footprint.

Increasing the reflux capabilities by purchasing two or three more EM6 hotplates, while possible, would not be economical or efficient regarding space, water, reagents (~10 mL MSFA/ sample), apparatus and above all, time as previously discussed. These examples highlight why an automated and accelerated chemical extraction (<1 hour) would be more efficient than chemical reflux extractions.

2.3.2.1.2 Microwave assisted chemical extraction

The aim of this study was to investigate different acids, concentrations, temperatures and extraction times to develop an accelerated method for the extraction and determination of SeMet in selenised yeast. The proposed method was subjected to a validation procedure [72, 73] based on predetermined guidelines [74] to confirm its application for SeMet analysis of SELM-1. The accelerated enzymatic extraction reduced the extraction time from 3 days to ~40 minutes using microwave technology. Microwave-assisted chemical extractions were also employed for selenium speciation analysis of yeast-based selenium food supplements in 2000 by B'Hymer *et al*. [55]. Using this extraction technique, the extraction time was 2 hours. Therefore, the microwave technique was investigated as a faster alternative to reflux extractions. On this occasion, to ensure the extraction method was applicable to any yeast strain, some screening and optimisation work was first carried out with YPA. As previously mentioned, this selenised yeast, while a different strain, has similar total selenium and selenomethionine quantities. Optimised conditions were then applied to SELM-1. Previous studies in the literature successfully utilised 4 M MSFA, under reflux conditions. Therefore, 4 M MSFA was used together with microwave energy to extract SeMet from YPA (Table 2.13).

Table 2.13: The effect of temperature on selenomethionine recovery.

Temperature (°C)	Mean Se as SeMet conc. ± S.D. (mg/kg)	Recovery ± S.D. (%)
130	641 ± 56	44.7 ± 3.9
140	999 ± 17	69.7 ± 1.2
150	1163 ± 22	81.1 ± 1.5
160	1212 ± 19	84.5 ± 1.3
170	1183 ± 10	82.5 ± 0.7
180	1145 ± 24	79.8 ± 1.7

Yeast product A (YPA) was extracted under microwave-assisted chemical extraction conditions of 4M MSFA for 20 minutes. Results are based on triplicate readings (n=3).

The data in Table 2.13 show SeMet recoveries ranging from ~45-85 % of YPA (YPA total Se as SeMet = 1434 ± 54 mg/kg Se as SeMet; personal communication from Dr. Cathal Connolly). It was noted that ≥80 % recovery of SeMet was achieved from extraction temperatures of 150 °C and above. Therefore, a higher temperature range study began at 150 °C increasing to 225 °C. Time was also explored as a parameter with microwave extractions set between 0 and 20 minutes (ramping to the set temperature took 2 minutes) (Figure 2.10).

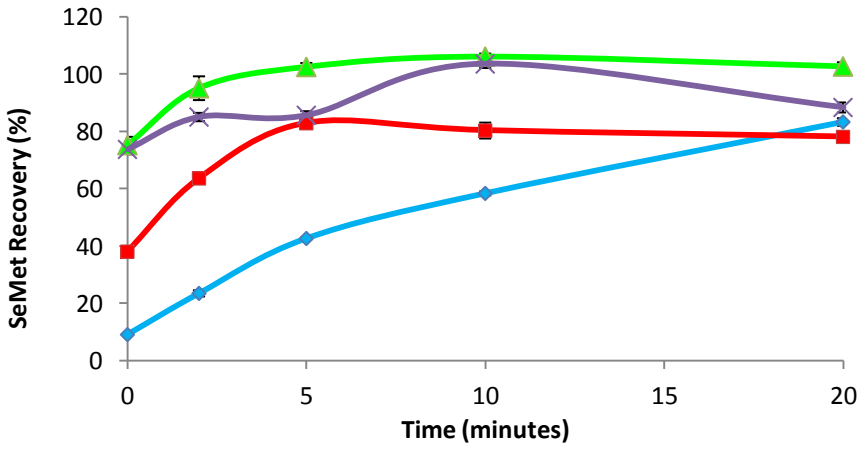

Figure 2.10: Effect of temperature and time on selenomethionine extraction efficiency.
Extraction carried out on yeast product A with 4 mL of 4 M MSFA for 0, 2, 5, 10 and 20 minutes at 150 (♦), 175 (■), 200 (▲) and 225 (×) °C. Results plotted are an average of three extracts (n=3) ± standard deviation.

The use of chemical reagents has found broad application for the extraction of SeMet from selenised yeast [1, 9, 42, 75]. Figure 2.10 illustrates the conditions that gave the best recovery of SeMet from YPA. The optimum conditions were 4 M MSFA at 200 °C and an extraction time of approximately 10 minutes. The primary aim was to

develop a quick chemical extraction method that was less than 1 hour, but looking at Figure 2.10, it was evident that a microwave chemical extraction of ~5 to 20 minutes long was possible. This was in line with earlier microwave extraction studies for SeMet determination [42]. Yang *et al.* reported a 20 minute microwave acid extraction for SeMet analysis by species specific isotope dilution by GC-MS [42]. This method, unlike the HPLC-ICP-MS approach in the current study, required SeMet derivatisation prior to analysis. Furthermore, the results in Figure 2.10 showed potential for an extraction time that was half that (~10 minutes) of other microwave extraction procedures. The optimised chemical extraction conditions were then applied to SELM-1 (Section 2.2.7.5), before making any attempt at method validation.

2.3.2.2 Optimisation of HPLC conditions for SeMet separation

The chromatographic column used for earlier enzymatic research was the 250 mm RX-C8, selected based on published work [1]. This column was selected again to begin the chemical extraction analysis. Even though the poroshell column worked well for the enzymatic extraction analysis, it was not selected for this work as this was a new study under which no assumptions were made. Therefore, the previously published reference point of the RX-C8 column was chosen as the starting point once more. While analysing samples of selenised yeast by the MACE method, a shoulder peak was observed on the SeMet analyte peak (Figure 2.11). With no access at the time to LC-MS/MS instrumentation for identification purposes, this small but interfering peak was investigated further. Firstly, reagent blanks were analysed to eliminate the possibility of contamination from the column, injection port and mobile phase.

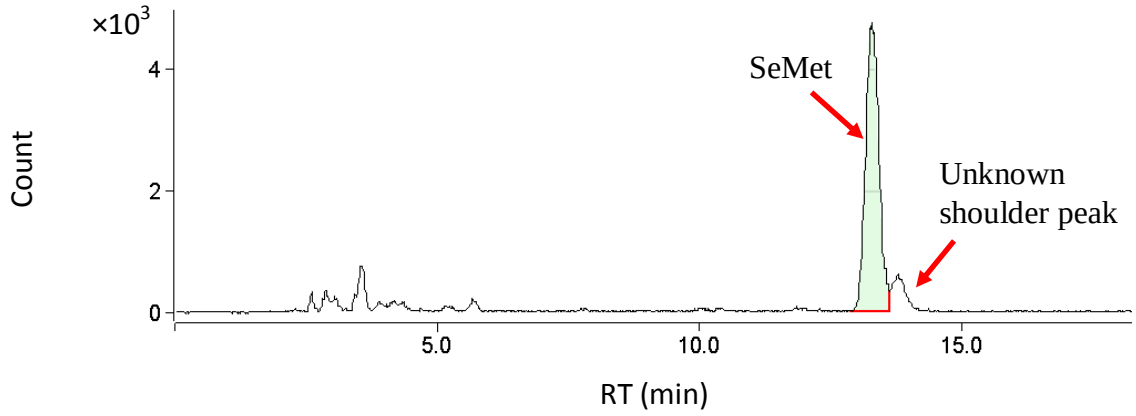

Figure 2.11: Chromatogram of selenomethionine extracted from selenised yeast product A.
YPA was digested with 4 M MSFA at 200 °C for 10 minutes and separated on Zorbax RX-C8 column.

Once the blank analysis revealed no interfering peaks, the investigation explored the possibility that the shoulder peak was a SeMet degradation product caused by the high temperature of microwave digestion combined with the acidic nature of MSFA. To determine if the shoulder peak was a SeMet degradation product, the stability of SeMet was investigated. A standard solution of L-SeMet (100 mg/kg) was made in 4 M MSFA and left for 24 hours. Upon analysis, there was minor degradation to form oxidised SeMet (Figure 2.12), suggesting that free SeMet was stable in MSFA under these conditions.

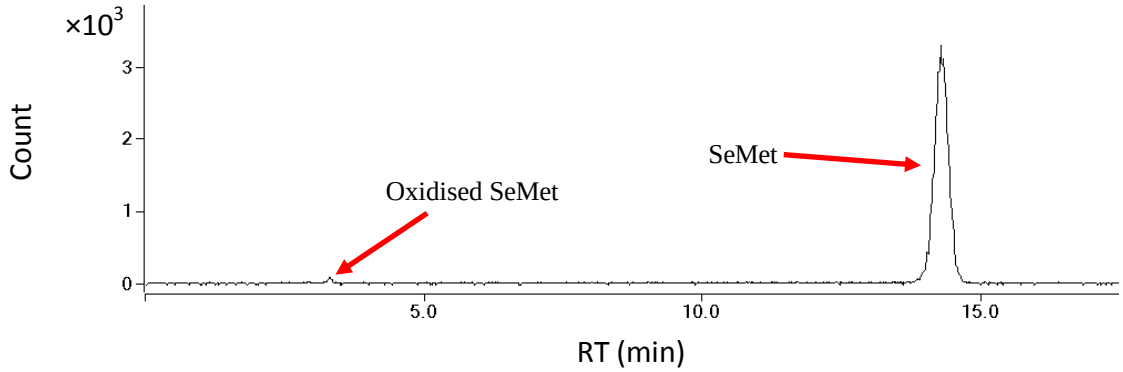

Figure 2.12: Chromatogram of 1.33 mg/kg selenomethionine standard solution in 4 M MSFA for 24 hours.

This analysis suggested that SeMet was stable in 4 M MSFA at room temperature over a period of 24 hours with recoveries of 100 ± 1.2 % from triplicate samples. However, when the same concentration of SeMet standard was digested under MACE conditions, SeMet was completely degraded, creating two separate peaks at a retention time of approximately 3 minutes (see Figure 2.13). The SeMet standard solution contained only SeMet and no other selenised reagent or product. Therefore, the two selenium-containing compounds detected at ~3 minutes were directly due to SeMet degradation with MACE and the dominant peak matched the retention time of oxidised selenomethionine. This level of degradation was not visible in Figure 2.11 during the MACE of selenised YPA. Since the only difference was the yeast matrix, this must have offered a protective effect to the liberated SeMet or the SeMet bound to peptides or proteins.

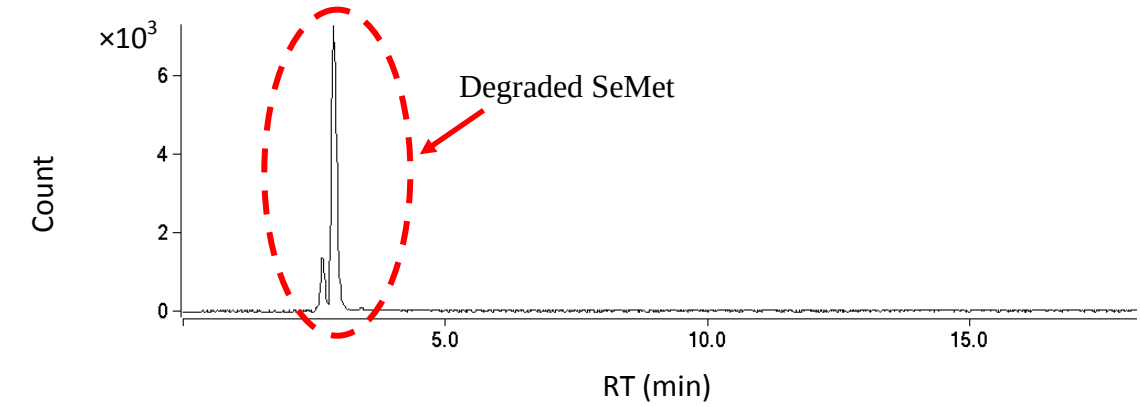

Figure 2.13: Chromatogram of 1.33 mg/kg selenomethionine digested in 4 M MSFA at 200 °C for 20 minutes.
The dominant peak was identified as oxidised SeMet due to retention time matching of SeMet that was oxidised by H_2O_2.

Fortunately, there was a placebo yeast sample of the selenised YPA. This placebo yeast went through the same fermentation process as the selenised yeast product, but in a selenium-deficient medium so that it should not contain measurable levels of selenium or selenium-containing compounds. This hypothesis was confirmed (Figure 2.14), when the placebo yeast was analysed following extraction under MACE conditions as no selenium compounds were detected. These results confirmed also that the MSFA reagent used was not the source of any interfering peaks and was void of any selenium.

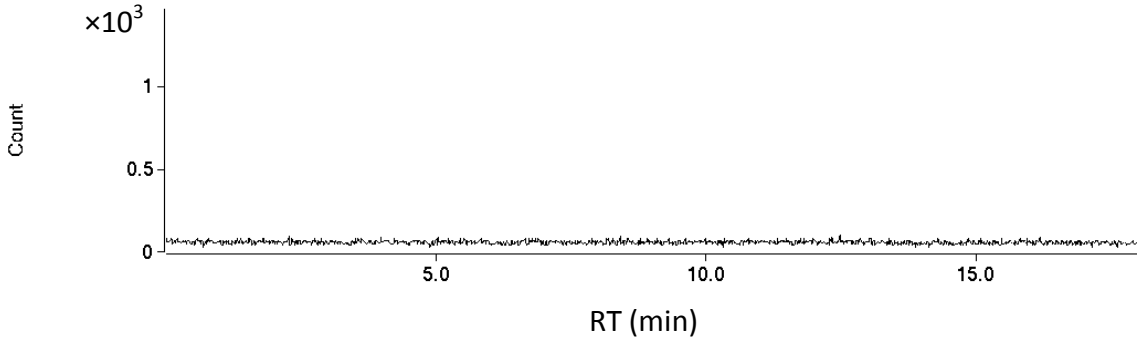

Figure 2.14: Chromatogram of placebo yeast product A extracted in 4 M MSFA at 200 °C for 20 minutes.

Free SeMet remained stable and unchanged over the course of 24 hours when it was placed in 4M MSFA at room temperature. However, it degraded completely when extracted by MACE in 4M MSFA, so similar degradation might occur when intracellular SeMet is liberated from the yeast cell during chemical extraction prior to analysis. This phenomenon was not reported in any of the earlier studies using MSFA

extraction under reflux [45, 48]. Therefore, it was proposed that the yeast sample matrix e.g. intracellular or cell wall components were having a protective effect against degradation of the liberated SeMet. To explore this hypothesis, the same concentration of free SeMet was spiked in on top of the placebo yeast product. Once the yeast and free SeMet were thoroughly mixed in MSFA, the yeast suspension was digested with the same MACE parameters. The placebo yeast product contained no selenium (Figure 2.14) which was also confirmed by total selenium analysis by ICP-MS, thus any selenium compounds detected in the extract can only be degradation or reaction products from the interaction of free SeMet and the yeast product. When analysed by HPLC-ICP-MS, the placebo yeast and free SeMet extract (Figure 2.15) had very similar chromatograms to that of a typical selenised yeast extract (Figure 2.11), with the free SeMet remaining intact and undegraded, eluting at the same retention time as before. This result suggested that the presence of the yeast sample matrix offered a protective effect to the liberated SeMet during extraction. It can also be reported that all peaks detected in Figure 2.15 were selenium compounds directly formed from the free SeMet standard, including the shoulder peak.

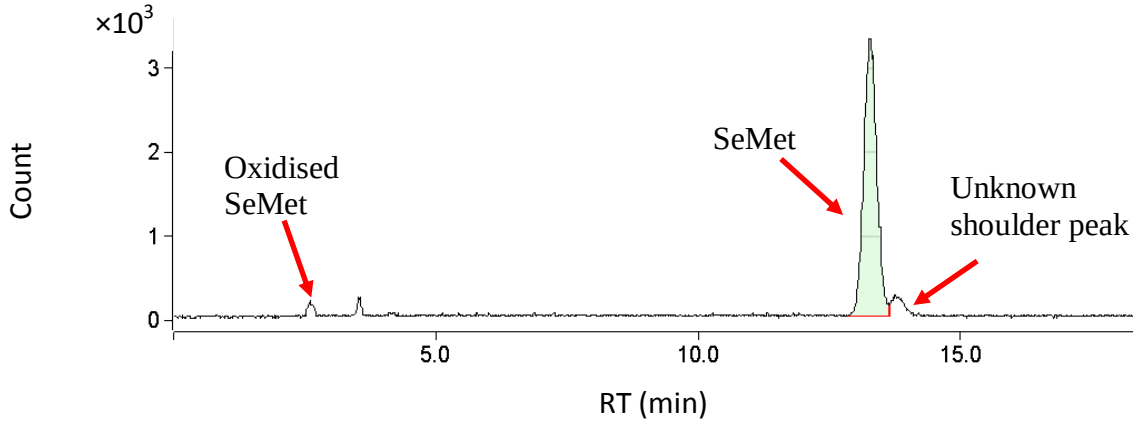

Figure 2.15: HPLC-ICP-MS chromatogram of placebo yeast product A (no selenium) spiked with selenomethionine.
SeMet (1.33 mg/kg) was spiked on top of the non-selenised yeast and digested with 4 M MSFA at 200 °C for 20 minutes.

The placebo and free SeMet chromatogram exhibited the same interfering peak that eluted just after SeMet. When the chromatogram of the placebo and spiked SeMet sample was overlaid with that of the extracted YPA (Figure 2.16), the shoulder peak profile matched in terms of retention time and peak shape.

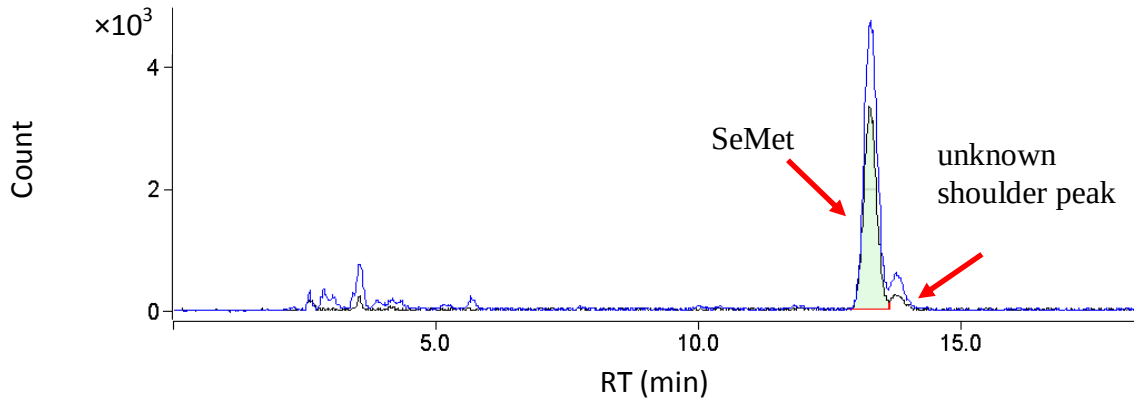

Figure 2.16: Overlaid chromatograms of selenomethionine extracted from selenised and non-selenised yeast product A.
Chromatograms of MSFA extracted selenised yeast product A and MSFA extracted free SeMet spiked on top of the placebo of yeast product A were overlapped to highlight similarities by retention time matching.

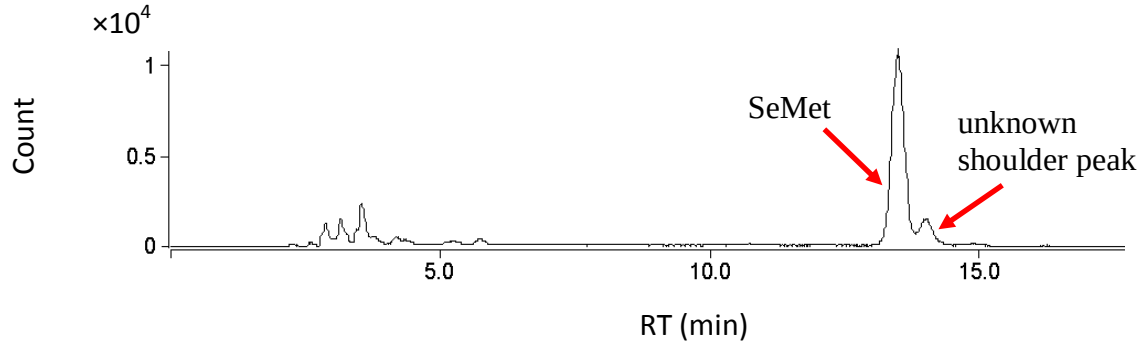

Figure 2.17: Chromatogram of selenomethionine extracted from SELM-1, also with unknown shoulder peak.

The shoulder peak highlighted in Figure 2.15-2.17, was primarily a product of SeMet or was SeMet bound to another compound. This unknown shoulder peak was observed in other selenised yeast products also. However, without sophisticated analytical techniques such as tandem mass spectrometry, the exact identity and structural composition of the compound could not be confirmed.

After ruling out all the reagents, blanks and instruments as potential sources of the interfering peak and discovering the shoulder peak was some form of SeMet product, the suitability of the column was investigated next. Analysis of peptides and amino acids like SeMet is often conducted at or below pH 2 [76]. However, HPLC generally does not use such acidic mobile phases because of the instability of silica-based stationary phases. Analysis carried out at pH <2 causes siloxane bonds to hydrolyze and allows the release of organosilanes from the surface of the stationary

phase. The deterioration of the bonded phase and creation of silanols translated directly to retention drifts and increased peak width [77]. Mobile phase conditions initially reported in 1999 by Kotrebai *et al.* [78] were utilised in many research studies since [1, 71, 79]. Therefore, method development included HPLC column selection to overcome these restrictions since samples chemically extracted with MSFA had a pH ~1.5 and the mobile phase had a pH ~1.8. Because of this the Agilent Poroshell 120 EC-C8 (pH range 2-9) was not utilised for this analysis like previous extraction work [71]. Instead, the YMC Triart C8 column was evaluated first to determine suitability. The Triart column stationary phase is made from a multilayered organic/inorganic hybrid C8-based particle. This column had an operational pH range of 1-12 which, when coupled with a temperature range up to 70 °C over a pH range of 1-7, allowed for SeMet analysis under the conditions of the chemical extraction assay (Figure 2.18 (a) and (b)). To check the suitability of the Triart column for this work, SeMet standards along with MSFA extracts of SELM-1 were analysed by HPLC-ICP-MS, leaving all other conditions unchanged. There was no shoulder on the SeMet peak as observed previously (Figure 2.15). Instead of eluting just after SeMet (~15 minutes), this small interfering peak eluted earlier, around ~12 minutes (see Figure 2.18 (a) and (b). Therefore, this column was deemed suitable for low pH separations and was selected for further optimisation and method validation.

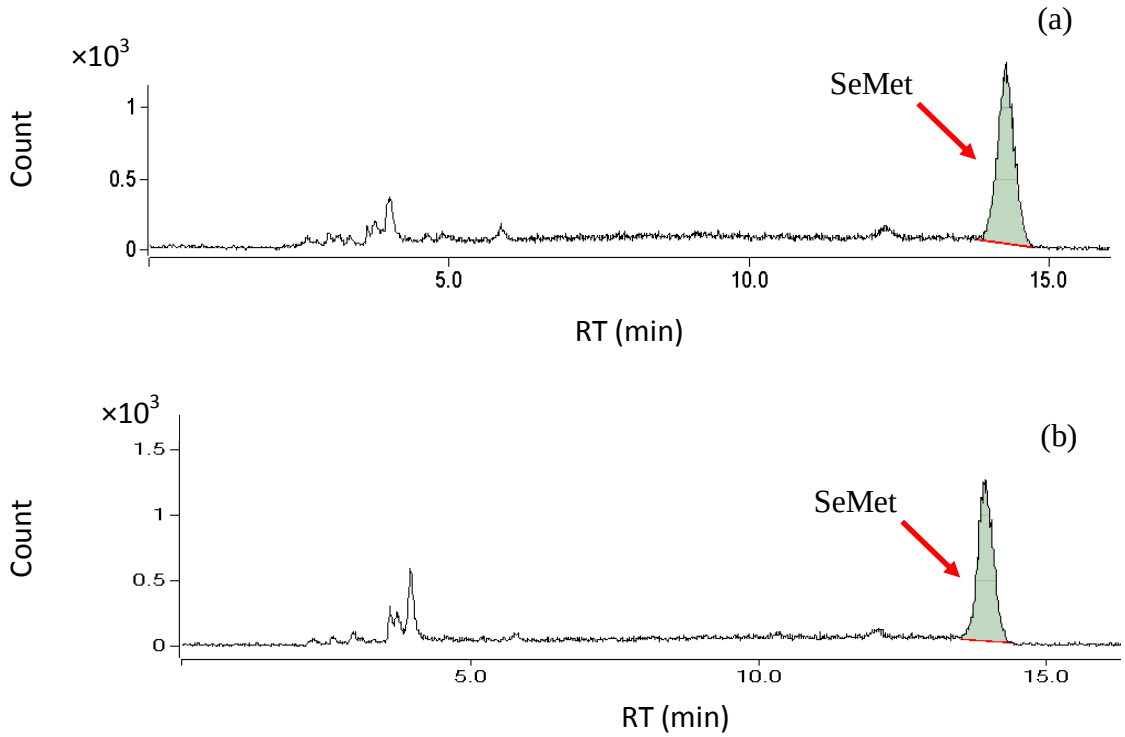

Figure 2.18 (a) and (b): Chromatogram of selenomethionine chemically extracted from YPA and SELM-1.
SeMet was extracted by MSFA under MACE conditions and separated using the YMC Triart C8 column. (a) YPA; (b) SELM-1.

2.3.2.3 Microwave chemical extraction optimisation

Screening of MACE conditions for time and temperature with 4 M MSFA (Section 2.3.2.1.2) suggested that extraction time of 5-20 minutes at 200 °C in 4M MSFA were suitable for SeMet determination in selenised yeast (Figure 2.10). High SeMet recoveries (103.8 %; 1488 ± 30 mg/kg Se as SeMet; n=9) were obtained under these conditions so it was decided to continue the method development and optimisation process with the certified reference material SELM-1. Optimisation for the extraction of SeMet from SELM-1 aimed to highlight what conditions worked best and could be examined further for validation of the extraction method. The 3 factors selected for further study were MSFA concentration, extraction temperature and extraction time.

Results from SELM-1 extractions (Figures 2.19, 2.20 and 2.21) propose that MSFA at 4 M and 6 M and at 200 °C (▲) and 225 °C (×) (Figure 2.20 and 2.21) liberated the most SeMet with 94.7-101.3 % of the certified reference value (1390 ± 54 mg/kg Se as SeMet). These figures show that both 4 M and 6 M MSFA reached close to 100 % recoveries almost instantaneously (after ramping over 2 minutes to the set

temperature) at 200 °C and 225 °C. Since maximum recovery was achieved within 10-20 minutes, it was decided not to investigate extraction times any further.

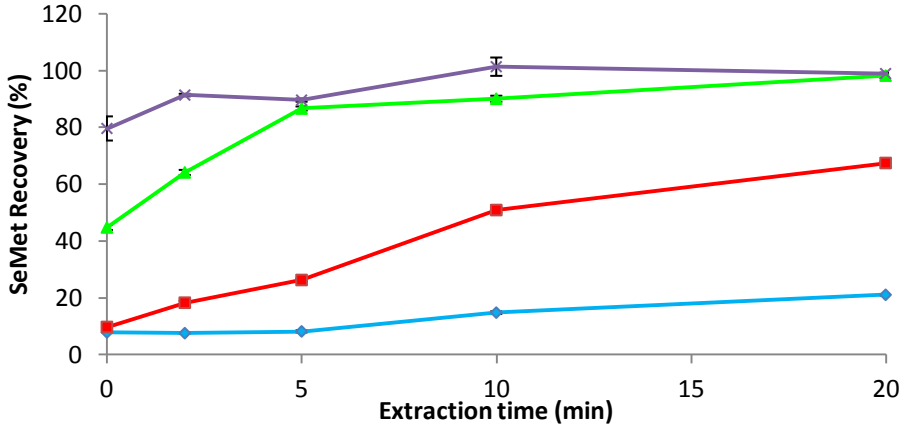

Figure 2.19: Effect of temperature and time on selenomethionine extraction efficiency.
Extraction carried out on SELM-1 with 4 mL of 2 M MSFA for 0, 2, 5, 10 and 20 minutes at 150 (♦), 175 (■), 200 (▲) and 225 (×) °C. Results plotted are an average of three extracts (n=3) ± standard deviation.

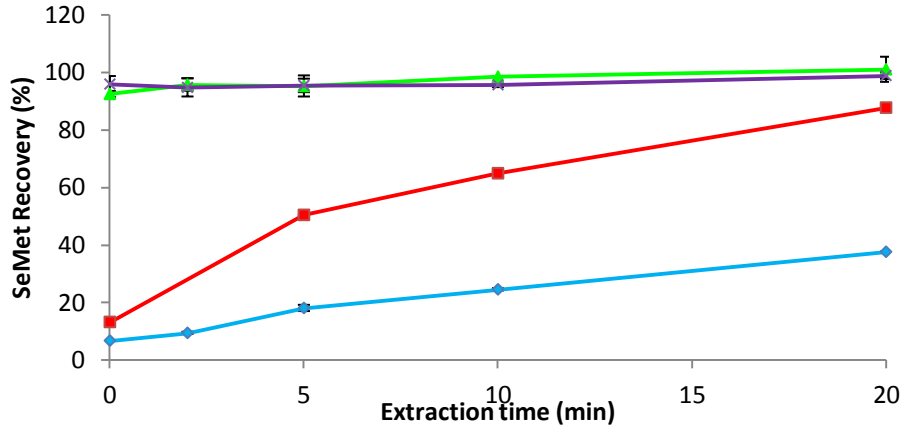

Figure 2.20: Effect of temperature and time on selenomethionine extraction efficiency.
Extraction carried out on SELM-1 with 4 mL of 4 M MSFA for 0, 2, 5, 10 and 20 minutes at 150 (♦), 175 (■), 200 (▲) and 225 (×) °C. Results plotted are an average of three extracts (n=3) ± standard deviation.

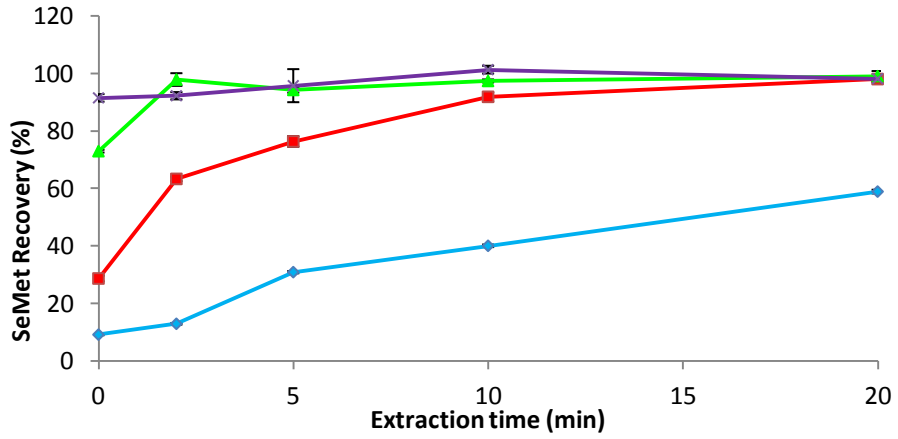

Figure 2.21: Effect of temperature and time on selenomethionine extraction efficiency.
Extraction carried out on SELM-1 with 4 mL of 6 M MSFA for 0, 2, 5, 10 and 20 minutes at 150 (♦), 175 (■), 200 (▲) and 225 (×) °C. Results plotted are an average of three extracts (n=3) ± standard deviation.

These extractions achieved 100 % recovery of SeMet from SELM-1. The results also complement previous research extractions with MSFA under reflux [75] while reducing extraction times by using microwave energy to accelerate the process similar to Yang *et al.*[42]. The 4 M MSFA at 200 °C for 10 minutes was chosen for the validation procedure (Tables 2.14-2.20), as it gave the best SeMet recoveries for SELM-1. Another benefit of choosing 4 M MSFA solution over 6 M MSFA was safety and also to reduce the acid concentration of the sample matrix on the column stationary phase before detection by ICP-MS. The lower temperature of 200 °C was selected over 225 °C as it was quicker to cool after microwave digestion and was safer when extracting in a pressurised microwave vessel, 200 °C resulted in a maximum pressure of 145 psi compared to 300 psi for 225 °C (Figure 2.22).

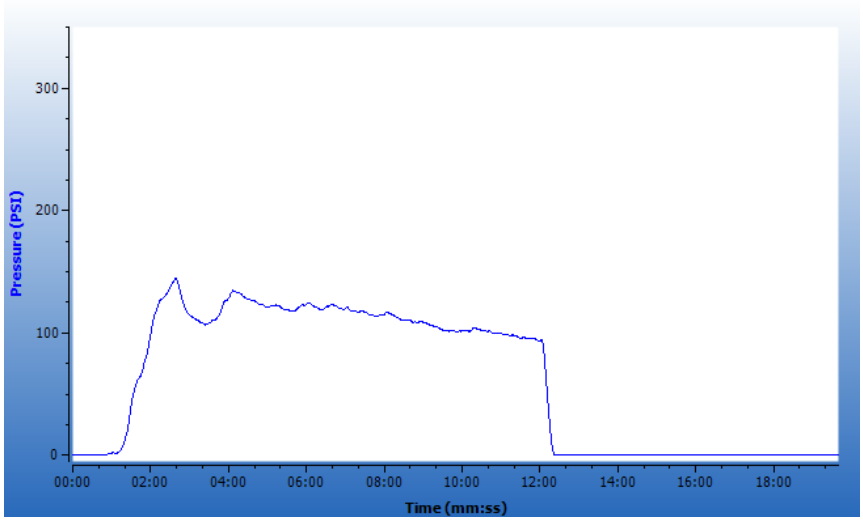

Figure 2.22: Pressure profile of sealed microwave vessel with no venting.
Pressure limit of microwave extraction program was 400 psi, since this maximum limit was not reached, no venting occurred during the extraction.

Therefore, the microwave extraction parameters of 4 mL of 4 M MSFA for 10 minutes at 200 °C were selected for method validation since it gave a recovery of 98.5 ± 0.2 % SeMet from SELM-1 (1369.2 ± 2.8 mg/kg Se as SeMet).

2.3.2.4 Optimised microwave assisted chemical extraction method

Samples of the CRM SELM-1 (0.04 g) were weighed into 10 mL microwave extraction vessels. Methanesulfonic acid (4 mL of 4 M MSFA) was added to each sample and vortexed (2 minutes). A microwave-safe stir bar (5 mm) was added to mix the

suspension during the microwave extraction. Once capped the 10 mL extraction vessels were placed into the CEM microwave autosampler. The microwave programme was set up to run for 10 minutes at 200 °C (ramping to the set temperature took 2 minutes). After the extraction and sufficient cooling (<40 °C), the extracted sample was diluted up to 50 mL with deionised water. The extract was filtered using 0.2 µm regenerated cellulose filters and further diluted (1 in 25: 40 µL in 960 µL deionised water) for analysis by HPLC-ICP-MS. Separation was carried out using the YMC Triart C8 column (4.6 × 250 mm, 5µm). Final HPLC and ICP-MS parameters were summarised in Table 2.1.

2.3.2.5 Method validation protocol

As per Section 2.3.1.7.

2.3.2.5.1 Precision and repeatability

As described in Section 2.3.1.7.1.

2.3.2.5.2 Limits of detection and quantitation

As before, these results were used to verify the LOD and LOQ calculations (Tables 2.14 and 2.15).

Table 2.14: LOD/LOQ results for 10 replicates of L-selenomethionine determination from deionised water samples (blank readings).

Replicate Number	SeMet (µg/kg)
1	0.71
2	0.77
3	0.56
4	0.91
5	0.73
6	0.65
7	0.71
8	0.72
9	1.07
10	0.98
Mean ± S.D.	0.78 ± 0.16

Table 2.15: LOD/LOQ results of L-selenomethionine.

Actual SeMet (µg/kg)	Measured SeMet (µg/kg)	Recovery (%)	R.S.D
1	1.42 ± 0.05	142.0	3.5
5	5.11 ± 0.13	102.1	2.6
10	10.00 ± 0.22	100.0	2.2
15	14.95 ± 0.16	99.6	1.1
20	20.05 ± 0.79	100.2	3.9
25	25.07 ± 0.59	100.3	2.4

Results are based on triplicate readings (n=3).

Using the equations in Section 2.3.1.7, the calculated LOD = 1.06 ng/kg SeMet (0.43 ng/kg Se as SeMet). Furthermore, the calculated LOQ = 3.21 ng/kg SeMet (1.29 ng/kg Se as SeMet), when S = 487.71 (from calibration curve).

The LOD and LOQ of SeMet were measured experimentally with analysis of the calibration standards decreasing from 25 µg/kg SeMet. The lowest measured SeMet standard was 1 µg/kg SeMet. The recovery of this 1 µg/kg SeMet (0.403 µg/kg Se as SeMet) standard was >140 % (1.42 ± 0.05 µg/kg). Therefore, this was deemed the experimental LOD with 5 µg/kg (2.015 µg/kg Se as SeMet) SeMet as the LOQ. This LOQ was selected since it was the lowest standard recovered (102.1 % recovery) with a relative standard deviation 2.6 % which was lower than the R.S.D determined by the Horwitz function of 35.5 %. Therefore, both precision and accuracy were acceptable.

2.3.2.5.3 Linearity and range

Solutions containing up to 150 times the LOQ for SeMet were used and the results are shown in Figure 2.23 and Table 2.16. The linearity and range data (R^2=0.9997) demonstrated that reliable results were achieved up to 750 µg/kg SeMet. When analysed by the fitted line plot for linear regression through Minitab, the standard error of the regression (S) was very low (S=4.91), indicating the precision of the data was acceptable.

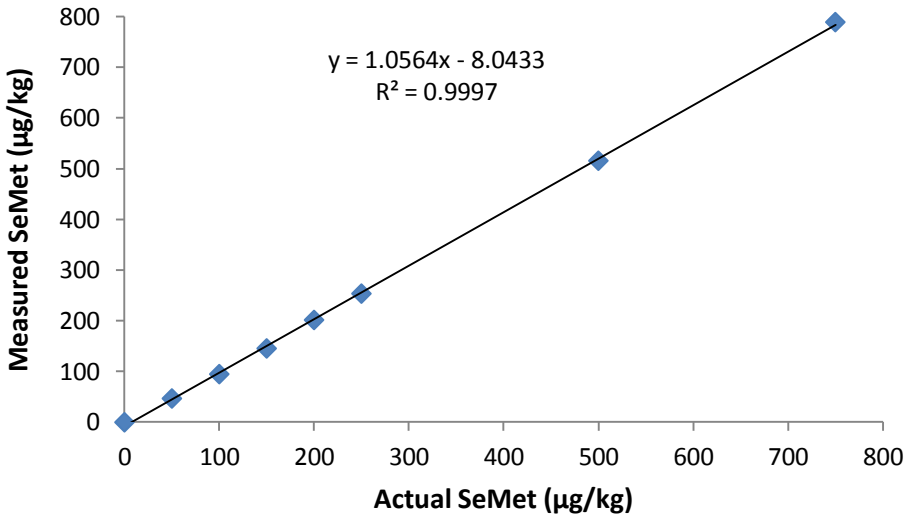

Figure 2.23: Linearity and range data plot.

Table 2.16: Linearity and range data for selenomethionine standard calibration curve.

SeMet (µg/kg)	Measured SeMet ± S.D (µg/kg)
50	47.94 ± 2.38
100	94.99 ± 2.52
150	145.63 ± 2.56
200	201.80 ± 0.93
250	253.79 ± 3.61
500	515.96 ± 20.79
750	789.38 ± 60.46

Results are based on triplicate readings (n=3).

The linearity and range were analysed as high as 750 µg/kg SeMet and found to be valid over this range.

Table 2.17: Method precision data for 9 replicates of selenomethionine extracted from SELM-1 in 4 M MSFA for 10 mins at 200 °C.

Replicate Number	SeMet (mg/kg)
1	3519
2	3388
3	3645
4	3455
5	3375
6	3359
7	3338
8	3250
9	3457
Mean ± S.D.	3421 ± 115

The low standard deviation between replicates and a relative standard deviation of 3.4 % provides evidence for the precision of the method. This was confirmed using the Horwitz function which calculated the acceptable R.S.D as 4.7 %. Repeatability was assessed by making 9 measurements of the SeMet content of the same test material (SELM-1) within a short time frame (2 days) in the same laboratory by the same operator using the same equipment. Results are shown in Table 2.18.

Table 2.18: Method repeatability for 9 replicates of selenomethionine extracted from SELM-1 in 4 M MSFA for 10 mins at 200 °C (days 1 and 2).

Replicate Number	Day 1	Day 2
	SeMet (mg/kg)	SeMet (mg/kg)
1	3585	3508
2	3697	3526
3	3623	3384
4	3598	3428
5	3535	3510
6	3606	3314
7	3557	3473
8	3404	3351
9	3413	3331
Mean ± S.D.	3557 ± 96	3425 ± 83

The results from Table 2.18 revealed that the extraction method was repeatable over separate days with an average recovery of 103.1 and 99.3 %, respectively, of the certified reference value of SELM-1. The relative standard deviation of the nine

replicates on day 1 and 2 were 2.7 and 2.4 %, respectively. These R.S.D's were both below the expected Horwitz function R.S.D's of 4.7 %, respectively.

2.3.2.5.4 Specificity and selectivity

As described in Section 2.3.1.7.4, standard SeMet solutions were used to spike samples at levels of 50, 100 and 150 % of the analyte concentration in the sample. The results are shown in Table 2.19.

Table 2.19: Specificity and selectivity data for selenomethionine samples spiked with 50, 100 and 150 % of the analyte concentration found in the sample.

Sample SeMet Value (µg)	SeMet Spike (µg)	Sample + Spike SeMet (µg)	SeMet Spike Recovery (%)
150.2	72.5	217.2	97.6 ± 2.7
148.6	139.9	279.8	97.0 ± 1.2
152.8	203.6	340.6	95.6 ± 2.3

Results are based on triplicate readings (n=3).

Results in Table 2.19 showed good recovery of the 50, 100 and 150 % spiked SeMet, with all mean recoveries >95 %. Therefore, the analyte of interest could be reliably determined in the presence of the yeast matrix after extraction.

2.3.2.5.5 Accuracy

Although there is only one batch of SELM-1 commercially-available, three replicates each of two different samples of SELM-1 were extracted and analysed and the results are presented in Table 2.20. These clearly illustrate method accuracy, with recoveries of SeMet averaging at 100.2 % of the certified reference value.

Table 2.20: Method accuracy data for selenomethionine extracted from SELM-1 in 4 M MSFA for 10 mins at 200 °C.

Sample	SeMet (mg/kg)	Certified SeMet (mg/kg)	Recovery of CRM (%)
SELM-1 (a)	3450 ± 72.6	3448 ± 146	100.1
SELM-1 (b)	3458 ± 85.7		100.3

Results are presented as SeMet. Selenium as SeMet accounts for 40.3% of the selenoamino acid. Results are based on triplicate readings (n=3).

2.3.2.5.6 Application of microwave assisted chemical extraction

The MACE conditions were applied to other yeasts (Table 2.21), to investigate if these optimum microwave extraction conditions worked. The validated chemical extraction

method was applied to commercially-available selenised yeast samples to determine assay suitability. Two non-consecutive batches of 3 different yeast products (YPB, YPC and YPD), were analysed for SeMet content using an enzymatic extraction method [71] and the chemical extraction method developed during the present study. The results showed good agreement (Table 2.21), thus further confirming the reliability of the accelerated MACE for the determination of SeMet in selenised yeast samples. There was no significant difference between the results from both methods ($p \geq 0.05$).

Table 2.21: Method application with non-consecutive batches of three commercially-available yeast products.

Samples	Extraction Method	Mean SeMet (mg/kg)	R.S.D	Recovery of Enzymatic Extraction (%)*
YPB B#1	Enzymatic	3238.5	1.0	
	Chemical	3181.6	3.5	**98.2**
YPB B#2	Enzymatic	2514.8	1.4	
	Chemical	2503.2	0.2	**99.5**
YPC B#1	Enzymatic	3049.1	0.3	
	Chemical	2992.3	4.1	**98.1**
YPC B#2	Enzymatic	3135.7	1.3	
	Chemical	3148.6	2.7	**100.4**
YPD B#1	Enzymatic	2882.8	0.7	
	Chemical	2791.1	1.7	**96.8**
YPD B#2	Enzymatic	3484.5	0.3	
	Chemical	3467.9	3.8	**99.5**

After extraction by the MACE method, 103.8 % SeMet was recovered from YPA when compared to the enzymatic extraction. * Average % recovery of enzymatic extraction = 98.8 ± 1.1 %. Results are based on triplicate readings (n=3). YP = Yeast product. B# = batch number.

The total selenium mass balances (as previously described in Section 2.3.1.5) were calculated for both extraction methods (Table 2.22). This comparison was applied to the same selenised yeast products including the CRM SELM-1. Total selenium was measured in the soluble and insoluble fractions of the different products following either enzymatic or chemical extraction. The close agreement between total selenium recovery using different extraction methods with each of the different products illustrates their suitability for sample preparation prior to SeMet analysis. It was essential that the developed extraction method was efficient for other yeast strains, i.e. universally applicable, since selenium varies in its deposition into individual protein and peptide containing fractions [79].

Table 2.22: Mass balance of non-consecutive batches of three commercially-available yeast products by an enzymatic extraction and compared to the chemical extraction.

	Enzymatic Extraction		Chemical Extraction	
Samples	**Mean % Se in Supernatant**	**Mean % Se in Pellet**	**Mean % Se in Supernatant**	**Mean % Se in Pellet**
YPB B#1	89.0	11.0	88.3	11.7
YPB B#2	83.0	17.0	84.7	15.3
YPC B#1	93.5	6.5	87.5	12.5
YPC B#2	85.0	15.0	85.9	14.1
YPD B#1	86.3	13.7	83.6	16.4
YPD B#2	82.4	17.6	81.0	19.0
SELM-1	87.5	12.5	85.6	14.4
YPA	89.0	11.0	88.1	11.9
Mean % Se*	**87.0**	**13.0**	**85.6**	**14.4**

Results are based on triplicate readings (n=3). *Numerical values are representative of average % selenium of all yeast product. YP = Yeast product. B# = Batch number.

The fractionation of the extracted selenium and the insoluble selenium remaining in the pellet (Table 2.22) was similar for both methods. This suggested that the chemical extraction liberated the same amount of selenium from selenised yeast as the enzymatic extraction. Therefore, both of these accelerated microwave extraction methods are suitable for SeMet analysis of Se-enriched yeast products.

2.4 Conclusion

The purpose of this study was to develop and validate an accelerated enzymatic and chemical method for SeMet determination in selenium-enriched yeast. The final conditions of the accelerated enzymatic extraction method were as follows;

Protease enzyme solution (500 µL) was added to 40 mg of the yeast sample. The sample suspension was ultrasonicated for 25 seconds at 80 % amplitude and the probe washed with 250 µL enzyme solution. The sample was microwave extracted for 15 min at a power output of 30 W. The extracted sample was centrifuged at 14,000 rpm for 3 min. The supernatant was filtered and diluted before analysis by HPLC-ICP-MS.

The analytical validation protocol examined specificity, linearity, range, accuracy, precision/repeatability, limits of detection (LOD) and limits of quantification (LOQ)

[54]. Extraction time of SeMet was reduced to less than an hour without compromising extraction efficiency. This was achieved by combining ultrasonication using an ultrasonic probe and enzymatic digestions with microwave extraction. The LOD and LOQ were 5 µg/kg SeMet and 15 µg/kg SeMet respectively. The method was linear up to 1,500 mg/kg SeMet. Method specificity and selectivity were satisfactory with an average recovery of spiked SeMet from the SELM-1 CRM matrix of 97.7 ± 2.0 %. The benefits of an accelerated enzymatic extraction for SeMet determination included an increase in sample throughput, improved sample analysis efficiency and the removal of antioxidants to prevent oxidation of SeMet. While all these benefits were substantial, enzymatic extractions were still a costly approach and would depend on the batch-to-batch consistency of the enzyme.

The SeMet recovery from SELM-1 via the accelerated enzymatic extraction confirmed the results of the published three day enzymatic extraction. In summary, the SeMet content of the certified reference material (SELM-1) measured after the accelerated enzymatic extraction, agreed well with the certified value (100.9 % recovery). Therefore, the methods described are suitable for accurate and precise determination of (SeMet) in Se-enriched yeast.

Primarily, an accelerated and validated enzymatic extraction was essential for SeMet analysis to rapidly improve on a time consuming 3 day extraction method, however, a second validated method was beneficial to confirm the first method. To reduce costs and improve method stability caused by enzyme batch-to-batch issues, a second extraction procedure was chemical based and void of any enzymes. Chemical solutions such as methanesulfonic acid (MSFA) were easier to store (room temperature) and did not degrade as quickly as enzymes or were subject to the use of buffers. Therefore, extraction stability should be improved for a chemical extraction method since the extraction solution can be made up in bulk and did not have any batch-to-batch issues unlike enzymes (Table 2.12). Furthermore, accelerated extractions minimised oxidation thus increasing method stability, this will be discussed in more detail in Chapter 3.

The goal of developing an accelerated chemical extraction for SeMet determinations was also achieved. Extraction time was reduced to 10 minutes without compromising extraction efficiency, thus providing an alternative method to the accelerated enzymatic method (~40 minutes), chemical reflux methods (8-16 hours) and

other MACE methods (20 minutes). An acid-tolerant YMC-Triart C8 column was utilised for the study and subsequent validation protocol. Validation of the method found that the extraction protocols were robust and fit for purpose. The experimentally-derived LOD and LOQ were 1 and 5 µg/kg SeMet, respectively. The method was linear from 0-750 µg/kg SeMet. Additionally, the first calibration point was 10 times higher than the experimental LOQ (5 µg/kg), starting at 50 µg/kg, increasing to the maximum standard calibration point of 250 µg/kg SeMet. Method specificity and selectivity were acceptable (≥95 % recovery) with an average recovery of spiked SeMet from the selenised yeast matrix of 96.7 %. The measured SeMet content of the certified reference material (SELM-1) agreed well with the certified value (100.2 ± 0.1 % recovery). The MACE method showed good agreement compared to the validated enzymatic extraction method with an average SeMet recovery of 98.8 ± 1.3 % across all the investigated yeast strain samples. Consequently, the MACE method confirmed the previously validated enzymatic extraction methods and was applicable to multiple yeast strains. Commercial selenised yeast samples have high concentrations of SeMet (SELM-1 = 3448 mg/kg SeMet). Therefore, with calculated LOQ's in the low parts per trillion range and linearity over the specified range, the method described was suitable for accurate and precise determination of SeMet in selenium-enriched yeast.

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Chapter 3: Development and optimisation of a High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry method for selenium speciation and chiral determination of water-soluble selenomethionine in selenised yeast

Abstract

Selenium-enriched yeast is traditionally characterised by its total selenium content or by the concentration of its dominant seleno-species, selenomethionine. Different yeast strains can have the same selenomethionine and total selenium contents and by traditional methods of characterisation, these yeasts may be identified as being the same. However, clear differences were seen in 15 minute water extractions of 5 selenium-enriched yeast products when analysed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). This assay determined the selenomethionine content as well as measuring the selenium content attributed to other seleno-species present in a water extract of selenised yeast. Enzymatic digestion of the aqueous extract distinguished between how much protein or peptide-bound selenomethionine was present. The water-soluble, peptide-bound selenomethionine along with unknown selenocompounds was found to vary from product to product and for various batches of the same products. A selenium mass balance calculation was carried out to account for the recovery of selenium in each fraction. Total selenium recovery ranged from ~98 % to ~102.5 %. Chiral investigations provided further information about the selenomethionine found in the selenised yeast products. A Chirobiotic T chromatography column was selected for the separation and quantification of D- and L-selenomethionine (SeMet) in water extracts of selenium-enriched yeast products. Once the analysis was optimised for the SeMet standards, it was then applied to the water-soluble fractions of selenium-enriched yeast products. This research highlights the different selenocompound that are evident after a 15 minute water extraction of these yeast products. The D,L-SeMet standard was calculated to be ~96 % of the L-SeMet standard by HPLC-ICP-MS. The total selenium content of the L-SeMet and D,L-SeMet standards was also confirmed by ICP-MS prior to enantioseparation, with recoveries of 101 and 99.7 % respectively.

3.1 Introduction

Selenium speciation analysis has developed considerably over the past several years from determining the total selenium content of the sample or supplement in question [1, 2]. The analysis of selenium species [3, 4], in dietary supplements is essential since significant differences were reported between selenium supplement products and their stated selenium contents [5, 6]. Regarding selenised yeast, speciation focused primarily on selenocompounds that were present, with the majority of attention given to the dominant organic selenocompound, selenomethionine [3, 7-11].

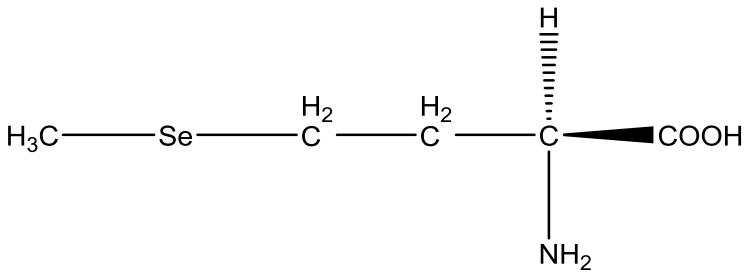
Selenium can be incorporated into proteins specifically as selenocysteine thus creating selenoproteins. However, when selenium in the form of selenomethionine is non-specifically incorporated into proteins, the resultant products are referred to as selenium-containing proteins [12, 13]. Numerous studies looked at different fractions when speciating or identifying selenocompounds in selenised yeast [14-17] with some focusing on the water-soluble selenium species [12, 17-19]. This water fractionation can contain as much as 25 % of the total selenium and because of the low costs and fast extraction, it is an important sample preparation technique used in speciation studies [15]. The water-soluble selenium fraction is comprised of multiple selenocompounds including selenomethionine [20, 21] and is from both intracellular and extracellular sources which will be discussed later in this chapter. Relevant research which examined the water-soluble, free and peptide-bound selenomethionine concentration in yeast suggested a knowledge gap addressed in this study. Further information was provided for biochemical profiling of commercially-available selenised yeast.

Selenium speciation development and improvement is always welcome, in particular the detection of additional selenocompounds, acceleration of extraction methods or simply providing more nutraceutical information about the commercial supplements available. However, very little chiral selenium speciation research has been carried out, a point also noted by other researchers [22]. In the last decade there have only been a few peer-reviewed papers investigating chiral speciation of selenised yeast [23-25].

Regarding chirality, a compound or molecule is said to be *chiral* if it is not superimposable with its mirror image (Figure 3.1). Macroscopic examples of this would be left and right-handed gloves, or simply your left and right hands. Therefore, the chirality of any object is linked to its symmetry. This configurational stereoisomerism is

titled enantiomorphism with both non-mirror image stereoisomers referred to as enantiomers. Furthermore, excluding glycine, all encoded protein amino acids have at least one chiral centre or possibly even two (e.g., threonine, isoleucine) [26].

(i)



(ii)

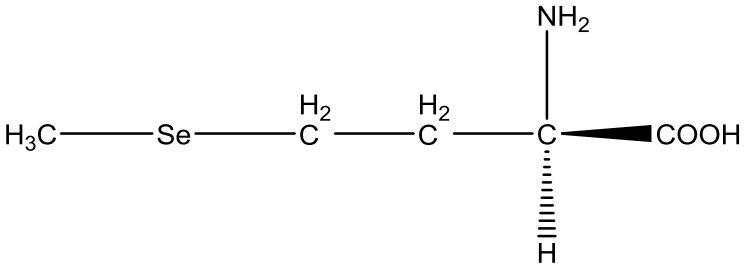


Figure 3.1 (i) & (ii): D- and L-selenomethionine, respectively.

Enantiomers are indistinguishable based on chemical and physical traits such as melting points and refractive index [22]. Originally, the only method for determination of which enantiomer was present was to examine their reaction to polarised light [22]. However, developments in analytical techniques led to more accurate determination of enantiomer presence and concentration [24]. Furthermore, when the racemic enantiomers are present in chiral environments, they can possess variations in biological activity or bioefficacy. While one enantiomer may have a desired positive effect, sometimes the corresponding enantiomer can have an entirely opposite effect or unwanted side-effects such as phocomelia experienced from racemic thalidomide in the 1960's [27]. In relation to dietary nutritional supplements and foods, the addition of D-enantiomers is forbidden and regulatory bodies prevent this inclusion [28-30]. Therefore, chiral speciation is essential for not only safety purposes but also for efficacy determination and regulatory purposes.

The dominant selenocompound in selenised yeast is selenomethionine [8, 9, 31]. Reports suggest that L-selenomethionine can be equally or less toxic than inorganic salts of selenium but the L-enantiomer is also more bioavailable than inorganic forms of selenium [32]. Studies outline that absorption of selenomethionine occurs in the small

intestine with efficiencies of 95.5 % recovery of an administered dose [33]. Selenomethionine can be sourced as a racemate containing 50 % D- and L-enantiomers or as the purified L- form. Throughout nature, the favoured amino acid enantiomer is the L- configuration, evident from mammalian proteins built exclusively from L-amino acids [34, 35]. However, conversion from the L-enantiomer to the D-enantiomer form can occur through various metabolic pathways or simply the processing conditions utilised by the food industry for example [28]. Futhermore, D-amino acids can be incorporated into peptides and can have varying activity [36-38].

Analytical methods for the determination of chiral compounds usually rely on either direct or indirect enantiomeric separation. The former is based on the separation of enantiomers by a chiral stationary phase (CSP) or via a chiral selector on an achiral stationary phase. The latter, an indirect method, is based on the reaction of the enantiomers with a chiral derivatising agent, followed by separation of these derivatives on an achiral stationary phase [39]. One of the first direct enantioselective methods was by GC in 1966 and utilised a CSP [40]. It took five more years before the first report of a direct method for resolution of chiral amino acids by HPLC [41]. Since then both GC and HPLC were popular methods for chiral separation and utilised a chiral stationary phase or employed derivatisation of the enantiomers to increase enantioselectivity [26, 34].

Commercially, there were only four chiral chromatographic columns utilised for the examination of selenoamino acids in publications up to 2017 [24, 42]. These included the Cyclobond 1 β-CD, Daicel Crownpak CR(±), Daicel Chiralpak AD-RH and the Chirobiotic T [23, 24, 42-44]. The Chirobiotic T column was selected for this present study due to its previously documented resolution of D- and L-methionine [45]. With similar chemistry to selenomethionine, the Chirobiotic T column should provide similar resolution to that of the methionine separation, see Figure 3.2. Other chiral selenoamino acids exist but were outside the time and scope allocated for this research.

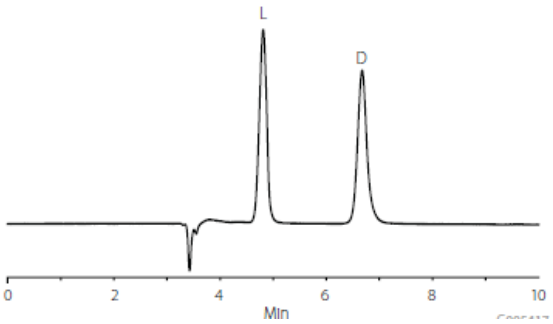


Figure 3.2: Chromatogram of D, L-methionine separation on Astec Chirobiotic T column. Taken from Claus *et al.* [45]

The bioefficacy (the fraction that has a positive effect on a functional parameter [46, 47]), of these selenium-enriched samples, is highly dependent on the chemical forms of selenium found in the samples. This research focused on the water-soluble extracts of selenised yeast to determine what species are present. The investigation was focused on what differences occurred between varying strains of commercially-available selenised yeast. Furthermore, to gain deeper insight about the dominant selenocompound - selenomethionine, its chirality was also investigated in the water-soluble extract of each selenised yeast product. Analysis of the water-soluble yeast fraction utilised HPLC-ICP-MS and compound independent calibration (CIC – discussed in detail later) to examine these selenocompounds. The main aim of this study was:

- to investigate the water-soluble selenium speciation profiles between multiple selenised yeast samples for potential differences.
- to investigate D- and L-selenomethionine differences between multiple water-soluble fractions of the selenium-enriched yeast products by direct enantioseparation.

3.2 Experimental

3.2.1 Instrumentation

Free and peptide-bound selenomethionine analysis was performed by HPLC-ICP-MS on an Agilent Zorbax RX-C8 4.6 × 250 mm (5 µm) column using an Agilent Technologies 1260 infinity series HPLC system connected to an Agilent Technologies 7700× series ICP-MS [48, 49]. The flow rate was 1 mL/minute with an injection

volume of 45 µL and the column temperature was set at 25 °C. Chiral speciation also utilised the same HPLC and ICP-MS system with an Astec Chirobiotic T (250 × 4.6 mm, 5 µm) column. A CEM Discover SP-D microwave equipped with an Explorer Plus 24/48 autosampler was used for sample extraction. Samples were filtered using Chromafil Xtra RC-20/25 0.2 µm regenerated cellulose (RC) filters. A Heinemann 130 W ultrasonic-homogenizer (HTU SONI 130, USA) equipped with a 3 mm double step titanium probe was used for sample preparation. The HPLC mobile phase was filtered with Whatman Glass Microfibre Filters. Samples were weighed out on Sartorius - MSE125P-100-DU and MSE6.6S-000-DM analytical balances.

3.2.2 Chemicals and reagents

A standard reference material, SELM-1, (2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet) was purchased from the National Research Council of Canada. Before analysis, four commercial yeast samples were randomly labelled as yeast product 1 to 4 (YP1-YP4). Total selenium was measured by ICP-MS after chemical digestion in nitric acid (Sigma, UK) and selenomethionine contents were determined by HPLC-ICP-MS following the accelerated enzymatic digestion (Chapter 2) and utilised protease XIV (Sigma, UK). Deionised water (>18 MΩ cm) was obtained from an ELGA Purelab Flex S7 system. L-SeMet and D,L-SeMet were purchased from Sigma (>98 % by TLC). These were used to prepare 100 mg/kg stock solutions in 0.1 M HCl. Aliquots (0.1 mL) of this solution were frozen and used fresh each day. The HPLC-ICP-MS analysis utilised a mobile phase of deionised water, methanol and trifluoroacetic acid (97.9: 2.0: 0.1). Both MeOH and TFA were supplied by Sigma, UK. The mobile phase for chiral separations consisted of deionised water, methanol and formic acid (50: 50: 0.02). All chemicals used in this work were of analytical-reagent grade or higher unless otherwise specified.

3.2.3 Instrumental calibration

The method was calibrated over the range of 50 to 250 µg/kg L-SeMet (20.15 to 100.75 µg/kg L-Se as SeMet). An aliquot (20 µL) of SeMet stock solution (100 mg/L) was added to 1.98 mL of deionised water to give a 1 mg/L working standard. Aliquots of 50, 100, 150, 200 and 250 µL of the working standard were made up to 1 mL with deionized water to produce the calibration standards. Each standard was analysed in

triplicate using an injection volume of 45 µL for free and bound selenomethionine analysis. The chiral analysis used D,L-SeMet and L-SeMet standards for chiral speciation with a 10 µL injection volume. Isotopes ^{76}Se, ^{77}Se and ^{78}Se were monitored. All calibration plots were linear with a correlation coefficient of ≥0.995.

3.2.4 Investigation of selenomethionine oxidation

A 100 mg/kg stock solution of L-selenomethionine was diluted to give a final concentration and volume of 200 µg/kg and 5 mL, respectively. Aliquots of this standard solution were transferred to five vials which were placed into the HPLC autosampler. This procedure was repeated to give similar concentrations of SeMet after both the enzymatic and chemical extraction methods, developed in Chapter 2. The extracts was filtered using Chromafil Xtra RC-20/25 0.2 µm regenerated cellulose (RC) filters and analysed by HPLC-ICP-MS. Separations were carried out on a Zorbax RX-C8 4.6 × 250 mm, 5 µm column. The isocratic mobile phase consisted of deionised water: MeOH: TFA (97.9: 2.0: 0.1). The mobile phase flow rate was 1 mL/min.

3.2.5 Extraction of water-soluble free selenomethionine

Approximately 0.5 g of selenised yeast sample was accurately weighed into 50 mL sterilin tubes. Deionised water (5 mL) was added. The sample was placed on an orbital shaker for 15 minutes at 300 rpm. The samples were then centrifuged at 8,500 rpm. An aliquot (2 mL) of the supernatant was filtered using Chromafil Xtra RC-20/25 0.2 µm regenerated cellulose (RC) filters and diluted in deionised water (1:25) before analysis.

3.2.6 Extraction of water-soluble total selenomethionine

A 500 µL aliquot of the water extract was added to 13.33 mg of protease XIV powder. Only protease XIV powder that previously gave ~100 % recovery of SeMet from SELM-1 by the accelerated enzymatic extraction was utilised for this extraction. The sample was ultrasonicated for 25 seconds at 80 % amplitude and the probe washed with 250 µL enzyme solution (6.667 mg protease XIV/250 µL) into the 1.5 mL tube. Some ice and water was placed into the microwave carousel and the microcentrifuge tubes were placed into the carousel holder (up to 14 tubes per run). The extraction program was run for 15 minutes at a power output of 30 watts. The extracted samples were centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a 15 mL

centrifuge tube. The volume was made up to 15 mL using water and mixed well before removing an aliquot (2 mL) for filtration (0.2 µm) and a further dilution in deionised water (1:25) was carried out before analysis.

3.2.7 Extraction of total selenomethionine from yeast sample

Approximately 0.04 g of yeast sample was accurately weighed into 1.5 mL microcentrifuge tubes. Exactly 500 µL protease enzyme solution (13.33 mg Protease XIV in 0.5 mL of Tris buffer (pH 7.5)) was added and vortex mixed for 2 minutes. The sample was ultrasonicated for 25 seconds at 80 % amplitude and the probe washed with 250 µL enzyme solution into the 1.5 mL tube. Some ice and water was placed into the microwave carousel and the microcentrifuge tubes were placed into the carousel holder (up to 14 tubes per run). The extraction program was run for 15 minutes at a power output of 30 W. The extracted samples were centrifuged at 14,000 rpm for 3 minutes. The supernatant was then transferred to a 15 mL centrifuge tube. The pellet was washed with deionised water (0.9 mL) and vortex mixed until completely suspended. The sample was centrifuged at 14,000 rpm for 6 minutes and the supernatant added to the 15 mL centrifuge tube. This step was repeated three times. The volume was made up to 15 mL using water and mixed well before removing an aliquot (2 mL) for filtration (0.2 µm) and dilution before analysis.

3.2.8 Analysis of free, bound and total water-soluble selenomethionine

The 2 mL aliquots of the sample extracts were filtered into 2 mL microcentrifuge tubes. Further dilutions were made using deionised water and transferred to 2 mL HPLC vials for analysis. For free selenomethionine analysis, the sample was diluted 1 in 50 up to a volume of 1 mL. Each sample was analysed in triplicate using an injection volume of 45 µL and monitored for isotopes ^{76}Se, ^{77}Se and ^{78}Se. Separation was carried out on Zorbax RX-C8 (4.6 × 250 mm, 5 µm) column.

3.2.9 Selenomethionine analysis of the yeast pellet after a water extraction

Samples of yeast were water extracted and centrifuged to remove the supernatant. The remaining yeast pellet was frozen and lyophilised. Once dried, samples were weighed out in triplicate for total selenium and total selenomethionine analysis as previously described.

3.2.10 Analysis of chiral selenomethionine standards and chiral water-soluble selenomethionine

The standards D,L and L-SeMet were analysed for their total selenium concentration by ICP-MS to verify their selenium concentration. The standards were weighed out on a 6 point Sartorius analytical balance. A stock solution of the standards was made-up in 0.1 M HCl and diluted to 200 mg/kg with deionised water. The water was sonicated to remove any air bubbles and to minimise dissolved oxygen. Aliquots of the stock solution (~500 µL) were transferred to 2 mL microcentrifuge tubes and frozen at -20 °C. A new standard was thawed daily for analysis.

The 2 mL aliquots of the sample extracts were filtered into 2 mL microcentrifuge tubes. Further dilutions were made using deionised water and transferred to 2 mL HPLC vials for analysis. Each sample was analysed in triplicate using an injection volume of 10 µL and monitored for isotopes ^{76}Se, ^{77}Se and ^{78}Se. HPLC-ICP-MS chiral analysis of the water-soluble extract measured both L- and D-SeMet in the samples. Analysis was carried out using the conditions outlined in Table 3.1.

Table 3.1: HPLC and ICP-MS conditions utilised for chiral analysis.

HPLC parameters prior to ICP-MS			
Liquid chromatograph	1260 Infinity series, Agilent		
Column	Astec Chirobiotic T (250 × 4.6 mm, 5 µm)		
Mobile Phase	A: H_2O: FA (99.8: 0.2)		
	B: MeOH: FA (99.8: 0.2)		
Mobile phase flow (mL/min)	0.50		
Column temperature (°C)	25		
Gradient	Time (min)	A (%)	B (%)
	0	50	50
	35	50	50
	40	30	70
	44	50	50
Injection volume (µL)	10.00		
ICP-MS plasma parameters			
RF Power (W)	1600		
RF Matching (V)	2.15		
Sample Depth (mm)	8		
Carrier gas flow (L/min)	0.28		
Option gas (%)	44		
Makeup gas (L/min)	0.20		
Nebuliser pump (rps)	0.4		
Spray chamber temperature (°C)	-5		
ICP-MS lense parameters			
Extract 1 (V)	0.0		
Extract 2 (V)	-170.0		
Omega Bias (V)	-80		
Omega Lens (V)	8.1		
Cell Entrance (V)	-40		
Cell Exit (V)	-70		
Deflect (V)	-0.6		
Plate Bias (V)	-60		
Collision cell parameters			
Gas	H_2		
H_2 flow (ml/min)	6.6		
Octopole Bias (V)	-18.0		
Octopole RF (V)	200		
Energy discrimination (V)	-3.0		

The ICP-MS torch was changed from one with an internal diameter of 2.5 mm for aqueous solutions to 1.0 mm for high organic solutions. This allowed higher concentrations of organic solvent to be introduced to the ICP-MS plasma without it being extinguished. Therefore, the torch position also needed optimisation of its x- and y-axis since the internal diameter changed.

3.3 Results and discussion

3.3.1 Selenomethionine oxidation

Before analysis of water-extractable free SeMet, peptide-bound SeMet or total SeMet determination, the stability of the SeMet analyte was investigated. Encinar *et al.* highlighted the issue of SeMet oxidation when carrying out fractionation and water extraction studies of selenised yeast [12]. To show how susceptible selenomethionine was to oxidation, standards were made and analysed over approximately 60 hours. The autosampler was cooled to 4 °C and temperature was controlled to help minimise oxidation of samples [50]. Selenomethionine standards were injected onto the HPLC system, one after another, over a period of 60 hours. This approach was repeated for selenised yeast extracted by the enzymatic and chemical methods developed in Chapter 2. The CIC technique (described in detail later, Section 3.3.6) was employed to quantify the oxidised selenomethionine and thus allowed oxidation to be monitored and measured over time (Figure 3.3).

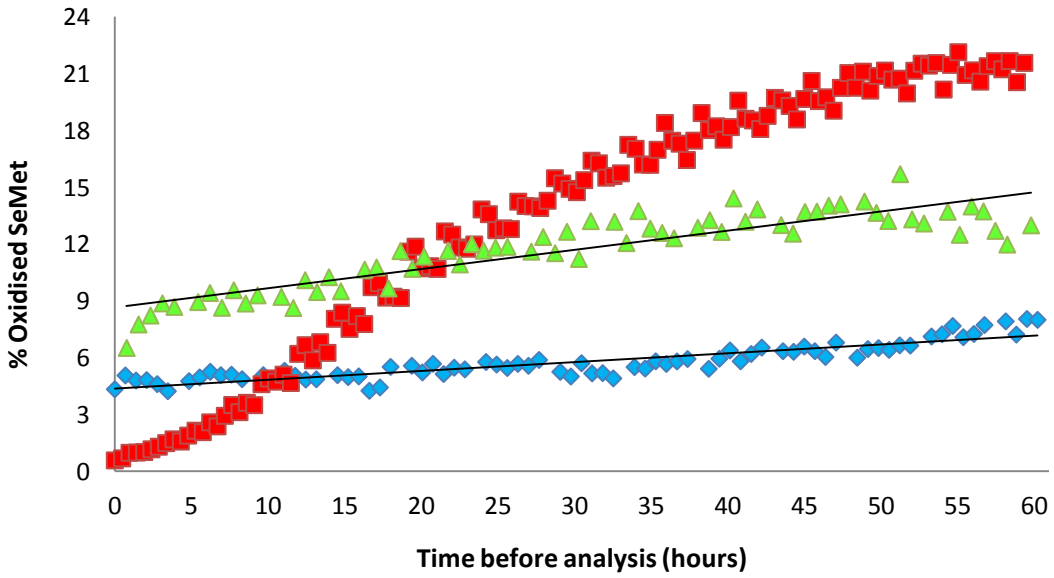


Figure 3.3: HPLC-ICP-MS analysis of oxidised selenomethionine over 60 hours.
♦ = enzymatic extraction of SeMet from SELM-1; ■ = SeMet standard; ▲ = chemical extraction of SeMet from SELM-1.

Many studies report the use of antioxidants such as β-mercaptoethanol and dithiothreitol (DTT) to minimise the oxidation of selenomethionine [50]. The reversal of oxidised SeMet to SeMet was described in Chapter 1, Section 1.3.3. The oxidised SeMet graph (Figure 3.3) showed that even after as little as ten hours, 5 % of the

selenomethionine standard (■) had oxidised. After 24 hours, there was roughly 2.5 times more oxidation. While the SeMet standards were made and injected for analysis straight away, the enzymatic (♦) and chemical extraction (▲) both had their specific extractions to go through first. On average there was less oxidation experienced by the enzymatic method compared to the chemical extraction method. This could be attributed to using a more labile extraction method which used enzymes where temperatures reached ~50 °C. This was in stark contrast to the chemical extraction which applied a 200 °C MSFA microwave extraction to determine selenomethionine in selenised yeast. According to the literature, time and temperature both have roles in oxidation of SeMet [50]. The results in Figure 3.3 display the varying rates of selenomethionine oxidation after different extraction methods. Furthermore, the figure highlights that after extraction of SeMet, analysis should take place as soon as possible to reduce these oxidation effects.

Loss of the selenomethionine standard to oxidation can be prevented by inclusion of reducing agents as previously mentioned or by ensuring analysis of the extracted sample takes place as soon as possible (analysis within 5 hours = 4-8 % SeMet oxidation; Figure 3.3). With advances in the speed of analytical separations by HPLC, carrying out analysis within this time period is plausible.

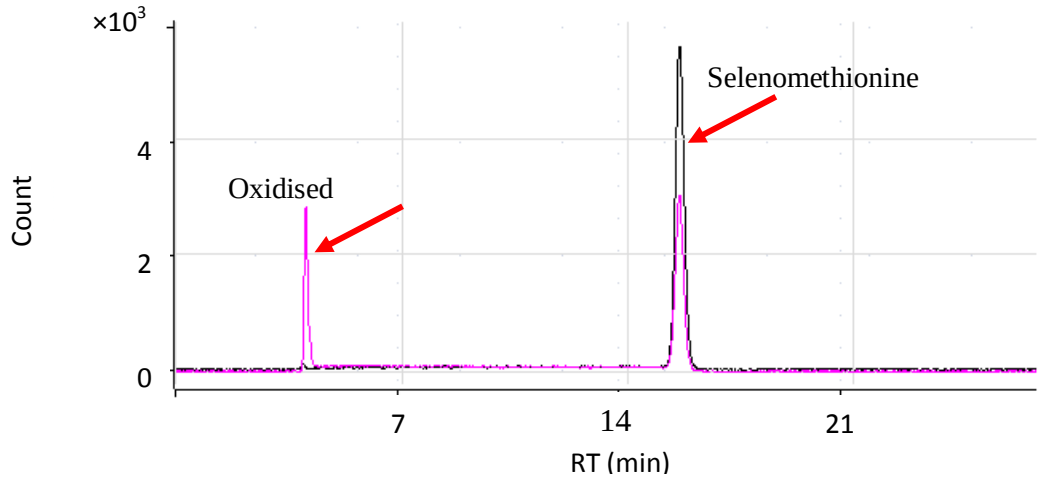


Figure 3.4: Overlapped chromatograms of selenomethionine and oxidised selenomethionine.
The chromatogram of the first 200 µg/kg standard, at time point zero (■), compared to the analysis of the same standard after 60 hours (■). This figure confirmed the retention time of oxidised SeMet.

Over the course of this experiment, ~20 % of the selenomethionine standard was oxidised (see Figure 3.3). Realistically, samples would not be analysed after such a long time (60 hours), instead, fresh extractions would be carried out. However, Figure 3.3

emphasised how important oxidation was to the degradation of the selenomethionine analyte. There was evidence in Chapter 2, Section 2.3.2.2, that when a SeMet standard was analysed on its own by HPLC-ICP-MS after microwave assisted chemical extraction (4 M MSFA at 200 °C for 10 minutes), it completely degraded to oxidised selenomethionine and another degradation product, possibly methionine selenone as discussed in Chapter 1, Section 1.3.3.

3.3.2 Quantification of free, bound and total selenomethionine

Organic selenium is primarily supplied as SeMet via selenium-enriched yeast, although numerous other organic selenocompounds have been detected in selenised yeast [21, 51-53]. The current research aims to demonstrate the effects of different extraction procedures on the release of selenium species from the yeast cell. An inactivated strain of *S. cerevisiae* was utilised in the fermentation process of commercial selenised yeast. While companies use this same yeast species, and while the dried powder may have a similar dark beige/cream appearance, each strain is species specific. This results in different selenium accumulation, creating varying selenocompounds and fluctuation of the concentration of these compounds [54]. Additionally, changes in the fermentation process can alter the selenium metabolism in the yeast cell [55-57]. Variants of yeast strains can lead to contrasts in not just selenomethionine content but the overall selenocompounds too. The method for determination of total selenomethionine was described elsewhere [8].

Table 3.2: Determination of total selenium and selenomethionine in different yeast products.

Samples	Mean Total Se (mg/kg)	S.D	R.S.D	Mean Se as SeMet (mg/kg)	S.D	R.S.D
YP1 B#1	2110	1	0.0	1102	19	1.7
YP1 B#2	2212	13	0.6	1189	35	2.9
YP1 B#3	1975	29	1.4	878	27	3
YP2 B#1	1973	57	2.9	1013	14	1.4
YP2 B#2	2052	28	1.4	1091	4	0.4
YP2 B#3	2040	17	0.8	1305	14	1
YP3 B#1	2178	5	0.2	1162	8	0.7
YP3 B#2	2030	17	0.9	1012	18	1.8
YP3 B#3	1934	38	1.9	874	23	2.6
YP4 B#1	2269	12	0.5	1404	4	0.3
YP4 B#2	2056	42	2.0	1264	16	1.3
YP4 B#3	2009	28	1.4	1229	4	0.3
SELM-1	2035	49	2.4	1405	20	1.4

YP = Yeast product. B# = Batch number. SELM-1 certified reference values: 2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet or 1390 ± 59 mg/kg Se as SeMet. Results are based on triplicate readings (n=3).

Table 3.2 outlines the total selenium and total selenium as selenomethionine contents for each yeast. This work identified how much selenomethionine was available in each sample before the water extraction experiment began. The highest relative standard deviation was 3 % which illustrated the good agreement between sample replicates. Based on the Horwitz function an acceptable R.S.D for total selenium determination was ≤5.1 %, while SeMet was ≤5.4 %.

Modification of the selenomethionine extraction method for the enzymatic digestion of the water extract enabled determination of the free and peptide-bound selenomethionine content. Figure 3.5 shows the average breakdown of selenomethionine in a water extract. Each bar represents the mean selenium as SeMet of three yeast batches for each product. ‘Water-soluble free SeMet’ represents selenomethionine that was unbound to any other compound, molecule or peptide that may be quantified without further extraction. ‘Peptide-bound water-soluble SeMet’ represents selenomethionine that was bonded to other compounds, molecules or peptides and was liberated by a further enzymatic extraction. ‘Total water extractable SeMet’ represents the separate enzymatic extraction and analysis of the water extract to determine all the SeMet present. Fundamentally, selenomethionine was liberated from larger selenomethionine-containing proteins or peptides thus producing free SeMet for detection by HPLC-ICP-MS [8]. When the extraction was applied across 5 selenised

yeast products, there were significant differences in the quantities of free, bound and total water-soluble selenomethionine ($p \leq 0.01$). The only exception was between the water-soluble peptide-bound selenomethionine in yeast products 2 and 3 ($p \geq 0.05$) and between the water-soluble free selenomethionine in yeast product 2 and SELM-1 ($p \geq 0.05$).

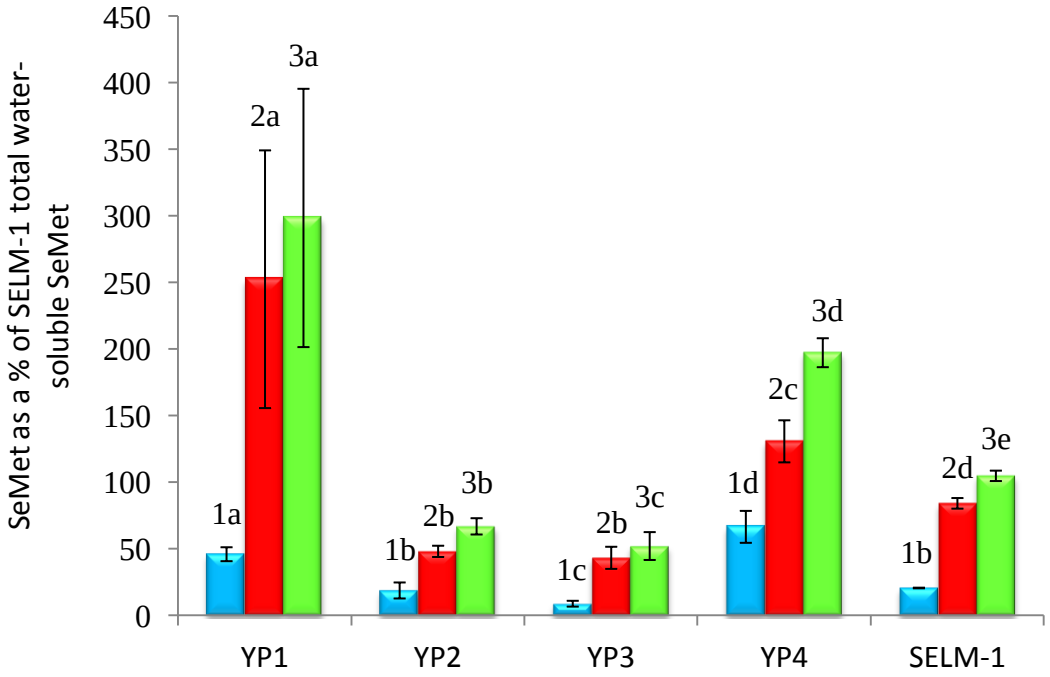


Figure 3.5: Partitioning of water-soluble selenomethionine from commercial selenised yeast products.

■ = water-soluble free SeMet; ■ = peptide-bound water-soluble SeMet; ■ = total water-extractable SeMet. [a,b,c,d,e] Values that lack a common superscript letter differ in significance ($p \leq 0.05$). YP = Yeast product. Results are based on nine data readings, triplicate analysis of three batches per yeast product (n=9).

Figure 3.5 contains the mean data for the three non-consecutive batches of each yeast product and demonstrates that intra-batch differences exist in the makeup of selenomethionine fractions in commercially-available yeasts. The error bars show variation between different batches of the same yeast product. Where error bars are much larger, there were greater differences. These differences can be caused by anything from different strains, strain mutations, differences in the fermentation process, age of the product or natural degradation [58, 59].

This study presents a water extraction and how it can show significant differences between commercially-available yeast products. Therefore, the final stages of the fermentation process for washing and drying the yeast must be considered as a large factor affecting water-soluble selenomethionine. The typical approach after a

successful fermentation of selenised yeast involves separation of the biomass from the culture medium by centrifugation, washing the yeast biomass with water more than once and finishing with a spray drying step [60]. The question remains if approximately 10-25 % of the total selenium content is liberated after a water extraction; how much selenium is removed during these washing steps? Previous reports have discussed the importance of the yeast cell wall and its binding potential of not just selenium but selenomethionine residues [59]. Research suggests that mannan in the YCW has increased sorption of selenium when compared to glucan and chitin [59]. This extracellular binding of selenomethionine and other selenocompounds residues to the yeast cell wall explains why so much selenium can be liberated from a water extraction. The intracellular accumulation of selenium occurs via transport of the ions across the cytoplasmic membrane [59], thus a stronger extraction method is essential to break open the YCW and release the intracellular selenium species.

Table 3.3: Summary of water-soluble free and peptide-bound selenomethionine.

Samples	Mean Free Se as SeMet (µg) (Mean ± S.D.)	Peptide-bound Se as SeMet (µg) (Mean ± S.D.)	SeMet liberated due to enzymatic extraction of water extract (%)
YP1 B#1-3	23.5 ± 2.8	128.8 ± 49.8	84.6
YP2 B#1-3	9.6 ± 3.1	24.5 ± 2.1	71.9
YP3 B#1-3	4.7 ± 0.1	23.2 ± 0.8	83.1
YP4 B#1-3	34.3 ± 6.0	67.4 ± 8.4	66.3

Results are based on nine data readings, triplicate analysis of three batches per yeast product (n=9). YP = Yeast product. B# = Batch number.

Table 3.3 is a summary of the average results from the three batches of the same yeast. It provides numerical data for Figure 3.5 and shows the amount of selenomethionine that was liberated by enzymatic extraction of the water extract. The free selenomethionine analysis was carried out before any enzymatic extractions. Clearly the free SeMet was loosely bound to the yeast cell, most likely extracellularly, and was extracted easily. However, more SeMet was bound to peptides or proteins (Table 3.3). This peptide or protein bonding could offer a protective effect to SeMet, which might explain why SeMet standards oxidised at a higher rate than enzymatically or chemically extracted SeMet (Figure 3.3). This effect was also observed in Chapter 2, Section 2.3.2.2. When a SeMet standard was chemically extracted on its own it was completely degraded and oxidised, but when SeMet was spiked in with a non-selenised

yeast and chemically extracted, there was very little oxidation experienced. This was also confirmed by the validation study of the microwave assisted chemical extraction method with ≥95 % recovery of the SeMet that was spiked on top of SELM-1 before the chemical extraction. A comparison was made to the peptide-bound selenomethionine which was determined after an enzymatic extraction of the original water extract. This selenium speciation showed that the extraction was essential to determine the true value of selenomethionine present in the water extract and its fractions. Regardless of which yeast was analysed, over 66-85 % more SeMet was liberated after the enzymatic extraction. This suggested that selenocompounds present were peptides that contained selenomethionine bound in one way or another.

3.3.3 Mass balance of water-soluble and insoluble fractions

A selenium mass balance calculation was carried out to account for the selenium in each fraction and recoveries of ≥98 % were consistently achieved. Data generated from the total selenium analysis of each yeast batch in Table 3.2 was utilised to determine the recovery of selenium after the water extractions. Both the soluble supernatant and insoluble pellet were analysed to complete this mass balance.

Table 3.4: Selenium mass balance of the water-soluble and insoluble fractions in yeast products.

Samples	Se in Supernatant (µg)	S.D.	R.S.D.	Se in Freeze dried pellet (µg)	S.D.	R.S.D.	Pellet + Supernatant Se (ug)	Total Se (ug)	Recovery For mass balance (%)
YP1 B#1	314.7	9.3	3.0	747.1	17.5	2.3	1061.8	1069.8	99.3
YP1 B#2	361.1	5.3	1.5	791.2	23.0	2.9	1152.3	1132.4	101.8
YP1 B#3	231.0	4.8	2.1	757.0	5.4	0.7	988.0	1005.2	98.3
YP2 B#1	155.5	0.7	0.5	826.9	25.6	3.1	982.4	1002.1	98.0
YP2 B#2	127.2	1.8	1.4	929.8	9.8	1.1	1057.0	1044.8	101.2
YP2 B#3	103.5	7.2	7.0	950.0	7.0	0.7	1053.5	1034.7	101.8
YP3 B#1	191.7	25.3	13.2	924.0	49.1	5.3	1115.7	1115.0	100.1
YP3 B#2	130.0	5.8	4.4	925.0	16.4	1.8	1055.0	1029.5	102.5
YP3 B#3	131.2	3.8	2.9	863.2	6.9	0.8	994.4	983.1	101.2
YP4 B#1	273.0	4.7	1.7	912.3	27.7	3.0	1185.3	1169.9	101.3
YP4 B#2	225.4	3.0	1.3	847.9	23.7	2.8	1073.3	1065.0	100.8
YP4 B#3	248.7	7.8	3.1	797.6	8.1	1.0	1046.2	1028.9	101.7
SELM-1	136.9	1.8	1.3	877.3	2.7	0.3	1014.2	1035.4	98.0

YP = Yeast product. B# = Batch number. Results are based on triplicate readings (n=3).

Furthermore, data generated from the selenomethionine analysis of each yeast batch in Table 3.2 was employed to determine a selenomethionine mass balance in Table 3.5.

Table 3.5: Selenomethionine mass balance of the soluble and insoluble fractions of yeast products.

Samples	Se as SeMet in supernatant (µg)	S.D	R.S.D	Se as SeMet in pellet (µg)	S.D	R.S.D	Se as SeMet in supernatant + pellet (µg)	S.D	R.S.D	Total Se as SeMet (µg)	S.D	R.S.D	Recovery of SeMet from supernatant and pellet (mass balance %)
YP1 B#1	141.6	1.0	0.7	449.5	10.2	2.3	591.1	10.2	1.7	559.1	16.2	2.9	105.7
YP1 B#2	214.5	4.6	2.1	446.7	6.7	1.5	661.2	8.4	1.3	608.5	8.0	1.3	108.7
YP1 B#3	100.6	4.0	4.0	383.4	2.5	0.7	484.0	6.4	1.3	446.6	11.5	2.6	108.4
YP2 B#1	34.3	2.0	5.8	442.2	7.7	1.7	476.5	6.9	1.4	515.3	9.4	1.8	92.5
YP2 B#2	37.1	1.9	5.1	489.3	13.6	2.8	526.5	15.5	2.9	555.5	11.8	2.1	94.8
YP2 B#3	30.7	0.7	2.4	584.2	18.4	3.2	614.9	19.1	3.1	662.0	14.2	2.1	92.9
YP3 B#1	32.0	0.4	1.3	539.2	25.2	4.7	571.2	25.6	4.5	594.8	13.3	2.2	96.0
YP3 B#2	27.9	0.8	2.9	449.2	4.2	0.9	477.1	4.8	1.0	513.5	8.0	1.6	92.9
YP3 B#3	19.8	0.5	2.6	412.9	4.1	1.0	432.7	4.6	1.1	444.5	12.2	2.8	97.3
YP4 B#1	108.5	3.2	3.0	607.8	4.0	0.7	716.4	5.0	0.7	724.0	5.9	0.8	98.9
YP4 B#2	96.6	1.0	1.1	512.3	10.8	2.1	608.9	11.5	1.9	654.5	7.6	1.2	93.0
YP4 B#3	100.0	1.4	1.4	521.5	11.0	2.1	621.5	10.9	1.8	629.3	1.4	0.2	98.8

YP = Yeast product. B# = Batch number. Results are based on triplicate readings (n=3).

Completion of a mass balance as mentioned previously (Table 3.4 and 3.5), allowed for confidence in the water-soluble selenocompound experimental data. All total selenium was accounted for by measuring total selenium in the supernatant and the pellet that remained after centrifugation of the water extracted yeast products. Comparison of the sum of selenium in these fractions to the overall total selenium quantification of the selenised yeast gave a mass balance. Additionally, completion of a selenomethionine mass balance by the same approach (selenomethionine in the supernatant + selenomethionine in the pellet = total selenomethionine quantified from the selenised yeast) showed precision in the recovery of selenomethionine from the experiment. The lowest recovery of selenomethionine was 92.5 % of the total selenomethionine.

3.3.4 Configuration of ICP-MS for chiral analysis of water-soluble selenomethionine

The ICP-MS (Agilent 7700× series) could only tolerate a certain percentage of solvent passing into the plasma before it would be extinguished (~30 %). This issue was also reported by Sanz Medel *et al.* [61]. The chromatographic method utilised high organic solvent, thus, the ICP-MS had to be modified from traditional analytical conditions that were employed for aqueous samples. The increase of MeOH from the usual 2 % to 70 % would deposit more carbon on the sample and skimmer cones [22]. To burn off this excess carbon, reduce the interference, keep the baseline from potential variation and reduce the amount of times the cones need to be cleaned, an additional gas module was installed into the ICP-MS. The extra gas was an 80:20 mix of argon to oxygen. The argon would not interfere with the existing plasma, which was also argon based, and the percentage of oxygen matched closely to that of atmospheric levels. To proceed with ICP-MS analysis, the standard nickel sample and skimmer cones were changed for platinum cones which are less reactive under these conditions [22]. A 1 mm internal diameter quartz torch replaced the 2.5 mm internal diameter torch. This allowed the MeOH concentration in the mobile phase increase to levels between 90 and 100 % [62]. Optimisation of the mobile phase went as high as 80 % MeOH with the final gradient parameters summarised in Table 3.1.

It was necessary to change the drain tubing and O-rings to organic resistant plastic versions to avoid degradation upon coming into contact with high organic

solvents. As part of the analysis optimisation, the argon:oxygen gas percentage was varied to minimise carbon deposits on the platinum cones. Optimisation involved selecting the right balance between the option gas and the make-up gas. The spray chamber temperature was reduced from 2 to -5 ℃, thus helping to reduce the vapour pressure of the high organic solvents used, which could extinguish the plasma. The carrier gas flow was increased until the solvent solution moved up the tubing while also decreasing the make-up gas by the same increments. This maintained an overall gas flow of 1.0 to 1.2 L/min. This optimisation approach gave a stable plasma for chiral analysis.

3.3.5 Total selenium and chiral selenomethionine standard analysis

The confirmation of selenium content in the D,L- and L-selenomethionine standard was essential to ensure the standards were reliable for analysis (Table 3.6).

Table 3.6: Recovery of total selenium in selenomethionine standards before analysis.

Standard	Mean Se (% Recovery)	S.D	R.S.D
100 mg/kg L-SeMet	99.7	1.0	1.0
100 mg/kg D,L-SeMet	101.0	1.0	1.0

Results are based on triplicate readings (n=3).

Once the argon:oxygen option gas supply was optimised, the standards were analysed for purity and concentration by HPLC-ICP-MS. The L-SeMet standard was analysed first as its retention time allowed for the determination of the enantiomers in the D,L-SeMet standard. When standards of D,L-SeMet were measured on the HPLC-ICP-MS (Figure 3.6), the recovery equalled more than 96 % of the equivalent L-SeMet standard. These calculations suggested that the SeMet standards utilised for this study were pure and reliable for analysis. Therefore, they could be utilised for D,L-SeMet quantification.

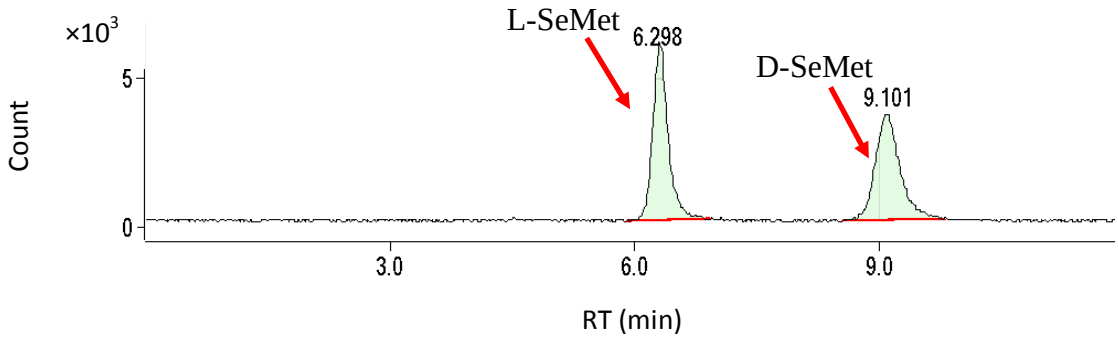
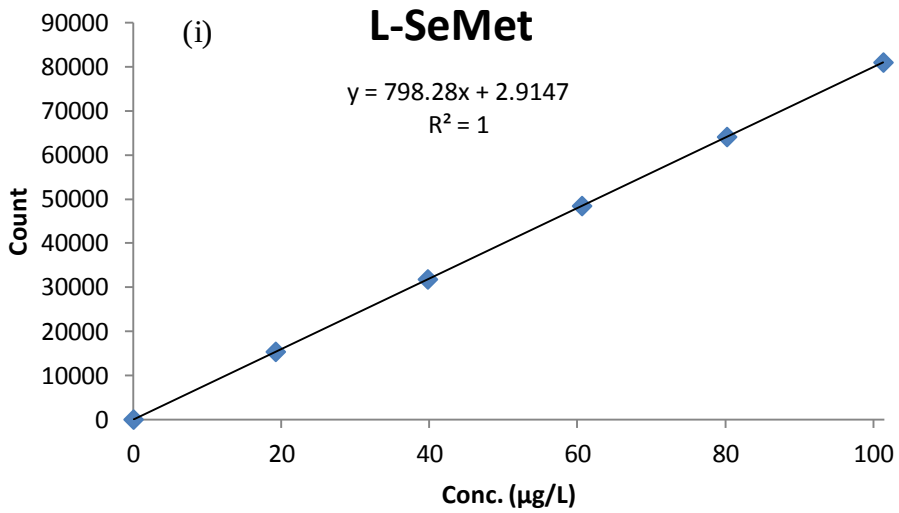


Figure 3.6: Resolution of D- and L-selenomethionine using an Astec Chirobiotic T column.



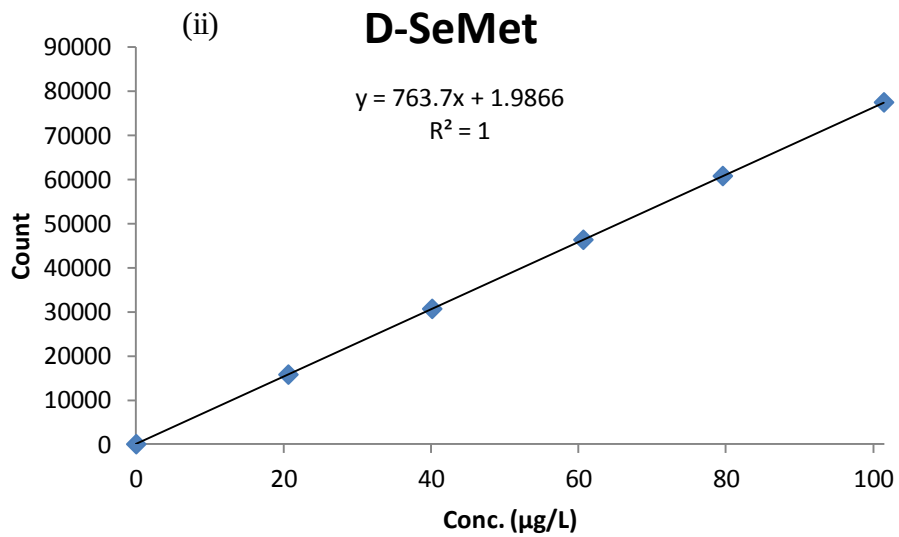


Figure 3.7 (i) & (ii): D,L-selenomethionine standard.
L-SeMet (52.09 %) and D-SeMet (47.91 %).

The D,L-selenomethionine standard was utilised for chiral speciation of both enantiomers. The standard concentrations were based on previous methods [8], but since it was a 52:48 ratio of L-SeMet to D-SeMet standard solution (Figure 3.7), twice as much of the D,L-selenomethionine standard was needed to produce the same calibration curve. Furthermore, to ensure the standards were reliable, the linearity and range were investigated and the results are shown in Figure 3.8 and Figure 3.9. Standards were made up to 800 µg/kg D- and L-Se as selenomethionine (~2,000 µg/kg selenomethionine).

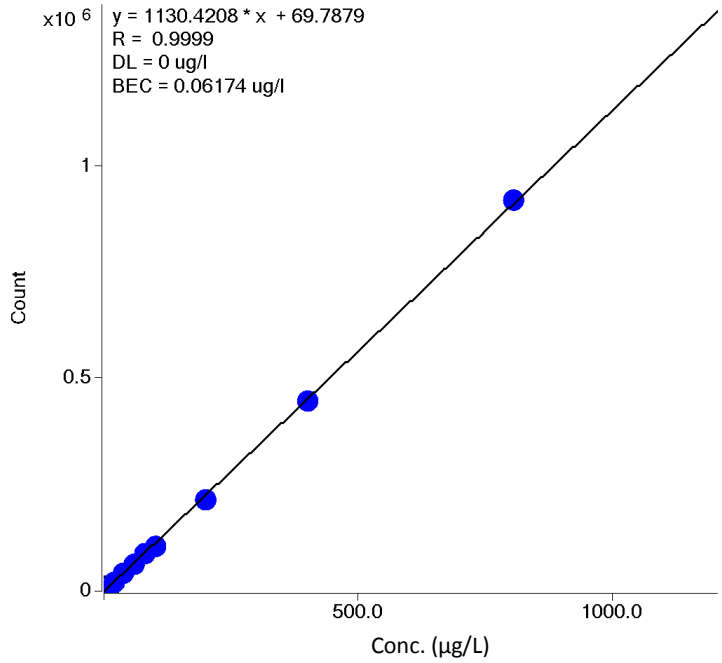


Figure 3.8: Linearity and range of Se as L-selenomethionine standards prior to chiral analysis.
The Se as L-SeMet was measured from the D,L-SeMet standard.

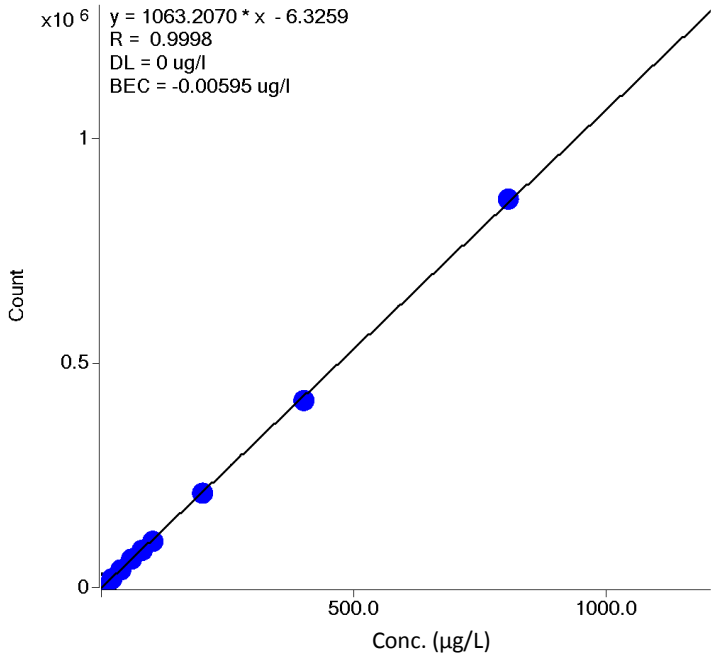


Figure 3.9: Linearity and range of Se as D-selenomethionine standards prior to chiral analysis.
The Se as D-SeMet was measured from the D,L-SeMet standard.

The calibration curve for each enantiomer was linear ($R^2 \geq 0.9998$) with a range eight times higher than the top SeMet standard of ~100 µg/kg [8]. Therefore, the objective to develop a method that can accommodate a higher concentration range in the test sample was achieved.

3.3.6 Quantification of D- and L-selenomethionine in water extracts of commercially-available yeast

Once chromatographic optimisation for the standards was complete, three batches of each of the four commercially-available yeast products were investigated for their water-soluble selenomethionine enantiomeric composition. While proteins of multicellular organisms are normally based on the L-configuration of the amino acid, the activity of the D-form can vary dramatically from that of its corresponding L-enantiomer [26]. The least concentrated selenomethionine enantiomer in selenised yeast, from current literature, was D-selenomethionine [24, 25, 63], with ratios <18/82 for D and L-selenomethionine respectively [24]. The present study also found L-selenomethionine to have the highest concentration of the two enantiomers in water-soluble extracts of all products except yeast product 3 (YP3). There was no significant difference between D and L-SeMet in YP3 ($p \geq 0.05$). This current research

followed on from previous investigations of water-soluble fractions of the selenium-enriched yeast products (Figure 3.5) which found large selenomethionine variations across yeast products after a water extraction. Clear differences were also observed when investigating the D- and L-selenomethionine concentrations of these water-soluble fractions, Figure 3.10.

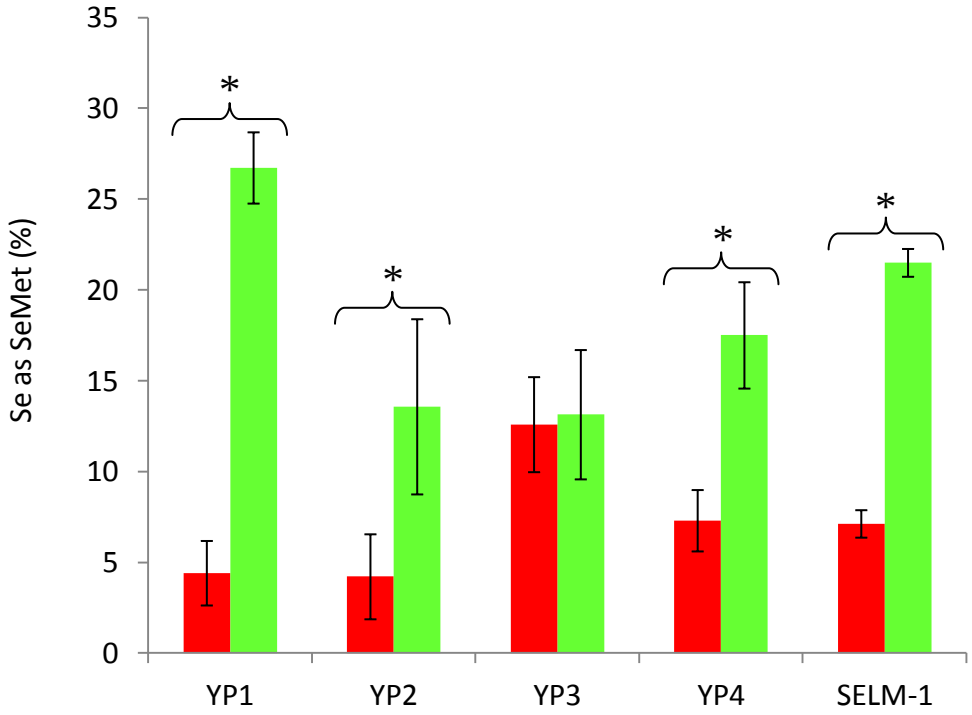


Figure 3.10: Mean chiral selenomethionine data of water-soluble yeast extracts.
Extracts of three non-consecutive batches from four different yeast products (YP) were analysed for chiral SeMet composition. ■ = D-Se as SeMet; ■ = L-Se as SeMet. SeMet results were expressed as a percentage of the total SeMet present in the water extract. SELM-1 was also included as a certified reference material (n=3). An asterisk [*] denotes a significant difference ($p \leq 0.05$) between D- and L-SeMet in yeast products 1 to 4 and SELM-1.

3.3.7 Compound independent calibration for selenium quantification of unknown selenocompounds

Compound independent calibration (CIC) proved beneficial for the analysis of the yeast water extracts. After quantifying the chiral, free, peptide-bound and total selenomethionine contents with available standards, approximately thirty additional unidentified selenocompounds were also detected. The CIC was essential to determine the concentration of selenium that was represented by each compound [64]. This approach allowed more understanding of selenium distribution and has helped other elemental research studies such as those concerning sulfur-containing biomolecules

which are closely linked to selenium [65]. Unknown species can be quantified based on their elemental concentration when compared to the elemental concentration of a known standard [66]. ICP-MS is ideally suited for analysis by CIC due to the complete atomisation that occurs in the plasma, independent of the compound in which the element is present. Therefore, the response of any selenium-containing compound or species is determined by the selenium signal, thus it is possible to calibrate based on selenium concentrations irrespective of the species. The main drawback to selenium speciation is the lack of access to affordable high purity selenium standards. Acquisition of a specific selenocompound standard for identification by HPLC-ICP-MS is possible. This approach identifies selenocompounds by matching the retention time of the calibration standards with that of the retention time of selenocompound peaks found in yeast samples. This calibration approach was employed for SeMet determination in Chapter 2. However, while it is possible to obtain such selenium compound standards through labs that perform custom synthesis such as companies like PharmaSe in Texas, these are expensive and would cost significantly more than commercial selenomethionine from traditional suppliers. Identification via tandem MS methods would be a lot more efficient and cost effective and would focus on fragmentation patterns rather than retention times. Another large issue would be in relation to the synthesis process. Selenium-containing compounds cannot be synthesised until an identity or structure is known, which presents one of the biggest challenges. Otherwise, trial and error analysis of commercially-available standards would be the main approach to see if the retention time matched any selenocompounds present in the samples. Situations like these rule out quantification by calibration standards but CIC allows researchers to overcome some of these barriers [64, 67].

As previously mentioned, selenium-enriched yeast is traditionally characterised by its dominant seleno-species, selenomethionine. To further distinguish between these yeast samples, other selenium peaks that were appearing in the chromatograms of the yeast water-extract were investigated. Retention times and the CIC were combined to estimate their concentration relative to each other. This approach was applied to determine any further differences.

When the data and graphs were summarised, ten of the approximately thirty selenocompounds, including selenomethionine, were detected in all batches of each yeast product. Their retention times were 2.76, 3.07, 3.57**,** 4.73, 5.38, 10.98, 17.57,

18.28 and 31.46 minutes. Figure 3.11 highlighted these similar water-soluble selenocompounds.

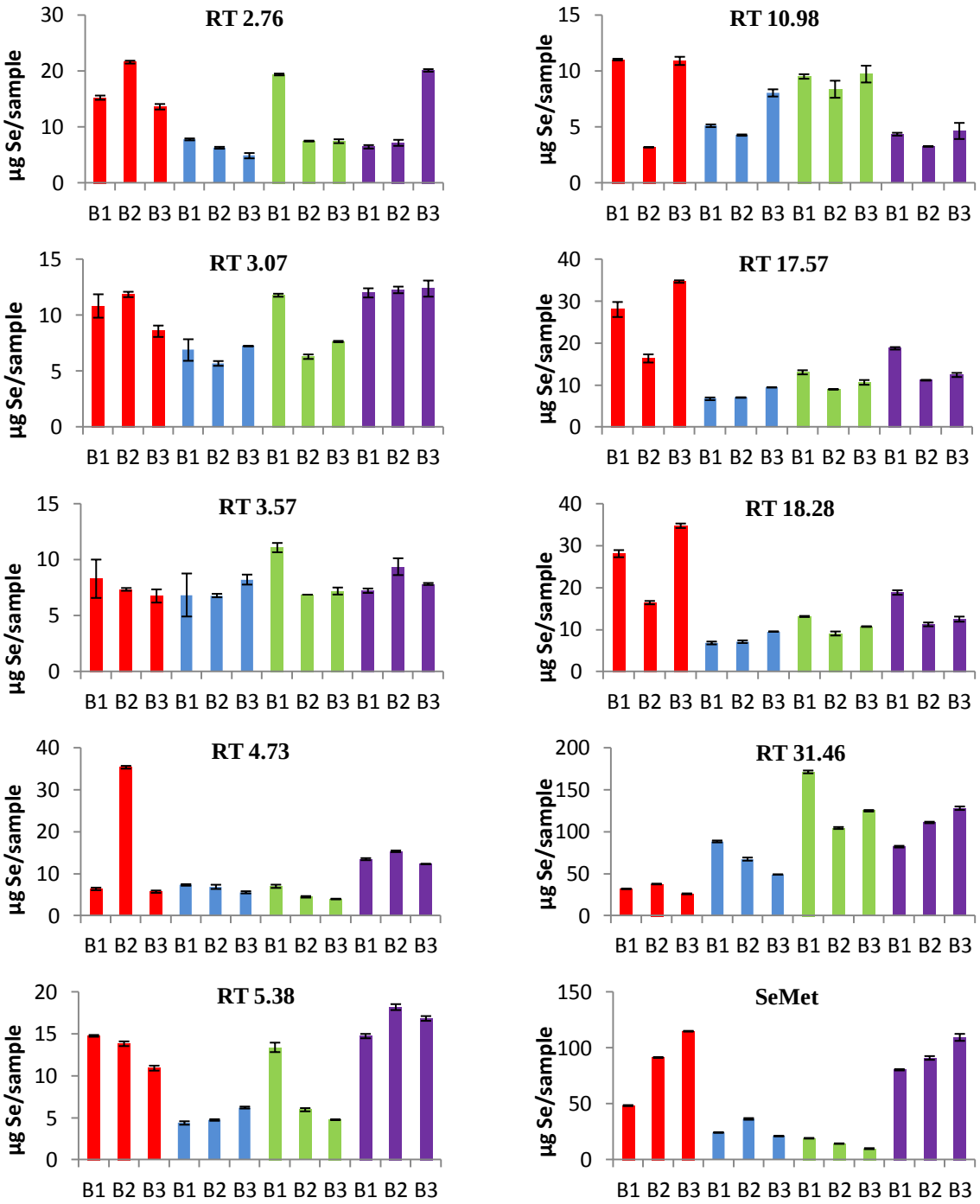


Figure 3.11: Selenocompounds detected in all commercial yeast products (YP) by HPLC-ICP-MS and quantified by CIC.

■ = YP1; ■ = YP2; ■ = YP3; ■ = YP4. YP = Yeast product. Consecutive batches were denoted by B1, B2 and B3. Retention time (RT)-2.76 min; RT-3.07 min; RT-3.57 min; RT-4.73 min; RT-5.38 min; RT-10.98 min; RT-17.57 min; RT-18.28 min; RT-31.46 min; SeMet, RT-15.05 min.

These ten selenocompounds were detected in all the selenised yeast products and their corresponding batches. However, differences were still evident in the amount of selenium represented by the selenocompound in each batch. This agreed with previous quantitative proteomic research that suggested that yeast strains will metabolise and accumulate selenium at varying rates, resulting in distinctly different selenised yeast products from different manufacturers [9]. Differences that occur across batches of the same product could be caused by variation in the fermentation process. Although 10 selenocompounds were detected in all the yeast batches, 20 other selenocompounds were not detected in all batches. Figure 3.12 clearly demonstrates these selenocompound differences across commercial selenised yeast products. The data shows that no two yeast products are the same in relation to selenocompound profile or concentration. Closer inspection of Figure 3.13 also shows a number of unique selenocompounds detected in specific yeast products only, for example, at retention time 2.38, 2.58, 3.72, 6.58 and 24.58 minutes. However, since this figure is based on an average selenocompound concentration of three non-consecutive yeast batches, it is possible that while some compounds are unique, they are not present in all batches of the yeast product (Appendix Figure A1).

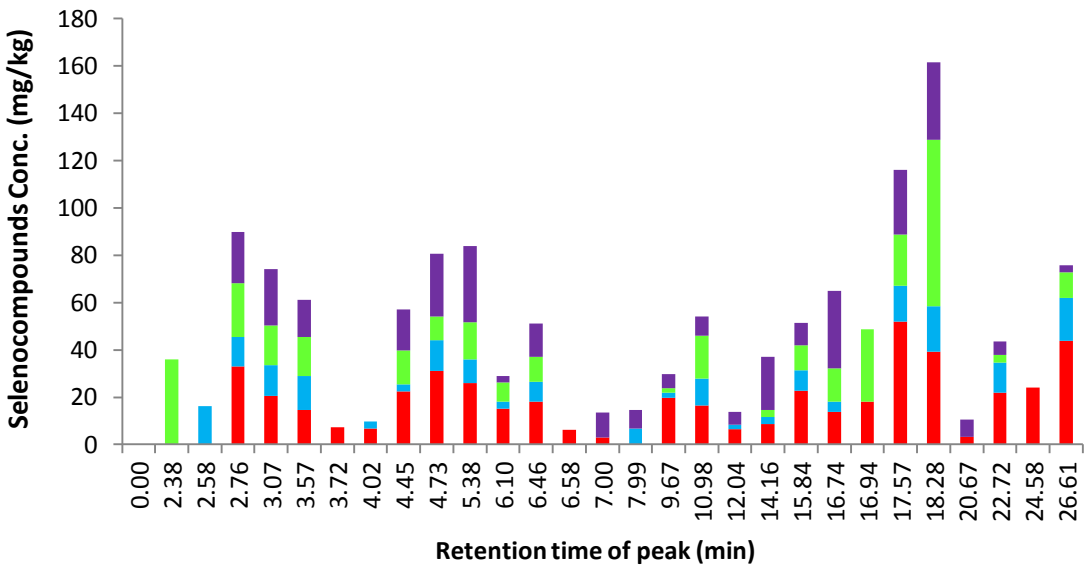


Figure 3.12: Summary of water-extracted selenocompounds detected in selenised yeast products.

■ = YP1; ■ = YP2; ■ = YP3; ■ = YP4. Analysis by HPLC-ICP-MS detected multiple selenocompounds in YP1-4. YP = Yeast product. Each yeast product is representative of the mean selenocompound recovery over three batches.

Discovery of a unique selenocompound(s) in all batches of a product, could be used as a selenium biomarker. Such biomarkers could be monitored in the manufacturing process for batch-to-batch reproducibility but could also provide a way to identify one commercial product from the next. An example of a potential biomarker present in all batches of one product was apparent in Figure 3.13. All batches of yeast product 1 and 3 contain the selenocompound with a retention time of 24.58 and 2.38 minutes, respectively. Although more batches of the yeast products would need to be analysed before definite biomarkers could be assigned. These examples suggest that it was possible to discriminate between selenised yeast products, especially due to continuously improving speciation techniques and the benefits of CIC. Advancement in analytical speciation techniques allows for much more comprehensive product discrimination. Information such as an individual unique fingerprint would be beneficial for manufacturers. These selenocompounds could be monitored for batch-to-batch stability and reproducibility. They could also be tracked to see if the selenised yeast supplement was included in feed products or by monitoring selenium metabolism in animals or trials [68-70]. The ability to distinguish products from that of a competitor is always advantageous for manufacturers and also for regulatory bodies. Moreover, since the selenocompound identity was unknown, its efficacy was also unknown. This could be investigated further if the compound was identified. Establishing an identity is possible using more advanced mass spectrometric techniques such as ESI-MS or QTOF-MS [21, 52, 54].

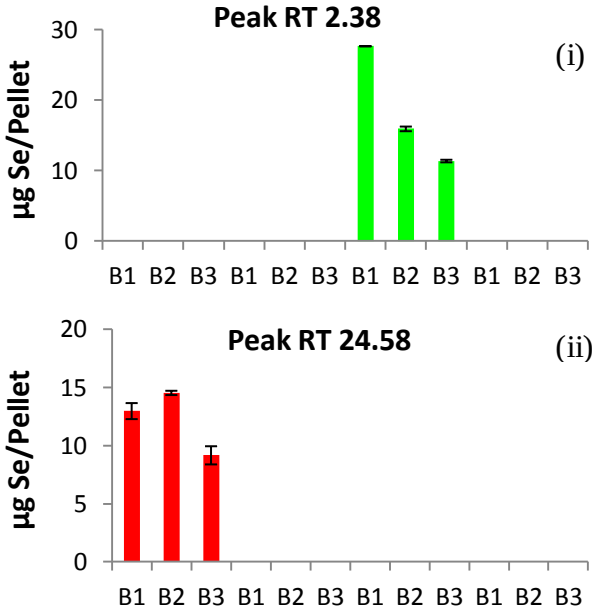


Figure 3.13 (i) and (ii): Selenocompound detected in all batches of yeast product 1 and 3, respectively.
Analysis by HPLC-ICP-MS detected selenocompounds at a retention time of 2.38 and 24.58, in YP1 and YP3, respectively. ■ = YP1; ■ = YP3. YP = Yeast product. Consecutive batches were denoted by B1, B2 and B3.

Determination of the accurate mass and elemental composition can combine to propose an identity of an unknown selenocompound [51]. The benefit of two mass analysers in tandem QTOF-MS, for example, allows the mass-to-charge ratio of the compound of interest to be filtered away from all other background matrix or interfering compounds. Once isolated, the ion of interest can then be fragmented by collision-induced dissociation (CID). This fragmentation breaks bonds and releases fragments of the ion. Certain bonds will break before others, i.e. C-N bond will break before a C-C bond. This is caused by the greater bond enthalpy between a C-C bond (348 kJ/mol) when compared to a single carbon nitrogen bond (308 kJ/mol) [71]. Combining the fragments will allow for structure elucidation. Once a structure was identified, it could then be synthesised. Therefore, larger quantities would be available to investigate the efficacy and potential benefits or dangers associated with the compound. Reports suggest that some selenocompounds are known for their anti-carcinogenic properties [19, 72]. If an unknown selenocompound was found to possess such traits, the fermentation process could be examined to try to produce more of this compound by altering the metabolism of the yeast cells.

While some selenocompounds were detected in all batches of the yeast products, the concentration of these selenocompounds varied from batch-to-batch. The figures

also highlighted some similarities between the selenised yeast products, but there was more that separated them than united them. A summary of the remaining selenocompounds detected in the yeast products was summarised in Appendix Figure 1A (a)-(r). While CIC cannot identify any of these selenocompounds, it showed that these selenised yeast products were distinct from each other when examined by a water extraction method. As previously mentioned, since retention time matching was not possible due to a lack of available selenocompound standards, identification of the differences between these products and batches will need to be investigated by a more advanced mass spectrometer. This could include tandem mass spectrometric techniques to allow fragmentation of the ions of interest and subsequent structure elucidation.

3.3.8 Issues with the compound independent calibration approach

The benefits of having CIC capabilities allowed for the quantification of selenium in unknown compounds. While identification would still need to be verified with a standard in these circumstances, CIC is a useful tool for HPLC-ICP-MS investigations. However, chromatography was a substantial limiting factor for CIC to succeed. Separation of the selenocompounds determined if adequate integration of the peak area could estimate the amount of selenium present. A relevant example of this would be the chiral speciation analysis. Separation of selenomethionine into its D- and L-enantiomers was only possible using a CSP or by derivatising the compounds beforehand [24]. Therefore, unless care was taken to resolve these enantiomers with the correct chromatographic conditions, they would co-elute. The speciation development carried out with C8 columns, for the enzymatic and chemical digestions of selenised yeast, resulted in a co-elution of D- and L-selenomethionine. This was only one example and it was possible for many other selenocompounds to co-elute. Since the identity of these selenocompounds was unknown, as was often the case with speciation techniques, minimal information about its chemical properties was available. Estimating the amount of selenium present in the forms of these unknown compounds is important. Nevertheless, the CIC technique can be used to demonstrate the distinction between yeast products and can also be utilised as another mass balance technique.

3.4 Conclusion

The objectives of this research were to show the SeMet, chiral SeMet and selenocompound differences between multiple selenised yeast samples using a water extraction. This investigation included water-soluble free selenomethionine, peptide-bound selenomethionine, total water-soluble selenomethionine and also the chiral composition of the free selenomethionine present. The chiral study aimed to employ a direct enantioseparation method for determination of D- and L-selenomethionine in water-soluble fractions of the selenium-enriched yeast products. It also intended to provide further speciation details about commercially-available yeast and their differences via water extraction.

The results highlighted the differences between aqueous extracts of different yeast products. Enzymatic digestion of the water-soluble compounds was essential to determine the true value of selenomethionine present. The method showed the larger selenomethionine-containing peptides were broken down thus liberating the selenoamino acid for detection. Overall there was a significant difference between the strains of commercially-available yeast. Yeast product 1 had the highest batch-to-batch variation in terms of SeMet content. There was no evidence to explain this large standard deviation, but it may be due to the age of the sample or from changes that were made to the fermentation process over time. However, regardless of the age of the sample or the fermentation processes that might have been altered, the free SeMet is still consistent between the three batches. This free SeMet consistency can be seen throughout all the yeast brands. Since free SeMet can be extracted relatively easily (a 15 minute water extraction), it raises the question about the final stages of the yeast fermentation process (i.e. washing/spray drying steps). Future work could investigate if multiple washing steps reduce the free selenomethionine available for detection.

The total selenium mass balance of the yeast products was accurate, the lowest mean total selenium recovery was ~98 % and the highest was ~102.5 %. In relation to the chiral speciation analysis, ICP-MS was used to confirm the selenium concentration in SeMet standards with a mean recovery of 99.7 and 101 % for L- and D-SeMet respectively. All yeast products varied in their D- or L-SeMet content (or both). The error bars in Figure 3.10 highlight batch-to-batch variation of individual products. As previously mentioned, this variation could be caused by anything from different strains, strain mutations, differences in the fermentation process, and possibly the age of the

product or natural degradation. Throughout the analysis, L-SeMet, as expected, was the dominant enantiomer across the yeast products, except for yeast product 3. In this case, the difference between D- and L-SeMet in yeast product 3 was negligible ($p \geq 0.05$). This extraction investigated the D- and L-SeMet content in water-soluble fractions of the selenium-enriched yeast products, again highlighting the potential for future work involving the final stages of the yeast fermentation process. Future work will not only involve identification of other selenocompounds with one or two chiral centres such as Se-methylselenocysteine (MeSeCys, one chiral centre), Selenoethionine (SeEt, one chiral centre) or Selenocystine ($SeCys_2$, two chiral centres), but also would examine if the later stages of the yeast fermentation could affect or alter the selenised yeast water profile for selenocompounds.

Compound independent calibration proved a useful tool, not just for estimation of selenium quantitation but as an additional approach to differentiate yeast products from each other. Retention time matching, along with exploiting the selenium concentration in the selenomethionine standards allowed a compilation of unknown compounds to be quantified. This accumulation of data emphasised further differences between the selenium-enriched products. Evidently, some selenocompounds were only discovered in certain yeasts. All batches of yeast product 1 and 3 contained a matching selenocompound at a retention time of 24.58 and 2.38 minutes, respectively. Such compounds could be useful as biological markers or investigated further with an aim to identify their structure via more advanced mass spectrometric techniques.

This work underlines the importance of selenium speciation to differentiate yeast products. While it is accepted that a water extraction only contains ~10-25 % of the total selenium in selenised yeast, this extraction assay is capable of determining significant variations. Since there is considerable diversity and plenty of unknown selenocompounds, it will be of interest to identify as many of these as possible. Therefore, techniques such as liquid chromatography electrospray ionisation mass spectrometry and time-of-flight mass spectrometry would build on this research.

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Chapter 4: Speciation of water-soluble selenium metabolites in commercial selenised yeast by Reversed Phase Liquid Chromatography-Electrospray Ionisation Quadrupole Time-of-Flight Mass Spectrometry

Abstract

Reverse phase liquid chromatography-inductively coupled plasma mass spectrometry (RPLC-ICP-MS) was utilised for the screening of organoselenium metabolites in the water extracts of selenised yeast products. The analysis was applied to four different commercially-available yeast products and the reference yeast standard, SELM-1. Total selenium and selenomethionine (SeMet) concentrations were determined by ICP-MS and RPLC-ICP-MS, respectively, using previously validated methods. Ultrafiltration using a 3 kDa molecular weight cut-off was used in the present study for sample clean-up. The water-soluble extracts were lyophilised to increase analyte concentration, resuspended in water and analysed by liquid chromatography electrospray ionisation quadrupole time-of-flight mass spectrometry (LC-ESI-QTOF-MS). Selenium-containing species were confirmed using selenium's unique isotopic pattern. This approach allowed for the detection of approximately 153 previously unreported selenium metabolites across four different commercially-available yeast products and the reference yeast standard, SELM-1. Determination of the elemental composition and proposed structure of 17 of these selenocompounds was advanced through MS^2 analysis and accurate molecular mass determination (Δ ppm<1.6 for all proposed structures).

4.1 Introduction

Numerous studies highlight the benefits of selenium supplementation for humans and animals [1-3]. The research by Clark *et al.* [4] on the cancer preventative properties of selenium-enriched yeast was the catalyst for further investigation of these products and their species. Since then, selenium compounds have been regularly investigated and reviewed for their efficacy regarding cancer chemopreventative activity [5-8], antioxidant effects [9], and even fertility [3, 10].

While it is widely accepted that selenium-containing compounds, peptides and proteins in commercially-available selenised yeasts are responsible for a range of health benefits, it has also been noted that these products vary not only in their organoselenium metabolite profile but also in the concentrations present [11, 12]. Studies have demonstrated that these selenoproteins and selenocompounds are deposited differentially in a strain specific fashion, in different locations in yeast cells, with some bound to the yeast cell wall (YCW) and others bound intracellularly [13]. Differential organoselenium deposition will result in product specific bioaccessibility [14]. As a consequence, the efficacy of individual selenised yeast preparations and their benefits will vary also.

Fermentation is one of the most efficient ways to produce selenised yeast and thus organic selenium [15, 16]. The assimilation of inorganic selenite, into *Saccharomyces cerevisiae* via the sulfur metabolic pathway [17], creates a selenised yeast containing a variety of organic selenium compounds. Accumulation of selenium in commercial *Saccharomyces* yeast can range between 500 and 3,000 mg/kg [7, 18]. Selenocompounds that account for this include selenoamino acids such as SeMet, selenocysteine and selenocystine in addition to numerous other organoselenium metabolites identified by other researchers. This includes the works of Preud'homme and Arnaudguilheim in 2012 and also Gilbert-López in 2017 [11, 19-25] who reported 49, 64 and 100 organoselenium metabolites respectively in selenised yeast products. The concentration and variety of the organoselenium metabolites can thus act as a fingerprint for the identification of specific yeast strains [11, 13, 26].

Research in ruminants has shown that supplemental SeMet has a superior bioavailability over selenite and has a rapid incorporation into proteins [3, 27]. However, determination of the total selenium concentration as well as organic selenium in the form of SeMet is only the beginning of speciation of selenised yeasts and

supplements [28, 29]. Speciation analysis of organoselenium metabolites from biological cells is highly dependent on favourable extraction conditions and also the stability of the extracted analytes. Furthermore, *in vitro* laboratory extraction does not always mimic exact biological conditions and thus, can only provide limited insight on what fraction of the total selenium would be bioaccessible under biological conditions [30]. These differences generate interest in the bioaccessibility of organoselenium species, and for this reason, there is a desire to speciate selenometabolites from different strains of yeast to determine the potential efficacy of the variants [11, 12].

Mass spectrometers are regularly coupled to separation techniques for increased selectivity and sensitivity in HPLC-ICP-MS [29, 31, 32], gas chromatography-mass spectrometry (GC-MS)/ GC-ICP-MS [29, 33, 34] and LC-MS/ESI-Q-TOF-MS/OrbitrapXL-MS [17, 19, 35, 36]. However, techniques such as HPLC-ICP-MS can only identify compounds if there is a standard for the analyte of interest. Other techniques for coupling HPLC to MS such as electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) allow molecular information about the analytes of interest to be determined [19, 37-41].

Following the introduction of ESI-MS by Yamashita and Fenn in 1984 [42, 43], this technique became popular for the analysis of polar and ionic compounds including organoselenium species [44]. Electrospray ionisation's success in coupling to HPLC arises from its capability of transferring the analyte to the MS under mobile phase conditions that promote analyte ionisation. This has proven helpful for identifying selenium peaks previously detected by HPLC-ICP-MS [45, 46]. Electrospray is also compatible with many mass analysers such as quadrupole, ion trap, double focusing and time-of-flight [47-49]. Tandem mass spectrometry, sometimes referred to as MS/MS or MS^2, is useful for amino acid, peptide and protein identification. The quadrupole rods optimise ion transmission based on pre-selected mass-to-charge ratios (*m/z)*. These ions continue to the hexapole collision cell for fragmentation. The fragmentation that takes place is usually achieved by collision-induced dissociation (CID). This approach utilises neutral atoms or gas molecules for collisional activation [50]. The product ions are then impelled through a time-of-flight unit before detection.

Time-of-flight mass spectrometry works on the principle of detecting the mass-to-charge ratio of ions accelerated at constant potential thus varying the time it takes to travel to the detector. Once ionised, an electric field, of known strength,

accelerates the ions. Any ions with the same charge will thus have the same kinetic energy. However, because the mass of the ions will differ, the velocity of the ions will vary since, for example, heavier ions with a certain charge will travel slower than ions of less mass but of the same charge. Additionally, if the ion has a higher charge state, this will result in increased velocity and shorter flight times. Consequently, the ions will travel the same distance but arrive at the detector at different times due to their different velocities. Therefore, the mass-to-charge ratio of the ions is calculated.

Identification and quantitative analysis of selenium compounds is also facilitated by Q-TOF-MS. This approach combines a quadrupole mass analyser with high performance time-of-flight technology, providing high mass accuracy and high resolution in both MS^1 and MS^2 modes capable of providing structure elucidation [21, 24]. Because the high resolution of these instruments provides isotope fidelity and <1ppm mass error, a molecular formula can be proposed and qualified.

The objective of the present study was to compare and characterise water-soluble extracts from commercially-available selenised yeast products. While yeast strains can have comparable total selenium concentrations and relatively similar amounts of SeMet, a simple extraction with deionised water reveals a multitude of different organoselenium metabolites. While other groups have carried out research to liberate and identify numerous selenocompounds from selenised yeast extracts [11, 19-21, 25] the present study has found a total of 153 selenium compounds that to date, to the author's knowledge, have not been reported previously.

4.2 Experimental

4.2.1 Instrumentation

Total selenium analysis was performed by ICP-MS. The SeMet analysis was carried out by HPLC-ICP-MS on an Agilent Zorbax RX-C8 4.6 × 250 mm (5 μm) column using an Agilent Technologies 1260 infinity series HPLC system connected to an Agilent Technologies 7700× series ICP-MS. A CEM Discover microwave equipped with an Explorer SP-D Plus 24/48 autosampler was used for sample extraction.

Determination of unknown organoselenium metabolites was investigated on two mass spectrometric instruments. The first was with a Dionex UltiMate 3000 RSLCnano system coupled to a Thermo LTQ OrbitrapXL. Separations were carried out on a

Thermo Accucore RP-MS C18 150 × 2.1 mm, (2.6 μm). The second MS utilised reversed-phase liquid chromatography-ESI-QTOF-MS analysis with an Agilent 1290 Infinity series High Pressure Liquid Chromatography system coupled to an Agilent 6545 quadrupole time-of-flight mass spectrometer. Separations were carried out on an Agilent rapid resolution HD Zorbax Eclipse Plus C8 column (2.1 × 150 mm, 1.8 μm). The mobile phase used in the HPLC separation coupled to the QTOF-MS was formic acid (>99 % HiPerSolv Chromanorm for LC-MS; VWR) in methanol and water (LC-MS Ultra Chromasolv; Honeywell). Frozen water extracts were lyophilised with a Christ Alpha 1-4 LD Plus freeze drier.

4.2.2 Chemicals and reagents

SELM-1 was acquired from the National Research Council of Canada and was included as a standard reference material (2059 ± 64 mg/kg Se, 3448 ± 146 mg/kg SeMet). Before analysis, four commercial yeast samples were randomly labelled as yeast product one to four (YP1-YP4). Total selenium and SeMet determination of each sample utilised nitric acid digestion and protease XIV enzymatic digestions respectively. Both reagents were acquired from Sigma [29, 31]. Deionised water (>18 MΩ cm) was obtained from an ELGA Purelab Flex S7 water system. The SeMet standard powder (>98 % by TLC) was obtained from Sigma. This was used to prepare a 100 mg/kg stock solution in 0.1 M HCl. Aliquots (0.1 mL) of this solution were frozen (-20 °C) and used fresh each day. The LTQ OrbitrapXL and QTOF-MS mobile phase utilised formic acid (>99 % HiPerSolv Chromanorm for LC-MS) supplied by VWR, with acetonitrile (ACN), methanol (MeOH) and water (LC-MS Ultra Chromasolv) all provided by Honeywell (LC and QTOF parameters were listed in Table 4.1). Agilent ESI-L tuning solution.

4.2.3 Sample preparation

4.2.3.1 Water extraction

Approximately 0.5 g of selenised yeast sample was weighed into a 50 mL centrifuge tube, and 5 mL of deionised water added. The sample was vortex mixed until homogenous (10 seconds) and shaken at 350 rpm for 15 minutes on an orbital shaker to complete a water extraction. This extract was centrifuged for ten minutes at 8,000 rpm. Filtration of the sample supernatant through Chromafil Xtra RC-20/25 0.2 μm

regenerated cellulose (RC) filters (Macherey-Nagel, Germany) was carried out into Amicon Ultra-15 3 kDa molecular weight cut-off (MWCO) membrane centrifugal filters (Millipore, Ireland) which were subsequently centrifuged at 4 °C for two hours at 4,000 rpm.

4.2.3.2 Lyophilisation of water extract

The yeast water extract was filtered as previously described. The resulting supernatant was frozen at -20 °C. Once completely frozen, the sample was placed into a Christ Alpha 1-4 LD Plus freeze drier. The lyophilised powder was stored at -20 °C until utilised for analysis.

4.2.3.3 Total selenium analysis

Sample preparation for total selenium analysis was similar to that used in previous studies [29]. Samples of selenised yeast (~0.2 g) were weighed into microwave vessels and digested in 10 mL HNO_3. The digests were made up to 50 mL with deionised water and diluted (1 in 10; 5 mL + 44 mL deionised water). Germanium internal standard (1 mL of a 5 mg/kg solution) was added, and analysis was carried out by ICP-MS. The ICP-MS used for selenium speciation included an octopole reaction system (ORS) for the introduction of H_2 to remove plasma-based polyatomic ions such as ArO and the Ar_2 dimer (molecular weight 80) [51], which interferes with the most abundant selenium isotope.

4.2.3.4 Selenomethionine analysis

SeMet analysis was carried out as previously reported [31]. Approximately 0.04 g of sample was mixed with protease enzyme solution (80 mg protease XIV in 0.5 mL of Tris buffer pH 7.5) and vortexed for 2 minutes. Samples were ultrasonicated for 25 seconds at 80 % amplitude. These samples were extracted for 15 minutes at a power output of 30 W. Extracts were centrifuged at 14,000 rpm for 3 minutes. The supernatant was transferred to a 15 mL centrifuge tube. The pellet was washed with water (0.9 mL) and vortex mixed until the suspension was homogenised. Samples were centrifuged again under the same previous conditions and the supernatant added to the 15 mL centrifuge tube. The volume was made up to 15 mL using deionised water and mixed well before removing an aliquot (2 mL) for filtration (0.2 μm) and dilution with

deionised water before analysis (injection volume 45 µL). The isocratic chromatography for this analysis had a mobile phase of 0.1 % TFA with 2 % MeOH in deionised water at a flow rate of 1 mL/min and the column temperature was set at 25 °C.

4.2.4 Set-up for HPLC-ICP-MS, LC-ESI-LTQ-OrbitrapXL and LC-ESI-QTOF-MS

4.2.4.1 HPLC-ICP-MS

This was previously described in Chapter 2, Section 2.2.3. However, selenocompound separations were performed on a YMC Triart C8, 4.6 × 250 mm (5 µm) as opposed to the Zorbax RX-C8 column. ICP-MS set up was the same as Chapter 2, Section 2.2.4.

4.2.4.2 LC-ESI-LTQ-OrbitrapXL

The LC system was a Dionex UltiMate 3000 RSLCnano system. The mobile phase was deionised water and ACN with a 60 minute run time. The analysis began with 5 % ACN for 5 mins followed by a constant gradient increase to 100 % over the remaining time. The injection volume was 20 µL and the flow rate was 50 µL/min. The chromatography column was a Thermo Accucore RP-MS C18 150 × 2.1 mm, (2.6 µm). Samples were cooled to 4 °C and the column compartment temperature was kept at 35 °C. The MS parameters were matched to that of work by Arnaudguilhem and co-workers and the scan range was between 100 and 900 *m/z* [20].

4.2.4.3 LC-ESI-QTOF-MS

Separations were carried out with an Agilent 1290 Infinity series LC system and utilised an Agilent RRHD Zorbax Eclipse Plus C8 (2.1 × 150 mm, 1.8 µm). Due to the success of the QTOF-MS (detection of >200 selenocompounds), the majority of the mass spectrometry analysis was carried out on this instrument and parameters were originally set to that of Arnaudguilhem *et al.* (Electrospay spray voltage = 3000 V; source and ion transfer tube temperatures = 300 and 350 °C, respectively) [20]. However, some optimisation with a SeMet standard was needed for this instrument and as such the LC and ESI-QTOF parameters are described in Table 4.1.

4.2.5 LC-ESI-OrbitrapXL-MS analysis

4.2.5.1 Fraction collection from HPLC prior to direct infusion

Selenised yeast samples for MS analysis were water extracted as described in Section 4.2.3.1. The supernatant was filtered through a 0.2 µm RC filter and diluted (1 in 50; 20 µL + 980 µL deionised water) for HPLC analysis. The samples were then injected onto a Zorbax RX-C8 column (4.6 × 250 mm, 5 µm) in an Agilent 1260 infinity series HPLC system. The mobile phase was increased from a 2 to 10 % MeOH gradient after 34 minutes. This gradient occurred over two minutes and was maintained at 10 % MeOH until the end (90 minutes). The flow rate was 1 mL/min and fractions were collected every minute for MS analysis. This resulted in 90 fractions per sample, across 5 selenium yeast products, resulting in 450 fractions. These fractions were flash frozen in liquid nitrogen and stored in the -20 °C freezer. When these samples were brought to the National Institute for Cellular Biotechnology (NICB) at DCU for LC-ESI-OrbitrapXL-MS analysis, it was discovered that the sample concentrations were too low for any substantial identification and were in the baseline noise range. To combat the low concentrations, freeze-drying was examined to concentrate the samples. Additionally, direct infusion of the sample was investigated without any previous separation via HPLC (see Section 4.2.5.2).

4.2.5.2 Freeze-drying to concentrate the sample before direct infusion

Direct infusion to the LTQ Orbitrap after concentrating the samples by lyophilisation, had sufficient sensitivity to detect compounds that were present. However, due to the complex matrix of yeast extracts, there were a lot of other compounds present which caused spectral interferences and made it difficult to detect any selenocompounds. To improve analyte separation and increase the chances of identifying selenium compounds, liquid chromatography was coupled to the mass spectrometer. Sample preparation for LC separations was kept the same as the sample preparation for the direct infusion analysis. After a water extraction, supernatants were filtered through a 3 kDa spin column to remove any large proteins or peptides. Freeze-drying was utilised to concentrate samples before MS analysis. The freeze-dried sample was resuspended in 500 µL deionised water and further diluted (1 in 3; 100 µL sample + 200 µL of deionised water) before injection into the LC system.

4.2.6 Water extraction for LC-ESI-QTOF-MS

Selenised yeast samples were water extracted (Section 4.2.3.1). This final supernatant was frozen and lyophilised. The lyophilised powder was stored at -20 °C and used to make a fresh sample for every analysis each day. The total freeze-dried powder was fully dissolved in 1 mL of deionised water for each sample and further diluted (1 in 5; 200 µL + 800 µL deionised water) before MS^1 analysis by LC-ESI-QTOF-MS. No further dilution was carried out for MS^2 analysis to avoid decreasing the concentration of the analytes before CID. Parameters for LC and also the MS are summarised in Table 4.1. Before making the experimental measurements, the QTOF was calibrated up to a mass range of 1700 *m/z* in both positive and negative polarity using a range of calibration compounds present in the Agilent ESI-L tuning solution (positive ions; 118.086255; 322.048121; 622.028960; 922.009798; 1221.990637; 1521.9714575; 1821.952313; 2121.933152; 2421.913990; 2721.894829; negative ions; 112.985587; 301.99819; 601.978977; 1033.988109; 1333.968947; 1633.949786; 1933.930624; 2233.911463; 2533.892301; 2833.873139). During the analysis two reference masses were continuously infused into the source, these were a purine (121.00509 *m/z*) and HP-921 (922.0098 *m/z*). The software monitored these masses and performed automatic mass correction during the data analysis. The acquisition mode for MS^1 included the *m/z* range 100-1,000 for organoselenium metabolites at a scan rate of one spectrum per second.

Table 4.1: LC and QTOF-MS parameters.

HPLC Parameters prior to QTOF-MS			
Liquid chromatograph	1290 Infinity series, Agilent		
Column	Agilent RRHD Zorbax Eclipse Plus C8 (2.1 × 150 mm, 1.8 µm)		
Mobile Phase	A: H_2O: FA (99.9: 0.1)		
	B: MeOH: FA (99.9: 0.1)		
Mobile phase flow (mL/min)	0.40		
Column Temperature (°C)	40 ± 0.8		
Gradient	Time (min)	A (%)	B (%)
	0	98	2
	5	98	2
	18	0	100
	20	0	100
	20.1	98	2
	23	98	2
Injection volume (µL)	1.00		
QTOF-MS parameters			
Ionisation method	Jet stream ESI		
Mode of operation	Positive		
Gas temperature (°C)	350		
Gas flow (L/min)	10		
N_2 nebuliser gas (psi)	60		
Sheath gas temperature (°C)	400		
Sheath gas flow	12		
Ion spray voltage (V)	3,000		
Nozzle voltage (V)	0		

4.2.7 Post-analysis data processing

4.2.7.1 Post HPLC-ICP-MS analysis

Once samples were analysed, the collected data were corrected for any constructive or destructive drift as per Chapter 2, Section 2.2.6. Adjustment for drift was carried out by analysis of an independent 120 µg/kg SeMet check standard that was tested every 6-8 samples. The samples were then corrected constructively or destructively based on the drift that this test sample underwent.

4.2.7.2 Post LC-ESI-LTQ-OrbitrapXL data processing

The Thermo Xcalibur software was employed to process the mass spectra data. Data acquisition was programmed to filter the most abundant compound based on its mass-to-charge ratio. Once isolated, by the first mass analyser, the parent ion was fragmented by CID to reveal an MS^2 fragmentation pattern. It was possible for the subsequent daughter ion to undergo CID also, producing an MS^3 spectrum for the granddaughter ion. This granddaughter ion could also be fragmented further to create MS^4 spectra. These spectra were only possible if there were sufficient counts detected after each fragmentation. Unfortunately, lack of sensitivity, low concentrations or poor ionisation of fragments of the compounds of interest prevented acquisition beyond MS^2. When the data were acquired for the most abundant ion, this compound was then excluded from the spectrum and the next most abundant compound was selected for fragmentation. This process continued until the injector syringe ran out of the sample and needed to be refilled for direct infusion and was subsequently repeated or until the end of the specified LC run time. Unfortunately, there was no automated way to identify selenocompounds on this software, so spectra were manually screened for a pattern based on the natural abundance of the selenium isotopes. Difficulties encountered with this analysis will be discussed later in Section 4.3.3.1.

4.2.7.3 Post LC- ESI-QTOF-MS

The data acquisition by LC-ESI-QTOF-MS was different to that of the OrbitrapXL. Firstly, the selenium compounds in the lyophilised yeast water extract were separated in the LC with a different column, the Agilent RRHD Zorbax Eclipse Plus C8 column (Table 4.1). The Agilent MassHunter Workstation software included two separate but beneficial programmes, Qualitative Analysis and the Isotope Distribution Calculator. Qualitative Analysis helped filter the mass spectra (MS^1 and MS^2). The accurate mass was utilised to apply fragment and compound composition based on the probability of matching with a theoretical compound. Another feature of this software was the ability to determine the overall elemental composition of the selenocompound with the aid of an elemental composition calculator. This option combined the accurate mass from MS^1 with the presence of one or two selenium atoms (determined by the selenium isotopic pattern) and a possible range (0-100 atoms) for C, H, O, N and S atoms to propose an elemental composition that best matched the selenocompound. Results were rated in

order of probability based on the difference between observed accurate mass and the theoretical mass of the proposed elemental composition. The ppm error was calculated using the following equation;

$$ppm\ error = \frac{(theoretical\ mass - accurate\ mass)}{(theoretical\ mass)} \times 10^6$$

Accurate mass = experimentally measured mass value
Theoretical mass = calculated mass based on the sum of atom masses in the proposed molecule (sometimes referred to as exact mass)

The Isotope Distribution Calculator presented a mass spectrum for the proposed compound or atom (see Figure 4.7 (a) and (b)). This tool was utilised to confirm the presence of selenium due to selenium's unique isotopic pattern. To reduce the number of compounds assigned for fragmentation, analyte ions were sent to the first mass analyser (MS^1) to determine the isotopic pattern for each parent ion. This was completed for each selenised yeast product and the spectra (>100,000; 1 spectra/compound) were recorded. Only samples with signal counts greater than 500 were selected for screening. Mass spectra of the MS^1 data were then filtered based on the presence of a selenium isotopic pattern. It was possible to distinguish between compounds that had one or two selenium atoms present due to their specific isotopic pattern (Figure 4.7 (a) and (b)). Regrettably, there was no automated approach to speed up this detection of selenium-containing compounds. Once a list of selenium-containing compounds was determined for all selenised yeast products, a targeted mass spectrometric approach was applied. The *m/z* for each selenocompound was recorded in the software and the compound ion went through the first mass analyser and was fragmented in the second mass analyser before proceeding to the time-of-flight module. Fragmentation patterns were analysed to propose an elemental structure, described in detail later.

4.3 Results and discussion

4.3.1 Total selenium and selenomethionine determination

Table 4.2 shows the analysis results for the SELM-1 certified reference material. The recovery of certified values for total selenium and SeMet were 98.8 ± 2.4 % and 101.1 ± 1.4 % respectively.

Table 4.2: Total selenium and selenomethionine results for SELM-1.

Sample	Mean Total Se (mg/kg)	S.D.	R.S.D.	Mean Se as SeMet (mg/kg)	S.D.	R.S.D.	Total Se as SeMet (%)	Recovery of Se from supernatant and pellet (% mass balance)
SELM-1	2035	49	2.4	1405	20	1.4	69.0	98.0 ± 0.2

Results are based on triplicate readings (n=3).

4.3.2 Purification of organoselenium metabolites

The first clean-up step for all yeast water extracts was filtration. The entire water extract was filtered with a 0.2 μm RC filter. Centrifugal filtration was investigated to remove any large proteins, polypeptides or other large matrix material. The molecular weight cut off of 3 kDa was selected as a means to reduce the sample preparation time and provide an alternative to size exclusion chromatography (SEC) fractionation and clean-up [20]. While this removed the possibility of fractionating the water-soluble yeast extracts into aliquots of varying selenocompound size and resulted in a more matrix rich filtrate, it prevented over dilution of the selenocompounds. An Amicon centrifugal unit was examined for ultrafiltration since it could hold volumes as high as 15 mL. Supernatants were analysed for SeMet content before and after filtration to determine if there was any loss of the analyte. The first water extract was not subjected to further filtration after the 0.2 μm filtration step. The second extract was applied to the 15 mL volume 3 kDa centrifugal unit.

The analysis of the filtrates was carried out by HPLC-ICP-MS. According to the literature, the primary organoselenium metabolite in selenised yeasts and their yeast water extracts is SeMet [13, 37]. This was confirmed in the present study, and as such, SeMet was monitored for any losses during sample handling. Therefore, the first supernatant extract (without any 3 kDa cut-off) was arbitrarily assigned a value of 100 % SeMet recovery. When the SeMet concentration was quantified in the post-MW

filtrate, the 15 mL volume 3 kDa filter yielded a ~86.3 % recovery. Furthermore, the sample chromatograms were overlapped (Figure 4.1) to verify that while there might be some recovery loss of the analytes, no apparent selenocompounds previously found by HPLC-ICP-MS were entirely lost after ultrafiltration. Yeast water extracts are known to be comprised of a complex mixture of salts and proteins [20, 52] which explained why some form of fractionation or filtration steps are needed. However, the approximately 14 % reduction in recovery of the main analyte, SeMet, provided some assurance that the method adequately removed large molecular weight interferences with minimal loss of already low concentration selenium compounds. This loss could be attributed to potential retention of analytes on the filter itself or blockage on the filter membrane caused by the complex, protein-rich water extract. There was approximately 300-400 µL (~10 % volume of the supernatant) of water extract left after centrifugation which could account for such loss. Lyophilisation was utilised for pre-concentration before analysis. This approach moved away from SEC, thus increasing the speed of sample preparation and decreased the number of steps needed to get a final lyophilised extract. Ultrafiltration steps were previously examined by other authors and recorded a considerable loss of selenium-containing compounds [20] which explained why SEC was a popular alternative [13, 19, 20]. The HPLC-ICP-MS analysis of a selenised yeast water extract with and without 3 kDa MWCO centrifugal filter is shown in Figure 4.1 with an enlarged inset illustrating sample complexity.

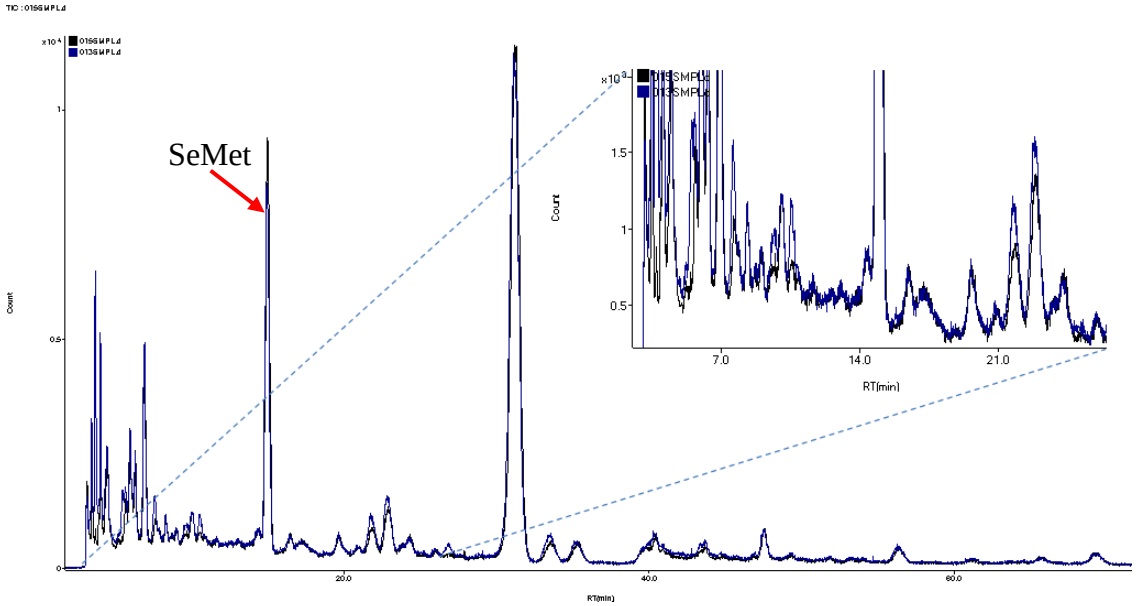


Figure 4.1: Over-lapped chromatogram of selenised yeast water extracts with and without 3 kDa MWCO centrifugal filter.
Mobile phase was 2 % MeOH and 0.1 % TFA in H_2O. (■) = with 3 kDa filter, (■) = without 3 kDa filter.

4.3.3 Identification of selenium compounds

4.3.3.1 LC-ESI-LTQ-OrbitrapXL

Direct infusion of samples involved an automated 500 µL syringe capable of injecting at flow rates between 3 to 25 µL/min into an OrbitrapXL MS with a standard ESI source. Electrospray ionisation conditions described elsewhere [20] were utilised for the analysis. The resolution was 100,000 with the *m/z* range examined between 100 and 900. A 10 mg/kg SeMet standard was investigated to determine if the ESI voltages successfully ionised the standard for detection by MS^1. The standard revealed the expected single selenium isotopic pattern (Appendix Figure A2). Fragmentation of this standard also matched previous fragmentation patterns for SeMet [47] and is included in Appendix Figure A3. Separations by LC were performed on a Thermo Accucore RP-MS C18 2.1 × 150 mm (2.6 µm), with samples prepared as previously described in Section 4.2.3. The complex yeast water extract matrix (consisting of proteins and peptides as previously mentioned) posed problems to adequately determine selenium isotopic patterns, evident from the overlapped chromatogram below, Figure 4.2, see Appendix Figure A4 to A11 for additional chromatograms.

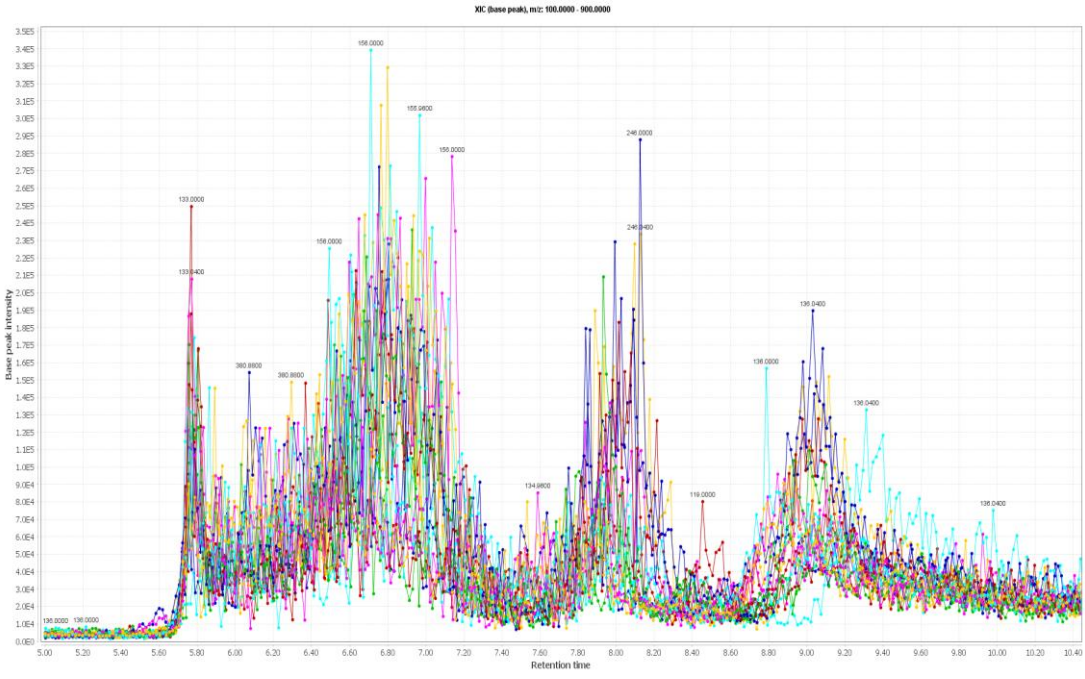


Figure 4.2: LC-ESI-OrbitrapXL-MS overlaid chromatogram of the yeast products.
Chromatograms shown were between 5.5 and 10 minutes. Individual chromatograms are available in the Appendix Figure A4 to A11.

However, some selenocompounds that were more abundant were detected based on the presence of the selenium isotopic pattern, see Figure 4.3 to 4.5 below.

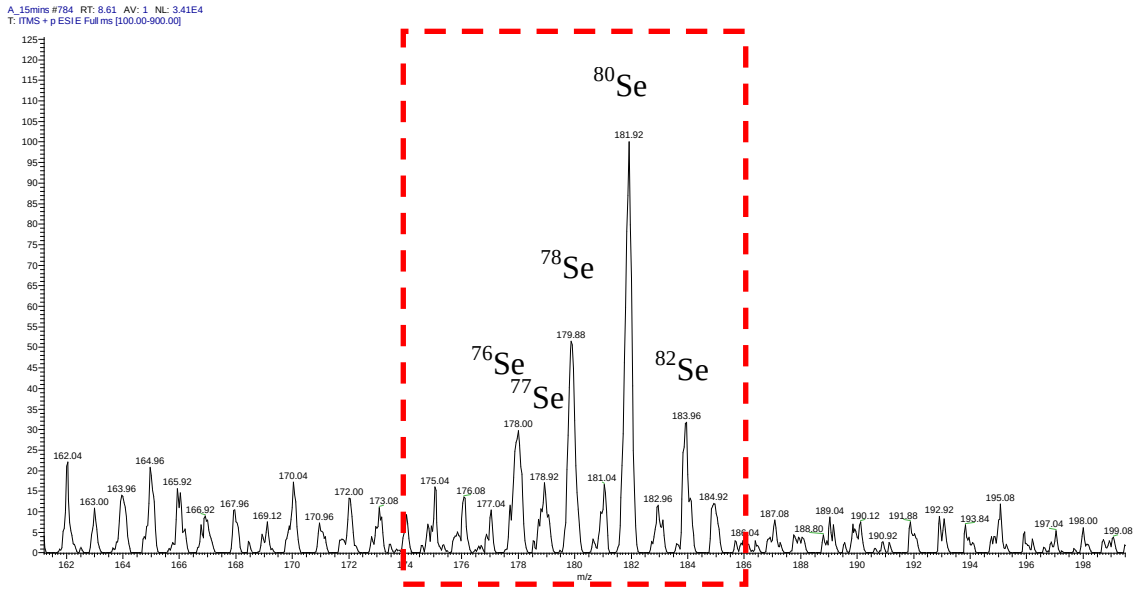


Figure 4.3: Mass spectrum of compound ion 181.92 *m/z* detected at retention time 8.61 minutes.
See Apendix Figure A12 for MS^2 spectra.

The ion at 181.92 *m/z*, Figure 4.3, was low in abundance but an MS^2 spectrum was acquired even though the compound ion was just above the baseline noise. Detection of the ion was possible since there were no other interfering peaks to mask it. Unfortunately, due to the mass accuracy of only two decimal places, the accurate mass was not good enough to propose elemental composition or structure for the compound ion 181.92 *m/z*. The more decimal places there are for the accurate mass determination, the easier it is to assign potential elemental composition. Typically, previous work has utilised instruments capable of between 4 and 5 decimal places for accurate mass determination [20-22, 25].

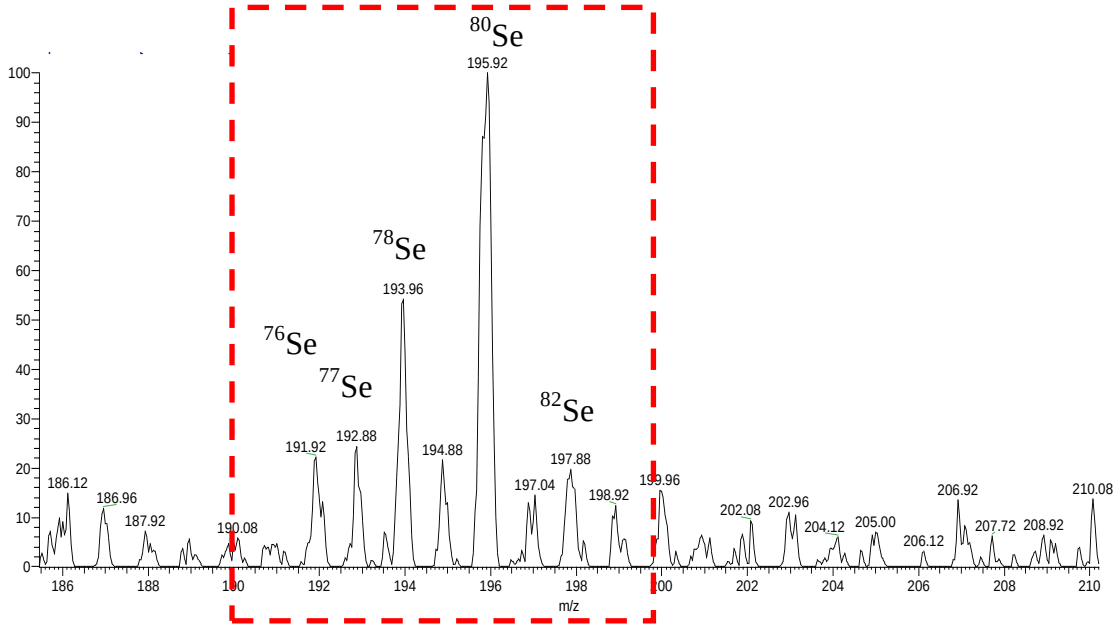


Figure 4.4: Mass spectrum of compound ion 195.92 *m/z* detected at retention time 9.47 minutes.

Similar to *m/z* 181.92, the selenium isotopic pattern for one selenium atom was detected in the ion at *m/z* 195.92. While it was not possible to categorically confirm the exact identity of *m/z* 195.92 from the accurate mass in MS^2 spectra, data from published research suggested the ion could be methyl-dehydrohomocysteine (see Appendix Figure A13) [20, 21].

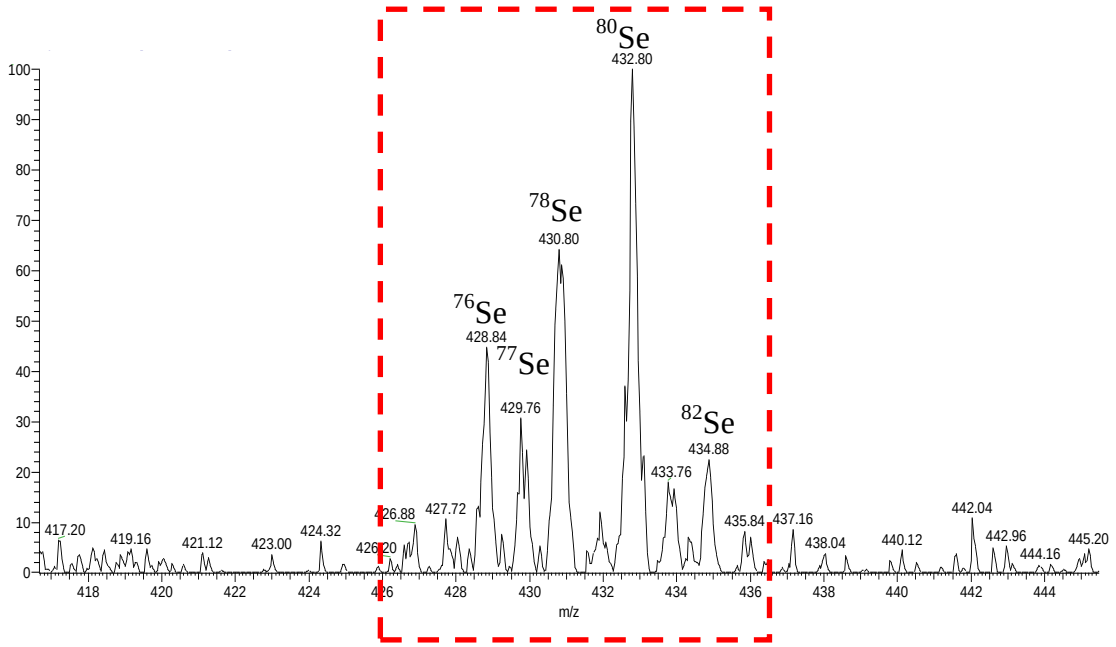


Figure 4.5: Mass spectrum of compound ion 432.80 *m/z* detected at retention time 8.58 minutes.

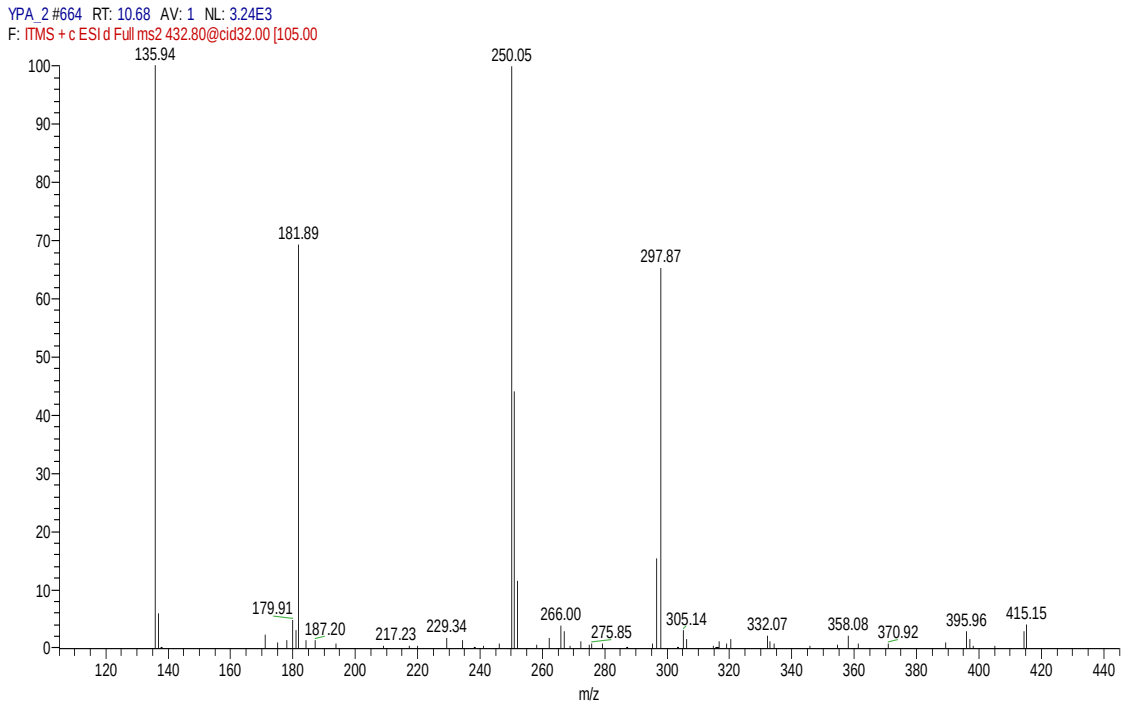


Figure 4.6: MS^2 spectra of ion 432.80 *m/z*.

Figure 4.5 and Figure 4.6 reveal the MS^1 and MS^2 spectra for ion *m/z* 432.80. The characteristic selenium isotopic pattern was evident in Figure 4.5. Confirmation of the presence of selenium was also evident from the change in the mass-to-charge ratio of the MS^2 spectra. Specific fragments decreased in *m/z* as MS^2 spectra were acquired for isotopes 76, 78 and 80 (see Appendix Figure A14-A16.). The accurate mass to two decimal places did not allow for identification. As the mass-to-charge ratio increased so too did the possibilities of ions that could match the particular fragment. Therefore, the smaller the molecule mass, the easier it is to try to identify it or at least propose a structure, especially if it contains a selenium atom of mass 80. Similarly, the larger the detected mass-to-charge ratio of a molecule, the more possibilities that could exist for the structure of that molecule.

4.3.3.2 LC-ESI-QTOF-MS

The benefits of the LTQ-Orbitrap MS allowed for the possibility of multiple fragmentation steps, as mentioned in Section 4.2.7.2. However, accurate mass sensitivity was only reliable to 1 or 2 decimal places. Unfortunately, this was not sufficient for identification. Therefore, having the potential for MS^2 to MS^4 capabilities was limited by this sensitivity. To build on this detection and identification work carried out on the LC-ESI-LTQ-OrbitrapXL, a more sensitive mass spectrometer system, the

LC-ESI-QTOF-MS, was investigated. This instrument provided a mass accuracy to 5 decimal places and therefore the possibility of proposing selenocompound compositions and structures based on the accurate mass. Selenium compounds were detected by a QTOF-MS which scanned for compounds between 100 and 900 Da. Qualification of a selenium-containing compound was based on the presence of the selenium isotopic pattern in the MS spectrum (Figure 4.7 (a) and (b)). By filtering the spectra based on these isotopic profiles, 213 selenium isotopic patterns were detected (see Appendix Figure A34), including 179 single selenium-containing compounds and 34 compounds with two selenium atoms per molecule. Sixty of those 213 were previously reported in the literature (Table 4.3) [11, 20-22, 25, 26, 53, 54]. Furthermore, the breakdown of novel organoselenium compounds specific to each product was summarised in Table 4.4. Yeast product 1 had 14 novel selenium compounds, YP 2 had 35, YP 3 had 17, YP 4 had 17 and SELM-1 had 5. However, of the remaining 153 unreported compounds, 17 were present in concentrations high enough to perform the MS^2 analysis. These compounds are summarised in Table 4.5. The elemental composition of these compounds was determined based on the MS^2 fragments (Figure 4.8 and 5.9). The accurate mass difference was below 1.6 ppm for all of the 17 compounds. Fragmentation patterns for the other 136 compounds were not considered due to low signal counts. However, the unique isotopic pattern of selenium was apparent throughout all of the spectra and was summarised in Appendix Figure A34. Furthermore, to the author's knowledge, these compound masses were never reported before in selenium-enriched yeast extracts.

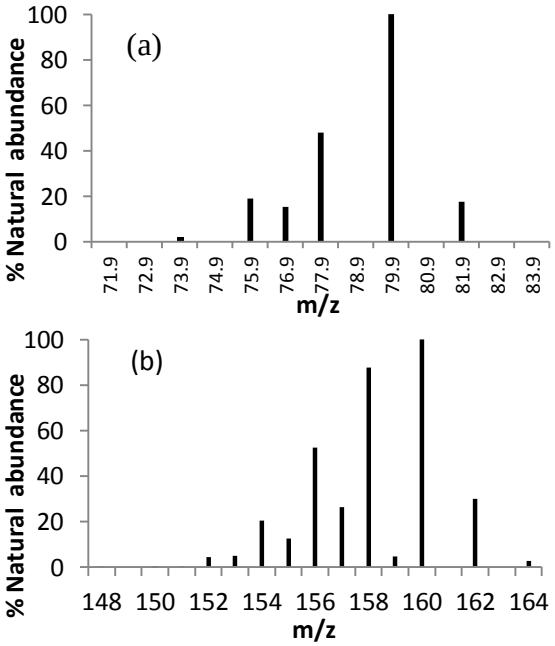


Figure 4.7: Relative abundance selenium isotopic patterns.
Isotopic patterns of a compound containing (a) one or (b) two selenium atoms, respectively (see also Figures 5.8 and 5.9).

Table 4.3: Selenium compounds previously reported in the literature (based on accurate mass and selenium isotopic pattern).

RT	Experimental *m/z*	Name	Elemental composition $(M+H)^+$	Theoretical *m/z*	Previous Experimental *m/z*	Y P1	Y P2	Y P3	Y P4	Y P5	
2.05	198.00290	Selenomethionine	$C_5H_{12}NO_2Se^+$	198.00278	198.00255	✓	✓	✓	✓	✓	[11, 20, 21]
6.13	272.00350	2,3-DHP-Se-methyl selenocysteine	$C_7H_{14}NO_5Se^+$	272.00317	272.0019				✓		[19, 22]
3.53	313.02990	γ-glutamyl-methyl selenocysteine	$C_9H_{17}N_2O_5Se^+$	313.02972	313.02972	✓		✓	✓	✓	[19-22, 24]
2.39	332.02550	Selenoadenosine	$C_{10}H_{13}N_5O_3Se^+$	332.02564	332.02550	✓	✓	✓	✓	✓	[20, 21]
7.92	345.02800	2,3-DHP-Selenolanthionine	$C_9H_{17}N_2O_7Se^+$	345.01965	345.01900			✓	✓	✓	[11, 20, 22]
8.42	345.00190	N-acetylcysteine-selenohomocysteine	$C_9H_{17}N_2O_5SSe^+$	345.00179	345.00222			✓	✓	✓	[20]
		N-acetylcysteine-selenohomocysteine	$C_9H_{17}N_2O_5SSe^+$	345.00179	345.00139			✓	✓	✓	[20]
		S-Se conjugate of N-acetyl-cysteine-selenomethionine	$C_9H_{17}N_2O_5SSe^+$	345.00179	345.00143			✓	✓	✓	[21]
9.33	346.04210	Selenomethyl-5'selenoadenosine	$C_{11}H_{16}N_5O_3Se^+$	346.04129	346.04123	✓	✓	✓	✓	✓	[21, 25]
1.21	359.03540	N-2,3-DHP-selenocystathionine	$C_{10}H_{19}N_2O_7Se^+$	359.03520	359.03506	✓		✓	✓	✓	[11, 19, 21, 22]
2.40	360.05600	Ethyl selenoadenosine	$C_{12}H_{18}N_5O_3Se^+$	360.05694	360.05617	✓					[20]
1.42	362.03650	5'-Se-methyl-oxide-selenoadenosine	$C_{11}H_{16}N_5O_4Se^+$	362.03620	362.03653		✓				[21]
9.34	362.03620	Seleno-adenosyl-Se(methyl)-selenoxide	$C_{11}H_{16}N_5O_4Se^+$	362.03620	362.03621	✓	✓	✓	✓	✓	[20]
3.41	370.05140	Methylselenoglutathione	$C_{11}H_{20}N_3O_6Se^+$	370.05118	370.05086	✓		✓	✓	✓	[19-22]
9.21	392.94700	N-acetylselenocysteine-selenohomocysteine	$C_9H_{17}N_2O_5SSe_2^+$	392.94649	392.94604		✓		✓		[20]
8.12	402.02350	Methylthioselenoglutathione	$C_{11}H_{20}N_3O_6SSe^+$	402.02325	402.0226	✓	✓	✓	✓	✓	[11, 20, 21, 35]
11.49	425.95830	S-Se conjugate of methylselenol-Se-methyl-5'-selenoadenosine	$C_{11}H_{16}N_5O_3Se_2^+$	425.95781	425.95874	✓	✓	✓			[21, 54]
2.42	433.07410	Seleno-adenosyl homocysteine	$C_{14}H_{21}N_6O_5Se^+$	433.07332	433.07349	✓	✓	✓	✓	✓	[11, 20, 21, 23, 25, 35, 45]
3.12	434.01390	2,3-DHP-selenocysteine-cysteinylglycine	$C_{11}H_{20}N_3O_8SSe^+$	434.01275	434.01275				✓		[11, 20, 21]
2.23	438.95230	2,3-DHP-selenocysteine-selenohomocysteine	$C_{10}H_{19}N_2O_7Se_2^+$	438.95129	438.95128				✓		[20]
3.09	438.95270	Se-Se conjugate of N-2,3-DHP-selenomethyl-selenohomocysteine	$C_{10}H_{19}N_2O_7Se_2^+$	438.95172	438.95309	✓	✓	✓	✓		[21]
7.32	448.02810	2,3-DHP-selenohomocysteine-cysteinylglycine	$C_{12}H_{22}N_3O_8SSe^+$	448.02874	448.02833		✓				[20]
2.40	449.06820	Seleno-hydroxy adenosyl homocysteine	$C_{14}H_{21}N_6O_6Se^+$	449.06823	449.06835					✓	[20, 25]
8.84	449.96820	Selenomethyl-selenoglutathione	$C_{11}H_{20}N_3O_6Se_2^+$	449.96771	449.9675	✓	✓	✓	✓		[20, 21]

Table continued

Table 4.3 continued

RT	Experimental *m/z*	Name	Elemental composition $(M+H)^+$	Theoretical *m/z*	Previous Experimental *m/z*	Y P1	Y P2	Y P3	Y P4	Y P5	
1.15	475.03960	Selenoglutathione-cysteine	$C_{13}H_{23}N_4O_8SSe^+$	475.03963	475.03975				✓		[11, 20]
1.46	489.05580	Glutathione-selenohomocysteine	$C_{14}H_{25}N_4O_8SSe^+$	489.05530	489.05476			✓			[20]
6.13	512.95193	Di-2,3-DHP-selenocysteine	$C_{12}H_{21}N_2O_{10}Se_2^+$	512.95211	512.95193		✓				[11, 20]
8.14	521.08960	N-2,3-DHP-selenoadenosyl-L-homocysteine	$C_{17}H_{25}N_6O_8Se^+$	521.08936	521.08929			✓		✓	[21]
8.83	526.96810	2,3-DHP-selenocysteine-2,3-DHP-selenohomocysteine	$C_{13}H_{23}N_2O_{10}Se_2^+$	526.96904	526.96812				✓		[20]
8.21	531.06580	Glutathione-N-acetylselenohomocysteine	$C_{16}H_{27}N_4O_9SSe^+$	531.06585	531.06548			✓	✓	✓	[20, 21]
2.50	532.06080	Glutathione-selenocysteinylglycine	$C_{15}H_{26}N_5O_9SSe^+$	532.06110	532.06052			✓	✓	✓	[11, 20, 21]
8.30	547.09930	γ-Glutamyl selenocysteine-γ-glutamyl cysteine	$C_{16}H_{27}N_4O_{10}SSe^+$	547.06076	547.06038	✓					[11, 20]
4.02	553.97900	γ-Glutamylcysteine-2,3-DHP-selenocysteine	$C_{14}H_{24}N_3O_{10}Se_2^+$	553.97866	553.97822				✓		[11, 20]
3.12	563.05630	Glutathione-2,3-DHP-selenocysteine	$C_{16}H_{27}N_4O_{11}SSe^+$	563.55680	563.05497	✓	✓	✓	✓	✓	[11, 20, 21, 26, 35]
7.40	577.07170	Glutathione-2,3-DHP-selenohomocysteine	$C_{17}H_{29}N_4O_{11}SSe^+$	577.07133	577.07062	✓	✓	✓	✓	✓	[11, 20, 21, 26]
3.13	585.03620	Glutathione-2,3-DHP-selenocysteine $[M+Na^+]$	$C_{16}H_{26}N_4O_{11}SSeNa^+$	585.03762	585.03789			✓	✓	✓	[21]
2.06	604.08280	Glutathione-γ-glutamylselenocysteine	$C_{18}H_{30}N_5O_{11}SSe^+$	604.08223	604.08164		✓		✓		[11, 20, 21, 26]
4.49	611.00050	Selenoglutathione-2,3-DHP-selenocysteine	$C_{16}H_{27}N_4O_{11}Se_2^+$	611.00013	610.99955	✓	✓	✓	✓		[11, 20, 21, 32]
8.01	625.01610	Selenoglutathione-2,3-DHP-selenohomocysteine	$C_{17}H_{29}N_4O_{11}SSe_2^+$	625.01578	625.01539				✓		[11, 20, 21]
8.10	637.09380	S-Se conjugate of glutathione-selenoadenosine	$C_{20}H_{29}N_8O_9SSe^+$	637.09379	637.09406		✓	✓	✓		[21, 25]
2.81	652.02660	Selenoglutathione-γ-glutamylselenocysteine	$C_{18}H_{30}N_5O_{11}Se_2^+$	652.02668	652.02631				✓		[11, 20, 21, 26]
2.50	661.10360	Selenoglutathione-glutathione	$C_{20}H_{33}N_6O_{12}SSe^+$	661.10304	661.10304			✓	✓	✓	[11, 20, 21, 35]
6.83	693.07670	Selenodiglutathione	$C_{20}H_{33}N_6O_{12}S_2Se^+$	693.07576	693.07518			✓			[11, 20, 21]
3.38	709.04860	Di-selenoglutathione	$C_{20}H_{33}N_6O_{12}Se_2^+$	709.04814	709.04768				✓		[11, 20, 21, 26]

YP = Yeast product 1 to 4. YP5 = yeast product 5 = SELM-1. RT = retention time in minutes. The presence of the selenocompound in each yeast product was denoted by a ✓.

Table 4.4: Previously unreported organoselenium compounds unique to each yeast product.

YP1		YP2		YP3		YP4		SELM-1	
RT	**$(M+H)^+$**	**RT**	**$(M+H)^+$**	**RT**	**$(M+H)^+$**	**RT**	**$(M+H)^+$**	**RT**	**$(M+H)^+$**
2.39	154.01370	6.48	206.96660	2.41	108.95500	7.19	244.99250	8.92	203.99010
9.33	210.98620	10.05	216.98760	2.42	135.96580	3.12	261.90580	11.42	235.96200
3.42	223.98220	9.44	222.00570	4.44	210.00300	1.93	274.53590	7.50	298.97120
5.34	265.00890	6.47	222.96160	4.15	210.00310	8.80	302.01460	4.48	358.00310
1.71	283.03060	11.49	242.98970	9.36	304.05630	5.88	314.05040	9.92	418.06260
6.64	292.01970	9.42	243.98480	18.6	313.67700	2.39	317.01370		
7.98	298.97080	11.90	249.00300	7.96	330.01040	8.14	326.99170		
10.89	299.02780	8.24	258.98714	9.73	384.98120	4.52	382.00100		
10.48	299.02950	3.70	272.98070	7.98	450.05220	2.12	383.95260		
7.50	336.09370	8.33	276.01370	2.42	471.02570	2.49	385.99400		
17.12	337.10620	11.34	286.99080	10.50	474.04450	2.14	414.04040		
1.55	354.10390	8.31	297.07104	9.92	474.04480	9.34	429.99380		
11.15	363.07950	8.39	343.02290	3.13	488.02300	2.08	522.06520		
2.40	388.05520	13.24	344.92538	10.69	488.06030	2.03	642.02990		
12.62	395.05200	10.31	373.90220	10.03	503.11520	2.49	699.05020		
2.05	467.97530	4.93	389.04730	7.10	530.12650	7.40	712.98690		
13.86	694.45780	15.00	409.11080	3.12	720.94950	2.03	739.99820		
		9.11	410.03400						
		4.93	411.02940						
		10.31	435.87250						
		4.95	443.02170						
		11.47	447.93990						
		10.44	448.01074						
		8.06	448.03598						
		7.23	452.96850						
		15.64	467.15260						
		10.19	468.07890						
		9.89	469.94820						
		7.42	476.03180						
		4.36	481.95742						
		4.32	505.05800						
		7.61	505.05800						
		8.43	546.00080						
		10.36	555.00050						
		10.28	604.08280						

YP = Yeast product 1 to 4. RT = retention time in minutes.

4.3.3.3 Similar selenium compounds

There were 13 organoselenium metabolites detected in all yeast products including the certified reference material SELM-1. These selenocompounds included some previously detected and identified compounds such as *m/z* 180.97606 which was potentially the loss of NH_3^+ from the dominant SeMet molecule [55], selenomethionine (*m/z* 198.00290), selenoadenosine (*m/z* 332.02550), selenomethyl-5'selenoadenosine (*m/z* 346.04210), seleno-adenosyl-Se(methyl)-selenoxide (*m/z* 362.03620), methyl-thioselenoglutathione (*m/z* 402.02350), seleno-adenosyl homocysteine (*m/z* 433.07410), glutathione-2,3-DHP-selenocysteine (*m/z* 563.05630) and glutathione-2,3-DHP-selenohomocysteine (*m/z* 577.07170). Additionally, novel selenocompounds, *m/z* 213.00278, 229.99362, 368.02295 and 407.96297 were detected in all selenised yeast samples. However, only selenocompound m/z 229.99362 had its elemental composition proposed due to MS^2 fragmentation. This composition was $C_6H_8N_5Se^+$ and potentially could be named as methyl-selenoadenine, a proposed molecular structure was presented in Appendix Figure A18 and Table A2.

4.3.4 Identification of elemental composition

The approach to determine the elemental composition of the most abundant previously unreported selenocompounds is portrayed in Figure 4.8. This particular approach was applied to the 17 selenocompounds that were selected for compound determination. While the compound information is summarised in Table 4.5, the complete data and figures are presented in the Appendix Figure A17 to A33 and Table A1 to A17.

Table 4.5: Elemental composition of novel selenocompounds detected in yeast water extracts.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δ ppm	RT (min)	YP1	YP2	YP3	YP4	YP5
218.01927	$C_7H_{12}N_3Se^+$	218.01910	0.78	3.96	✓		✓	✓	✓
229.99362	$C_6H_8N_5Se^+$	229.99394	-1.39	9.28	✓	✓	✓	✓	✓
275.02972	$C_{10}H_{15}N_2O_2Se^+$	275.02933	1.41	10.73	✓	✓	✓		
277.91972	$C_5H_{12}NO_2Se_2^+$	277.91930	1.51	7.74	✓	✓			
292.01968	$C_9H_{14}N_3O_3Se^+$	292.01949	0.65	6.56	✓				
326.99171	$C_9H_{15}N_2O_4SSe^+$	326.99123	1.47	8.04				✓	
336.09366	$C_{11}H_{22}N_5O_2Se^+$	336.09332	1.01	7.41	✓				
351.91930	$C_7H_{14}NO_5Se_2^+$	351.91969	-1.11	10.21	✓	✓	✓	✓	✓
379.05161	$C_{12}H_{19}N_4O_5Se^+$	379.05152	0.24	1.64	✓		✓	✓	
389.04729	$C_{12}H_{17}N_6O_4Se^+$	389.04710	0.49	4.76		✓			
390.03151	$C_{12}H_{16}N_5O_5Se^+$	390.03112	1.00	8.09	✓	✓	✓		
405.07904	$C_{13}H_{21}N_6O_4Se^+$	405.07840	1.58	3.09	✓				✓
442.03741	$C_{14}H_{16}N_7O_5Se^+$	442.03726	0.34	7.00		✓	✓		
452.96872	$C_{12}H_{17}N_6O_3Se_2^+$	452.96871	0.02	7.18		✓			
476.07956	$C_{15}H_{22}N_7O_6Se^+$	476.07913	0.90	8.06	✓				✓
490.03932	$C_{14}H_{24}N_3O_9SSe^+$	490.03930	0.04	7.79			✓	✓	
520.04977	$C_{15}H_{26}N_3O_{10}SSe$	520.04986	-0.17	7.48			✓	✓	

Δ ppm = ppm error. RT = retention time in minutes. YP = Yeast product 1 to 4. YP5 = SELM-1.

Separation was carried out by liquid chromatography and the separated peaks were then analysed by mass spectrometry. Once the selenium isotopic pattern, with either one or two selenium atoms per molecule, was detected, the *m/z* value for each selenocompound was recorded along with the spectra. This list of selenocompounds was then used for targeted screening. The sample was analysed once more and the selenocompounds were isolated in the quadrupole and fragmented by CID. The accurate masses of the fragment ions were determined by time-of-flight mass spectrometry. Detection of these fragments gave an insight into the possible structure of the selenocompound. Examples of compounds containing one or two selenium atoms per molecule are shown in Figures 5.8 and 5.9 for comparative purposes. These unique selenium isotopic patterns agree with the isotopic patterns described in Figure 4.7 (a) and (b).

With the aid of this information, the elemental composition of each compound was proposed. Determining the presence of one or two selenium atoms allowed a fixed value for selenium to be entered into an elemental composition calculator. This software created a list of potential results in compliance with established values [54]. While the

number of selenium atoms is important, filtering the maximum and minimum potential number of atoms and thus types of atoms further aids accuracy. Primary parameter selection in the software included C, H, O, N and S atoms, with the option of others and was previously described elsewhere [54]. When this software was combined with the accurate mass of the selenocompound, it helped remove various possible elemental compositions, leaving only a few probable outcomes. The summary of possible compositions was ranked by score and ppm error. The further the proposed *m/z* value was from the accurate monoisotopic mass, the larger the ppm error and the lower its score. The elemental composition calculator was also utilised for fragment composition and gave more confidence in the proposed elemental composition. The proposed structure and composition for all 17 selenocompounds were presented in the Appendix Figure A17 to A33 and Table A1 to A17.

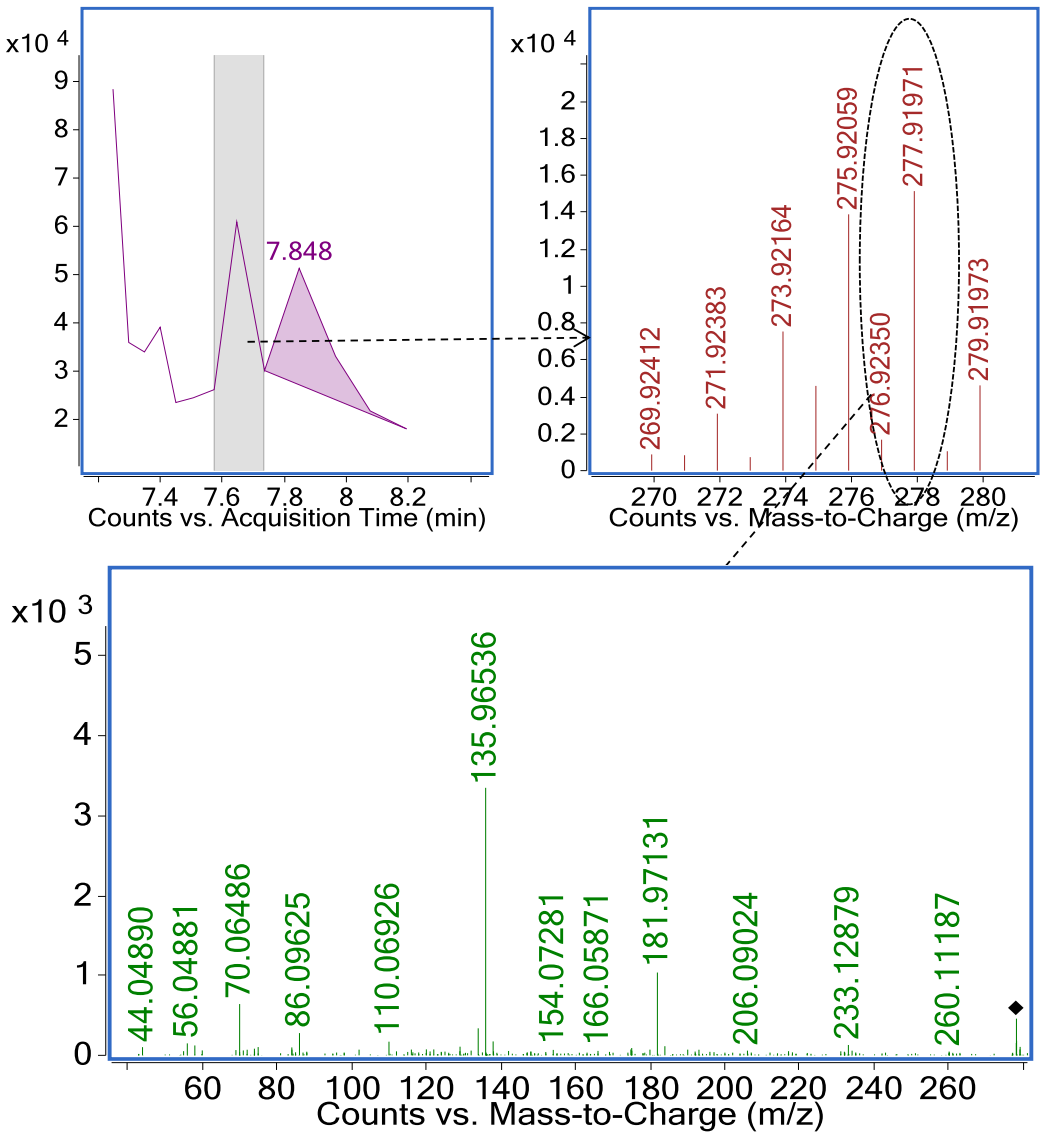
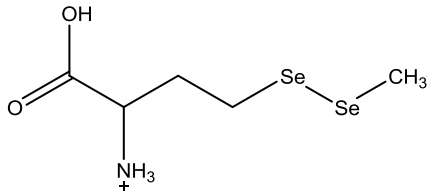


Figure 4.8: Chromatogram of *m/z* 277 selenocompound followed by MS[1] and MS[2] analysis.

Table 4.6: Elemental composition and proposed structure of *m/z* 278 based on exact mass, MS[2] and two selenium atom isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
277.91972	$C_5H_{12}NO_2Se_2^+$	277.91930	1.51	7.74	$C_4H_8NO_2Se^+$ (loss of CH_4Se)	181.97131 (-0.93)
					$C_3H_6NSe^+$	135.96536 (-4.71)
					$C_5H_{12}N^+$	86.09625 (-2.09)
					$C_4H_8N^+$	70.06486 (-3.85)

Δ ppm = ppm error. RT = retention time in minutes.

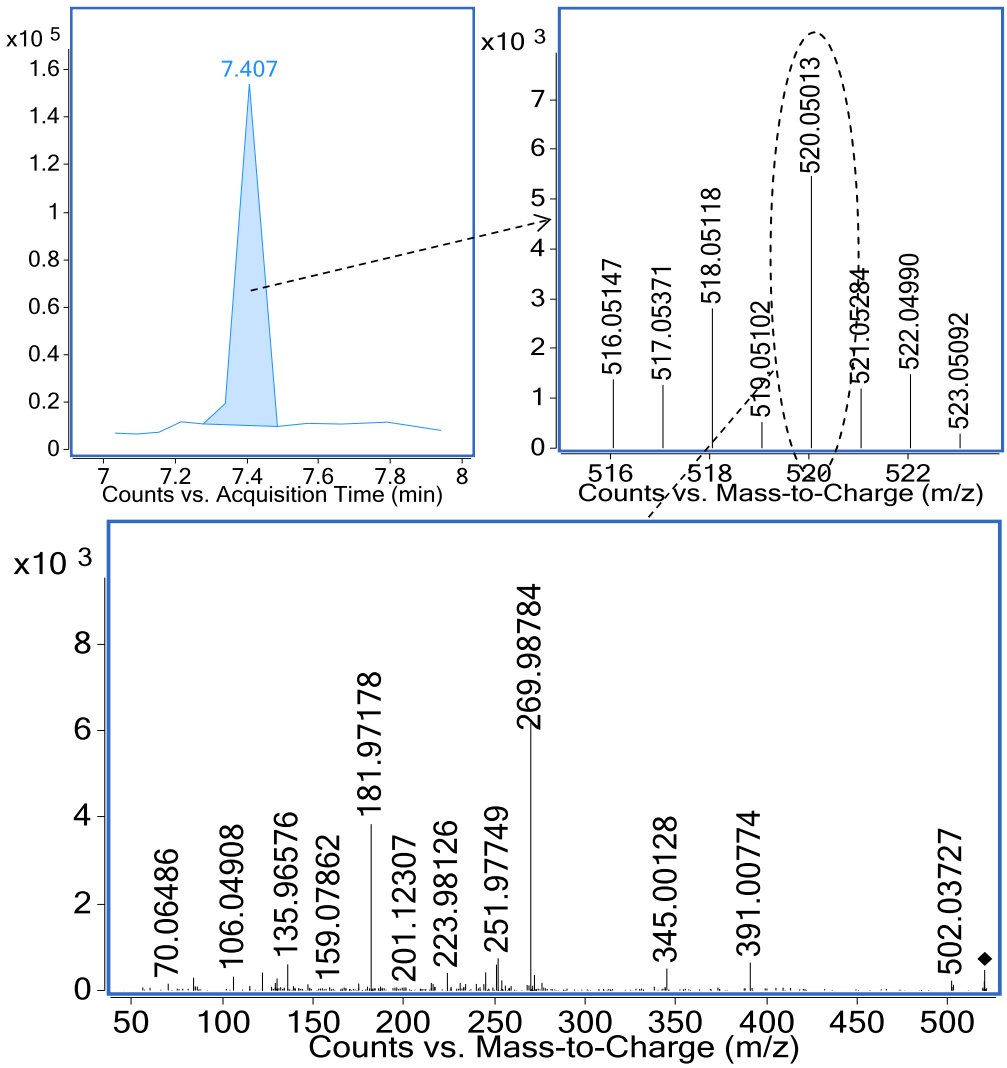
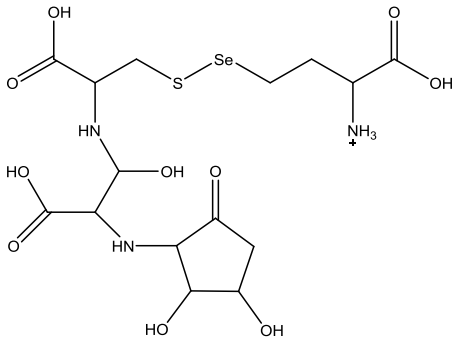


Figure 4.9: Chromatogram of *m/z* 520 selenocompound followed by MS[1] and MS[2] analysis.

Table 4.7: Elemental composition and proposed structure of *m/z* 520 based on exact mass, MS[2] and single selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δ ppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
520.04977	$C_{15}H_{26}N_3O_{10}SSe^+$	520.04986	-0.17	7.48	$C_{10}H_{19}N_2O_7SSe^+$ (loss of $C_5H_7NO_3$)	391.00774 (1.20)
					$C_9H_{17}N_2O_5SSe^+$ (loss of formic acid, N-acetylcysteine-SehomoCys frag)	345.00128 (-1.48)
					$C_7H_{12}NO_5Se^+$	269.98784 (1.19)
					$C_4H_8NO_2Se^+$ (SehomoCys frag)	181.97178 (1.65)
					$C_3H_6NSe^+$ (loss of formic acid from SehomoCys frag)	135.96576 (-1.77)
					$C_3H_8NO_3^+$	106.04908 (-7.45)

Δ ppm = ppm error. RT = retention time in minutes.

The structures of the 17 selenocompounds were proposed based on the following details.

m/z 218.01927

This compound was identified by its selenium isotopic pattern which indicated the presence of only one selenium atom. Two elemental compositions were plausible, $C_9H_{14}OSe^+$ and $C_7H_{12}N_3Se^+$. The overall elemental composition was proposed to be $C_7H_{12}N_3Se^+$ with an error of less than 1 ppm to that of the theoretical value. This composition was based on the MS^2 fragments, *m/z* 123.07947 ($C_6H_9N_3^+$) and *m/z* 108.05514 ($C_5H_6N_3^+$), which had approximately a 3 ppm and -4.44 ppm error respectively. Since the only proposed compositions for *m/z* 108.05514 contained nitrogen, this ruled out $C_9H_{14}OSe^+$. The proposed structure also took into account the loss of CH_3Se and coupled with the double bond equivalent calculation of at least 3 double bonds, an aromatic compound was favoured. See Appendix Figure A17 and Table A1 for corresponding data.

m/z 229.99362

Compound *m/z* 229.99362 was allocated an elemental composition of $C_6H_8N_5Se^+$ with less than 1.5 ppm error. The MS^1 spectra confirmed the existence of one selenium atom in the compound due to its isotopic profile. Therefore, the potential candidates were filtered based on an inclusion of selenium and also on the accurate mass. MS^2 analysis of this compound presented an adenine fragment ($C_5H_6N_5^+$) of *m/z* 136.06132 and an error of -3.31 ppm. This fragment agreed with previous detections by Arnaudguilhem *et al*. [20] and could only match the stated elemental composition. While there was a fragment ion of *m/z* 86.09696 (-6.16 ppm) which would correspond to $C_5H_{12}N^+$, this was not plausible with the current elemental composition. Upon closer investigation of the chromatographic and spectrometric data, there was another saturated compound also of *m/z* 229.9939 which eluted just before $C_6H_8N_5Se^+$ and was possibly responsible for this fragment. See Appendix Figure A18 and Table A2 for corresponding data.

m/z 275.02972

An MS/MS fragmentation produced ions of 181.09712 and 158.05985. The best match for the *m/z* 181.09712 fragment was $C_9H_{13}N_2O_2^+$, evident from a low ppm error of -0.17, while *m/z* 158.05955 had a ppm error of -1.20 for a composition of $C_{10}H_8NO^+$.

The mass difference between these ions was 23.03727 Da and corresponded to a sum loss of H_5NO and a gain of elemental carbon which equated to a theoretical mass of 23.03727 Da. Taking into account these fragments, coupled with a single selenium atom isotopic pattern, the proposed elemental composition was $C_{10}H_{15}N_2O_2Se^+$ with approximately a 1.4 ppm error. This *m/z* 181.09712 fragment also corresponded to a loss of CH_2Se (93.9326 Da) from the overall elemental composition and thus matched the proposed $C_{10}H_{15}N_2O_2Se^+$. These fragments helped to put forward the structure, Appendix Table A3 (Appendix Figure A19).

m/z 277.91972

This compound displayed the isotopic pattern for two selenium atoms (Figure 4.8; Table 4.6). With a relatively low mass, selenium accounted for approximately 57.5 % of the total compound mass thus reducing the elemental composition possibilities. Therefore, the only plausible outcome was $C_5H_{12}NO_2Se_2^+$ (1.51 ppm). Ions produced by MS^2 fragmentation included the selenohomocysteine residue 181.97131 ($C_4H_8NO_2Se^+$; -0.93 ppm). The detection of this ion accounted for the loss of CH_4Se from the proposed elemental composition since there had to be a second selenium atom according to the MS^1 spectra. The suggested structure can thus be elucidated as such fragments were recorded before by Dernovics *et al.* [19]. Further fragmentation of the selenohomocysteine residue resulted in a loss of formic acid and was confirmed by the presence of the *m/z* 135.96536 ($C_3H_6NSe^+$; -4.70 ppm) also previously detected by Dernovics *et al*. (Appendix Figure A20, Table A4).

m/z 292.01968

The ion responsible for fragment *m/z* 181.06097 was selected as $C_8H_9N_2O_3^+$ (1.11 ppm). The accurate mass loss from the overall compound was *m/z* 110.95871 and matched the single isotopic profile found in the mass spectra as it had to contain selenium. This accurate mass loss corresponded to CH_5NSe as the next option for a selenium-containing compound was over 100 ppm error away. Simple addition would suggest an overall elemental composition of $C_9H_{14}N_3O_3Se^+$ (-1.23 ppm). This was confirmed by calculating the composition based on the overall accurate mass. The double bond equivalent/ level of saturation for $C_8H_9N_2O_3^+$ was 5.5 and suggested the

presence of a ring structure. See Appendix Figure A21 and Table A5 for corresponding data.

m/z 326.99171

The isotopic profile of the compound indicated the presence of a single selenium atom. The MS/MS spectrum showed an ion at *m/z* 169.95368 and corresponded to $C_3H_8NSSe^+$ (-0.24 ppm) which was also detected by Arnaudguilhem *et al*. [20]. The inclusion of at least one sulfur atom was also confirmed with ion fragment *m/z* 74.00592 ($C_2H_4NS^+$; 0.32 ppm). Furthermore, a nitrogen atom and two oxygen atoms were also detected based on the ion fragment 126.05511 ($C_6H_8NO_2^+$; 1.23 ppm) When the presence of a single selenium atom was factored in with the accurate mass, in conjunction with the fragmentation data, an elemental composition of $C_9H_{15}N_2O_4SSe^+$ (1.47 ppm) was proposed. See Appendix Figure A22 and Table A6 for corresponding data.

m/z 336.09366

The MS/MS spectrum had two prominent fragment ions of *m/z* 277.04468 and 181.09662. A loss of a seleno-methyl group (CH_3-SeH) created the latter fragment ion. These ions were confirmed as $C_{10}H_{17}N_2O_2Se^+$ (-1.08) and $C_9H_{13}N_2O_2^+$ (-2.93), respectively. Fragment ion *m/z* 181.09662 was also previously detected in compound *m/z* 275.02972. With the MS spectrum only indicating the presence of a single selenium atom from its isotopic profile, the elemental composition was recorded as $C_{11}H_{22}N_5O_2Se^+$ with a ppm error of 1.01. See Appendix Figure A23 and Table A7 for corresponding data.

m/z 351.91930

Examination of the MS spectrum identified the selenium isotopic pattern for two selenium atoms. With a combined mass of 159.83249 Da, selenium alone accounted for ~45 % of the compound mass. The loss of H_2O from the SeCys ($C_3H_6NO_2Se^+$, 2.50 ppm) fragment ion *m/z* 167.95625 corresponded to ion *m/z* 139.96077 ($C_2H_6NOSe^+$, -1.00 ppm) and agreed with previously detected fragments by Arnaudguilhem *et al.* [20]. Additionally, the ion detected at *m/z* 255.97315 ($C_6H_{10}NO_5Se^+$, 5.00 ppm) corresponded to a 2,3 DHP SeCys fragment [20]. This all combined to confirm an elemental

composition of $C_7H_{14}NO_5Se_2^+$ (-1.11 ppm) and subsequently propose a structure for the compound. See Appendix Figure A24 and Table A8 for corresponding data.

m/z 379.05161

Fragment ions *m/z* 122.07124 and 123.07924 were separated by the mass of a single hydrogen atom and were calculated to be $C_6H_8N_3^+$ (-0.28) and $C_6H_9N_3^+$ (-1.15), respectively. Selenium was also calculated to be present in *m/z* 204.00338 - $C_6H_{10}N_3Se^+$ with sub-ppm error (-0.34).Based on its exact mass, fragment ions and a single selenium isotopic profile, only one formula was below 1 ppm error - $C_{12}H_{19}N_4O_5Se^+$ (0.24 ppm). See Appendix Figure A25 and Table A9 for corresponding data.

m/z 389.04729

A large adenine fragment ion was present, *m/z* 136.06114, along with a dehydroxy-ribofuranose fragment ion, *m/z* 97.02799. Both fragments were previously detected in other studies [20] and the ppm error was -4.63 and -4.33 respectively. Based on accurate mass, fragment ion *m/z* 162.96547 was confirmed as $C_5H_7OSe^+$ (-1.17 ppm). Similarly, *m/z* 218.95413 in the MS/MS spectrum was calculated to be $C_5H_5N_3O_2Se^+$ with a ppm error of -0.09 and a loss of –CO formed *m/z* 190.95940. One selenium atom was verified to be in the compound by the isotopic profile thus the elemental composition was established to be $C_{12}H_{17}N_6O_4Se^+$ (0.49 ppm). See Appendix Figure A26 and Table A10 for corresponding data.

m/z 390.03151

As previously encountered with *m/z* 389.04729, dominant adenine and dehydroxyribofuranose fragment ions were detected. The dehydroxyribofuranose fragment ion *m/z* 97.02843 gains a selenium atom to become *m/z* 176.94588. This selenium atom is the only one present in the compound and was confirmed by screening with the first mass analyser. Building the proposed structure was possible by confirming the elemental composition created by these fragments. The overall elemental composition was concluded to be $C_{12}H_{16}N_5O_5Se^+$ with a ppm error of 1. See Appendix Figure A27 and Table A11 for corresponding data.

m/z 405.07904

An MS/MS spectrum showed numerous fragment ions such as the heteroelement-free homocysteine residue $C_4H_8NO_2^+$ (*m/z* 102.05477) that were also detected by Dernovics *et al.* [19]. A loss of CO from fragment ion *m/z* 201.08711 ($C_8H_{13}N_2O_4^+$; 0.65 ppm) formed *m/z* 173.09186 ($C_7H_{13}N_2O_3^+$; -1.21 ppm). Furthermore, with an error < 1.3 ppm, the fragment *m/z* 155.08035 was confirmed to be $C_5H_9N_5O^+$. The mass spectrum was examined for the selenium isotopic pattern and confirmed one selenium atom was incorporated in the compound. Therefore, the selenium compound was determined to be $C_{13}H_{21}N_6O_4Se^+$ with a ppm error of 1.58. See Appendix Figure A28 and Table A12 for corresponding data.

m/z 442.03741

Only one selenium atom was present in this compound with confirmation based on the selenium isotopic pattern in the mass spectra. A large adenine fragment ion was present, *m/z* 136.06206, along with a dehydroxyribofuranose fragment ion, *m/z* 97.02899, and were also previously detected in other studies [20]. Other fragment ions included *m/z* 192.95067 ($C_4H_5N_2O_2Se^+$; -2.12 ppm) and 325.98896 ($C_9H_8N_7O_2Se^+$; -2.94 ppm). Uniting these fragments together allowed for structure elucidation and for the compound composition to be determined as $C_{14}H_{16}N_7O_5Se^+$ (0.34ppm). See Appendix Figure A29 and Table A13 for corresponding data.

m/z 452.96872

The isotopic profile of this compound highlighted the presence of two selenium atoms in the molecule. This accounted for a large part of the mass of the compound (~35 %). Combined with known fragment ions; *m/z* 181.97238 - selenohomocysteine fragment ($C_4H_8NO_2Se^+$; 4.95 ppm), *m/z* 135.96624 - loss of formic acid (CH_2O_2) from the selenohomocysteine fragment ($C_3H_6NSe^+$; 1.77 ppm) and *m/z* 56.04982 - loss of formic acid and selenium from the selenohomocysteine fragment ($C_3H_6N^+$; 6.14 ppm) [19] the elemental composition of the compound was implied to be $C_{12}H_{17}N_6O_3Se_2^+$ (0.02 ppm) and subsequent structure proposed. See Appendix Figure A30 and Table A14 for corresponding data.

m/z 476.07956

The previously documented selenohomocysteine ($C_4H_8NO_2Se^+$; -0.60 ppm) and adenine fragments ($C_5H_6N_5^+$; -1.98 ppm) were identified as *m/z* 181.97137 and 136.06150, respectively [20, 21]. Fragment ion *m/z* 224.97711 was calculated to be $C_5H_9N_2O_3Se^+$ (-0.80 ppm) and matched what was previously detected by [20]. There was one single selenium atom present and was confirmed by the selenium isotopic pattern. With all this information the overall elemental composition was determined to be $C_{15}H_{22}N_7O_6Se^+$ (0.90 ppm), and the fragment ions helped propose the structure of the selenium-containing compound. See Appendix Figure A31 and Table A15 for corresponding data.

m/z 490.03932

Characteristically, a single selenium atom was determined by its unique isotopic pattern. The fragment ions *m/z* 130.04954 ($C_5H_8NO_3^+$; -2.54 ppm), corresponding to the γ-Glutamyl fragment, and *m/z* 112.03957 ($C_5H_6NO_2^+$; 2.41 ppm) which was the loss of water from the γ-Glutamyl fragment. These fragments agreed well with previous studies by Arnaudguilhem *et al*. [20]. The presence of at least one sulfur atom was suggested by fragment ion *m/z* 74.00563 ($C_2H_4NS^+$; -3.65 ppm). The fragment ion *m/z* 257.96888 had a mass difference of exactly 127.91934 Da from the γ-Glutamyl fragment (*m/z* 130.04954) which was a loss CH_4SSe (0.00 ppm). Therefore, ion *m/z* 257.96888 was determined as $C_6H_{12}NO_3SSe^+$ and agreed with elemental composition calculations (-3.41 ppm). Other fragment ions *m/z* 214.92697 (-2.75 ppm) and 360.99606 (-1.80 ppm) were determined to be $C_4H_7O_3SSe^+$ and $C_9H_{17}N_2O_6SSe^+$, respectively. Combined with the accurate mass of the compound and the previously identified and unidentified fragments, an overall elemental composition of $C_{14}H_{24}N_3O_9SSe^+$ (0.04 ppm) was suggested, along with proposed structure elucidation. See Appendix Figure A32 and Table A16 for corresponding data.

m/z 520.04977

The isotopic profile detected by the first mass analyser clearly represented a compound with a single selenium atom (see Figure 4.9; Table 4.7). However, the larger the compounds, the harder it is to determine the elemental composition and there were over 25 possible compositions with <5 ppm error. Fragmentation patterns become even more

important under these circumstances. Fragment ion *m/z* 345.00128 was proposed to be $C_9H_{17}N_2O_5SSe^+$ (-1.48 ppm) and corresponded to N-acetylcysteine-selenohomocysteine which was also identified by Preud'homme *et al*. [21] Additionally, the fragment ion *m/z* 269.98784 was determined to be $C_7H_{12}NO_5Se^+$ (1.19) and was detected by Dernovics *et al*. [19]. Other fragment ions which were previously detected in this study and by Arnaudguilhem *et al*. [20] were ions *m/z* 181.97178 ($C_4H_8NO_2Se^+$; 1.65 ppm) and 135.96576 ($C_3H_6NSe^+$; -1.77 ppm) which represented selenohomocysteine and the loss of formic acid from the selenohomocysteine fragment, respectively. Finally, the fragment ion *m/z* 106.04908, also detected by Arnaudguilhem *et al*, was calculated to be $C_3H_8NO_3^+$ (-7.45 ppm) and corresponded to the 2,3-DHP and NH_3^+ fragment. All of this information combined to suggest the elemental composition of $C_{15}H_{26}N_3O_{10}SSe^+$ (-0.17 ppm), (see Appendix Figure A33, Table A16).

4.4 Conclusion

Two types of mass spectrometer instrument were utilised in the course of this study for the purpose of identifying selenocompounds across different commercially-available selenised yeast. These were the LTQ-Orbitrap from Thermo and the QTOF-MS from Agilent. Due to the complex nature of the sample matrices and also sensitivity issues caused by low analyte concentration, identification by Orbitrap was unsuccessful while the QTOF-MS instrument allowed for the detection of >200 selenocompounds. While it was difficult to determine the isotopic pattern of parent ions from MS^1 data with the Orbitrap, the QTOF-MS, through its software, recorded every isotopic pattern for every parent ion it detected, across the specified mass-to-charge range. This allowed each ion to be individually screened for the selenium isotopic pattern. Although this was a manual screening process, the Thermo software did not allow for this capability. Therefore, the overall MS^1 spectra generated by the Orbitrap had to be visually inspected and manually recorded for any isotopic patterns. Unfortunately, there were also multiple ions detected in the Orbitrap MS^1 spectra, thus making it more a challenging process to determine selenium isotopes. However, the Orbitrap had the capabilities to investigate down to the MS^4 spectra if required, while the QTOF-MS was limited to MS^2.

The majority of selenocompound identification was carried out by using data from the QTOF-MS. There were 213 organoselenium metabolites detected by the

unique single (179) or double (34) selenium isotopic ratio. To the author's knowledge, 153 were previously unreported selenium-containing species. Elemental compositions were identified for 17 of the 153 compounds due to accurate mass and MS^2 capabilities. A total of 136 of these species were too low in concentration (~500 counts) to perform MS/MS analysis. Future developments may overcome this difficulty and allow for further investigations to continue. When the 153 compounds were grouped by selenised yeast product, the results suggest that there were more differences than similarities between them. YP 1 had 17 novel selenium compounds, YP 2 had 35, YP 3 had 17, YP 4 had 17 and SELM-1 had 5. There were only 13 selenium-containing compounds, including SeMet, common to all five yeast products. Elemental composition and identification were previously determined for 8 of those 13 selenocompounds [11, 20, 21]. It was possible to propose an elemental composition for one of the remaining four new selenocompounds.

Having so many differences in selenocompound distribution between these yeast products highlights the plausibility of varying efficacy under physiological conditions. The 153 novel selenocompounds detected in this current study will have varying chemical structures. These different structures will behave very differently during digestion processes *in vivo*. While the investigation of these physiological functions was outside the remit of this study, these selenocompounds could be investigated in the future for potential efficacy. Therefore, the importance of selenium speciation analysis is clearly evident, especially when marketing selenised yeast products for commercial markets. Different selenium-enriched yeast products, produced by similar methods using different yeast strains result in a variety of selenocompound compositions [11]. The analysis of aqueous extracts of these products allowed for the identification of a vast number of hydrophilic selenium-containing compounds without the need for SEC or other preparation steps. Therefore, it is possible to use this as a fingerprinting tool to make comparisons between commercially-available selenised yeast products and this work has potential for other applications such as product reproducibility investigations.

4.5 References

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Chapter 5: Final conclusions and future work

The focus of the study was the development and validation of extraction and analytical methods for speciation analysis of commercially-available selenium-enriched yeast products. It is a common misconception that all selenised yeast products are the same especially if the yeast products have similar total selenium content. Improved analytical and extraction capabilities allowed quantification of the dominant organic selenoamino acid, SeMet, but also of different organoselenium species. These analytical speciation improvements served to reveal significant selenocompound differences across the yeast products.

A primary goal for SeMet determination was to reduce extraction and analysis times. Before this research began, the method of choice involved three sequential proteolytic extractions. All three enzymatic extractions took ~17 hours each. Inherently, this three day extraction was protracted for a high sample throughput industrial setting. Therefore, one of the initial aims of this study was to develop an accelerated extraction method for SeMet analysis. A HPLC-ICP-MS method was employed for separation and detection of the SeMet analyte. The instrument was employed through every phase of the research either directly or in a supporting role and allowed for detailed quantitation of SeMet in supplements. The feed supplement in this study was selenium-enriched yeast. To accelerate the SeMet extraction process, enzymes, microwave energy and sonication were investigated.

Incomplete SeMet recoveries by microwave digestion were improved upon by modification with sonication. Microwave assistance was employed for SeMet extraction and worked in conjunction with enzymes. A combination of protease XIV and driselase was assessed due to the previous success of the enzyme cocktail to liberate intracellular SeMet from selenium-enriched yeast [1]. An assessment was made of the microwave power output parameters over varying times. The conditions that gave the best recovery of SeMet were 30 W of fixed power for 15 minutes.

Examination of a sonication method utilised an ultrasonic probe capable of sonicating minimal sample volumes (500-1,000 µL). Sonication (25 seconds at 80 % amplitude) of the yeast/enzyme solution in conjunction with microwave extraction gave recoveries of ≥95 % SeMet for the certified reference material, SELM-1. The results suggested that protease XIV, on its own, with no other enzyme was sufficient to extract SeMet from selenised yeast. When applied to the CRM, the SeMet recovery was ≥95 % (1326.3 ± 47 mg/kg Se as SeMet). Furthermore, by decreasing the time before analysis,

oxidation was minimised, thus allowing more accurate and precise determination of SeMet from selenised yeast. The extraction and analytical method for SeMet determination were subsequently validated.

Validation employed the ICH guidelines for analytical procedures [2]. The analytical validation protocol examined specificity, linearity, range, accuracy, precision, repeatability, limit of detection (LOD) and limit of quantification (LOQ). The measured SeMet content of the certified reference material (SELM-1), by the accelerated enzymatic extraction, agreed well with the certified value (100.9 ± 0.7 % recovery). Therefore, the methods described are suitable for accurate and precise determination of SeMet in selenium-enriched yeast. This research culminated in the publication of the procedure in a peer-reviewed journal [3].

Although the development of an accelerated and validated enzymatic extraction for the determination of SeMet was a primary goal of this study, a second validated chemical extraction method was also developed as it enabled the avoidance of enzyme use and any variation that may be caused by enzyme activity fluctuations. Chemical extraction of selenium-enriched yeast products involved a lengthy 8-16 hour reflux with methanesulfonic acid. To accelerate the chemical reflux extraction, microwave energy was explored once more.

The success of previous microwave chemical extractions (2 hours) demonstrated its potential to accelerate SeMet extraction from selenised yeast. Therefore, the technique was applied to the development of an accelerated chemical extraction. Methanesulfonic acid successfully extracted SeMet from selenised yeast [4-6], thus it was selected for further investigation. Acid concentration, time and temperature were all examined to determine the conditions that would liberate ≥95 % of the certified value of SeMet from SELM-1. A closed vessel microwave chemical extraction allowed temperatures inside the microwave vessel to reach 225 °C without any venting which could potentially lead to some sample loss. Ultimately, after developmental progress, the chosen conditions (4 mL of 4 M MSFA with 0.04 g of selenised yeast extracted for 10 minutes at 200 °C) were validated as per ICH guidelines and reliably recovered 100.2 ± 0.1 % (1392.78 ± 1.4 mg/kg Se as SeMet) SeMet from SELM-1. Comparisons of SeMet results from both the accelerated chemical and enzymatic microwave extractions showed no significant difference ($p \geq 0.05$). Therefore, the accelerated

chemical extraction procedure for SeMet determination was accurate, precise and could be applied to any yeast strain.

With the ability to rapidly quantify SeMet from selenised yeast, the focus of the study moved towards the fractionation of SeMet present in water extracts of four commercial selenised yeast products (YP1-YP4) and a selenised yeast certified reference material, SELM-1. The total SeMet content in selenised yeast is well documented, thus the water-soluble SeMet fraction was investigated next. Once the water-soluble fraction of the commercial selenised yeast products was extracted, the newly developed enzymatic method was applied to determine the free, peptide-bound, total water-soluble and chiral SeMet contents in the water extracts. The purpose of this work was to apply a simple water extraction to selenium-enriched yeast to show what variation existed in the free and peptide-bound SeMet contents of selenised yeast products. Additional comparisons of chiral SeMet enantiomers across the water-soluble extracts of commercial yeast products indicated significant differences existed and that L-SeMet was the favoured enantiomer. However, while L-amino acids are expected to be the dominant enantiomer, D-SeMet was also present yet variable across the different selenised yeast products. Speciation results showed that the products differed in free SeMet and peptide-bound SeMet composition, but also it was possible to discriminate between commercial selenium-enriched yeast products by their unique selenocompounds.

As part of this thesis, a study was conducted into the selenocompounds extracted by water from selenium-enriched yeast. This work detected ~30 selenocompounds by HPLC-ICP-MS, but compound independent calibration (CIC) also showed that the quantities varied widely with some selenocompounds present only in certain products or batches. The findings of this experimental work illustrated the benefits of selenium speciation and the differences between yeast strains on the market. However, it also highlighted the numerous other selenocompounds, aside from SeMet, that were liberated from a simple water extraction.

Further investigations on the water extracts were completed using more powerful analytical instrumentation such as liquid chromatography-electrospray ionisation-quadrupole time-of-flight-mass spectrometry (LC-ESI-QTOF-MS). Ultrafiltration and lyophilisation were employed as a sample cleanup and preconcentration step. Ultrafiltration only allowed the isolation of compounds ≤3 kDa in

size. Additionally, this approach would avoid size exclusion chromatography which could over-dilute the selenium species. Unique selenocompounds were found in all yeast products tested. This included 17 selenium compounds exclusive to yeast product 1, 35 in yeast product 2, 17 in yeast product 3, 17 in yeast product 4 and 5 unique selenocompounds in the certified reference material, SELM-1. Additionally, there were 179 selenocompounds detected by the unique single selenium isotopic pattern and 34 by the selenium isotopic pattern created by the presence of two selenium atoms. Elemental composition was identified for 17 of the 153 previously unreported selenocompounds due to accurate mass and MS^2 capabilities. This information was utilised to propose structures for the discovered selenocompounds. These compounds were the first of their kind to be reported in selenised yeast research, clearly demonstrating the wide and various differences between commercially-available yeast products.

In conclusion, two new accelerated extraction methods were developed for the determination of SeMet in selenised yeast. These methods utilised both enzymatic and chemical approaches. There was 98.8 % agreement between SeMet recoveries when commercially-available samples of selenium-enriched yeast were extracted using each of these methods. The enzymatic hydrolysis was applied to water extracts of commercial yeast products and helped determine the fractionation of SeMet into free, peptide-bound and total water-soluble SeMet. A direct HPLC enantioseparation method was applied for chiral analysis of the water-soluble SeMet enantiomers from selenised yeast. This direct HPLC assay avoided the need for derivatisation and supplied further details about the water-extractable fraction of selenised yeast. Finally, using ultrafiltration as a preparative step, the water-extract from commercial selenised yeast products were investigated for novel and unique compounds. Over 200 selenium-containing compounds were detected, of which ~153 were reported for the first time. Proposed structures for 17 of these novel compounds were possible due to LC-ESI-QTOF-MS capabilities. The method could be utilised to distinguish novel selenium biomarkers between commercially-available selenised yeast products and possesses the potential for product batch-to-batch reproducibility and process consistency investigations. The method may also be applied for product characterisation and discrimination studies as demonstrated throughout this research.

5.1 Future Work

Figure 5.1 visually represents the work carried out throughout this Ph.D. thesis. This guide should make it easier to understand the flow and results of the thesis.

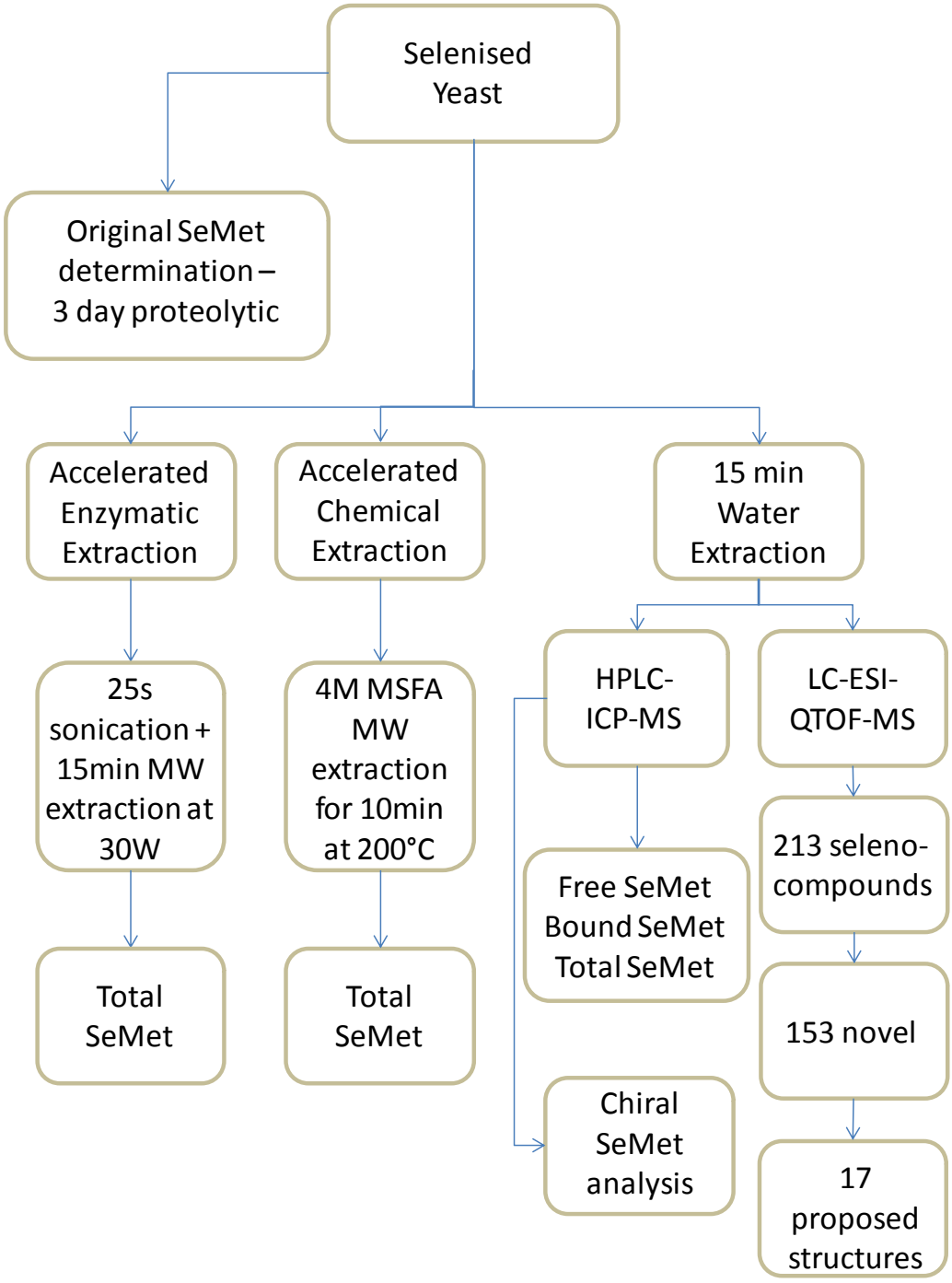

Figure 5.1: Flow chart summarising work carried out throughout this Ph.D. thesis.

To build on the discoveries found in this research, future studies could explore any avenue of the chiral and novel selenocompound results. Identification of the remaining novel selenocompounds would be the first challenge before chiral or selenocompound efficacy studies could begin. The primary selenoamino acid of interest throughout this PhD research was SeMet. However, as mentioned throughout the chiral analysis, numerous other chiral selenoamino acids exist, with some containing more than one chiral centre [7]. Some of the L-conformations of these amino acids were detected in selenised yeast compounds before, thus future research could also investigate for the presence of the D-conformation.

The novel selenocompounds detected have the potential to be investigated for efficacy and benefits such as possible antioxidant, anti-carcinogenic or even anti-Alzheimer characteristics. Prior to any investigations, the elemental composition and identification of these selenocompounds will be a considerable challenge. Only then could their synthesis be possible which could lead to studies of their relative stabilities and the kinetics of their uptake and mechanism. However, these interesting novel selenocompounds have the potential to be exploited for health benefits in the human and animal supplement industry but also in the area of medicine. Therefore, the discovery of these selenium-containing compounds could be investigated further. While it was not possible to draw conclusions about efficacy, there were some selenocompounds that were composed of beneficial functional groups or compounds that had been previously documented and detected [8-13]. An example of these would be adenine and adenosine fragments detected by MS^2 analysis. These fragments were found in the proposed structures of 5 of the 17 novel selenocompounds. Adenine is essential for the formation of nucleotides and binds with thymine to create the A-T base pair in DNA. Adenosine is also well known for its composition in adenosine triphosphate (ATP) which supplies energy to biological processes via phosphorylation.

Another finding of this thesis was the considerable variation of selenocompounds between commercial selenium-enriched yeast products. No two yeast products were the same in their selenocompound composition (Chapter 4, Table 4.3). While it was reported that some methylated selenocompounds have anti-carcinogenic properties [14, 15], another dominant property was protection against oxidation which can be provided by glutathione peroxidases where glutathione acts as a reductant [8, 9].

Twenty such glutathione containing compounds that were previously detected were also found in these yeast products. However, the presence of these compounds was not common to each selenised yeast product. Therefore, it raises the question of which selenised yeast products might be more efficacious in terms of specific biological processes such as anti-oxidative effects. These compounds could be examined in future studies to determine possible health benefits.

This PhD research has provided an insight into the benefits of selenium speciation. The information and data provided in this study highlighted how susceptible selenomethionine was to oxidation and also how simple it was to extract selenomethionine and other organoselenium species from yeast via a water extraction (~10-25 % of total selenium in selenised yeast). With such a large amount of selenium liberated from a water extraction, future work could also look at the yeast fermentation process and examine any secondary outputs or waste for selenium and selenocompound content. The yeast itself is grown in a selenium-enriched environment and numerous questions remain to be addressed to see if the cells lose SeMet or other selenocompounds to the media or washing steps.

5.2 References

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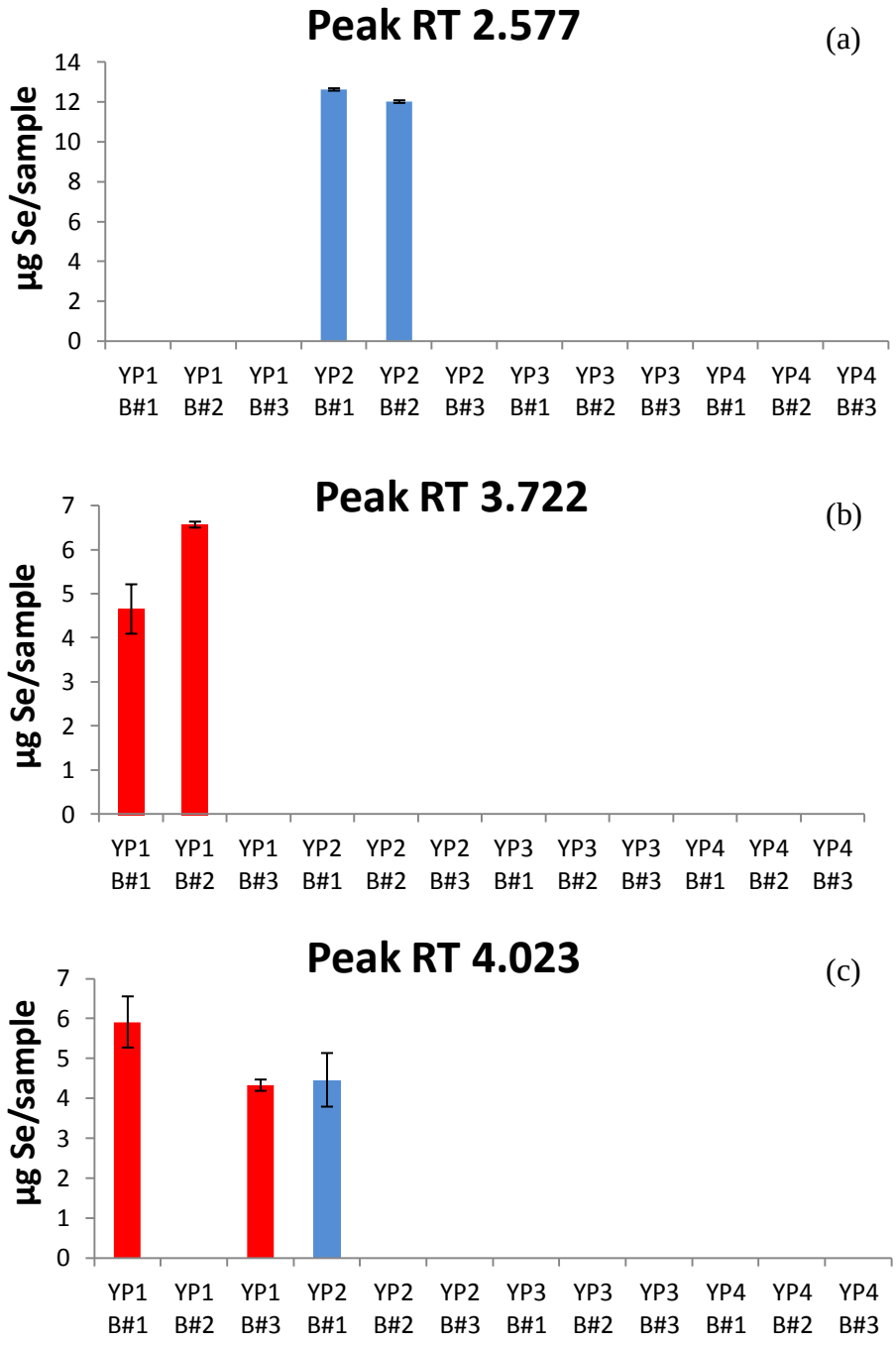
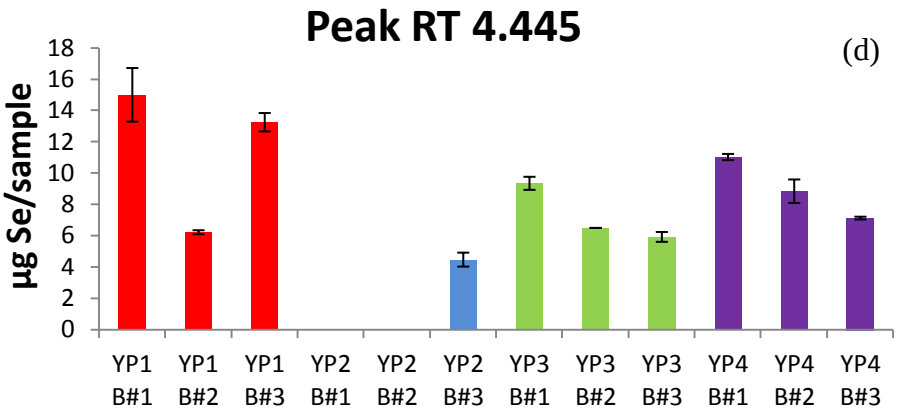
Appendix

Unknown selenocompounds detected by HPLC-ICP-MS.

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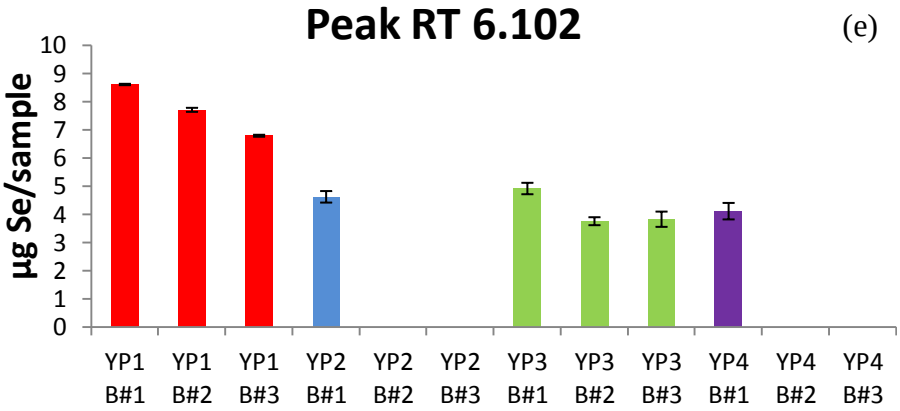
YP4 B#1
YP4 B#2
YP4 B#3

(d)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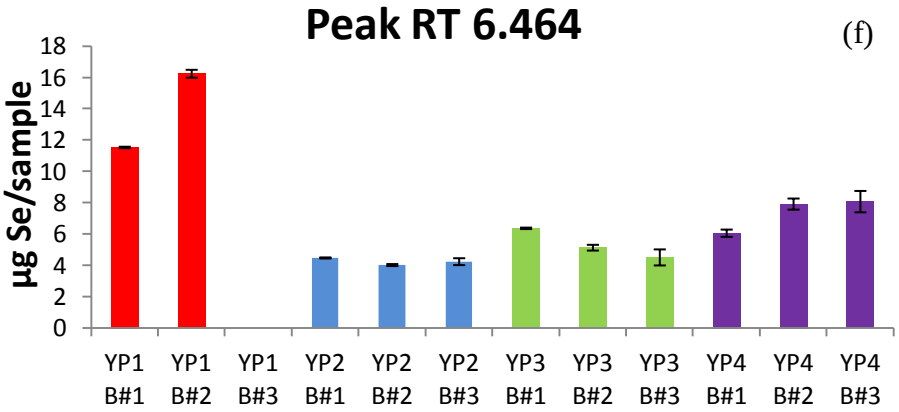
YP4 B#2
YP4 B#3

(e)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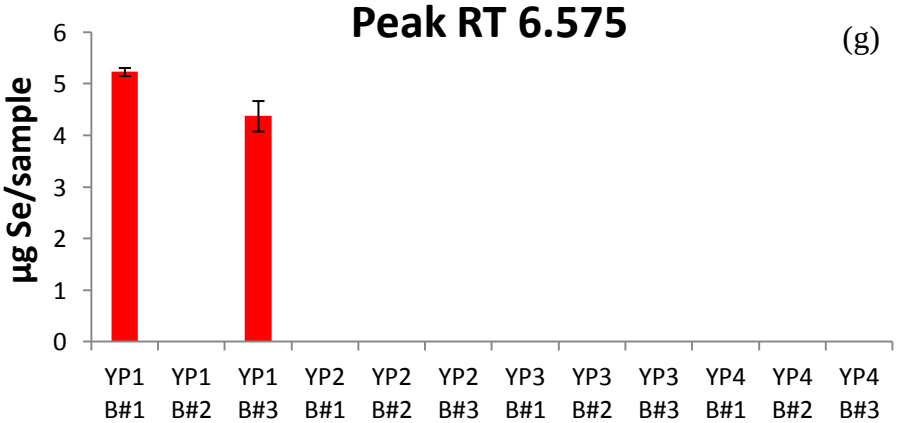
YP4 B#1
YP4 B#2
YP4 B#3

(f)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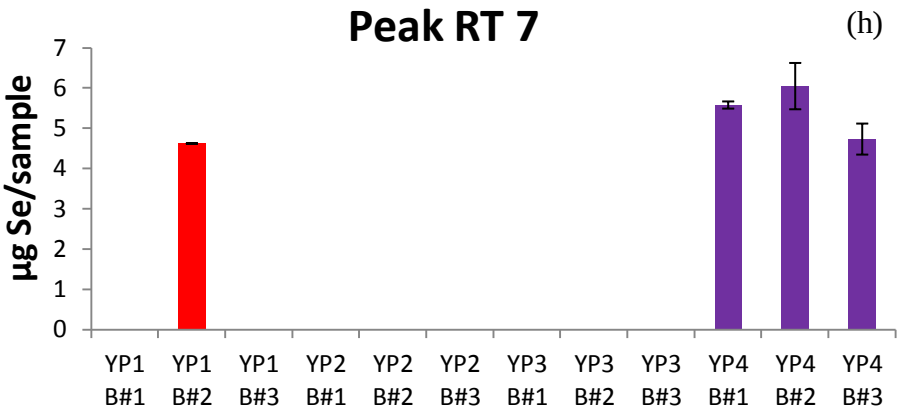
YP4 B#2
YP4 B#3

(g)

(h)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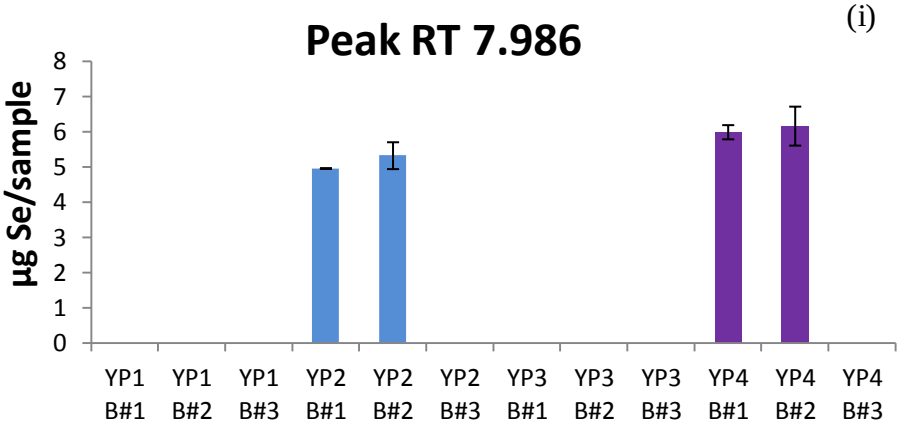
YP4 B#2
YP4 B#3

(i)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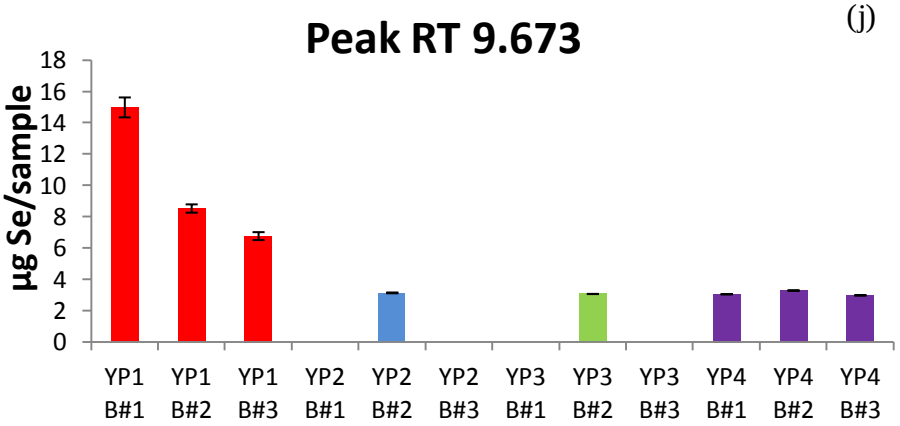
YP4 B#3

(j)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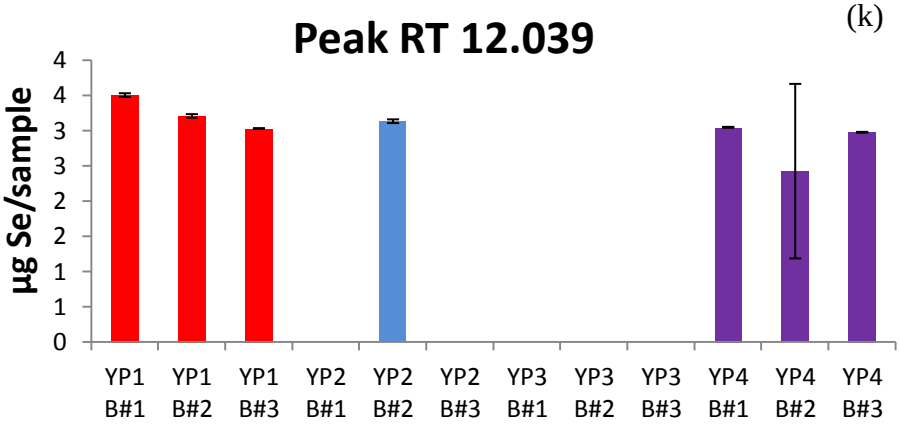
YP4 B#3

(k)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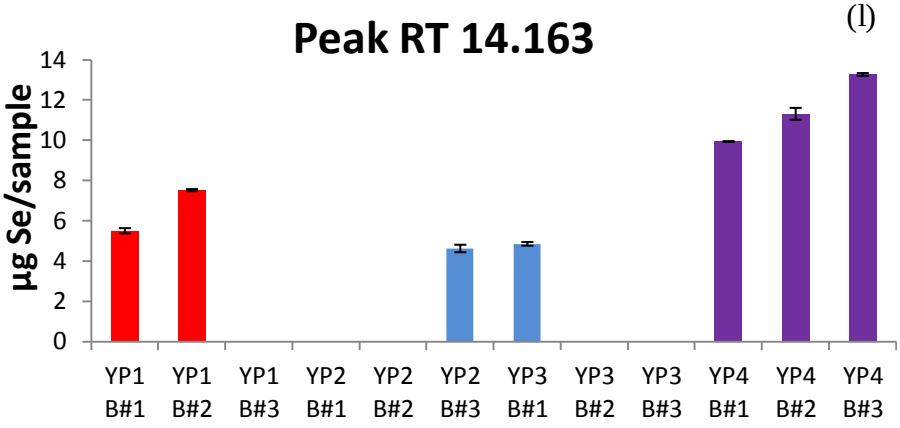
(l)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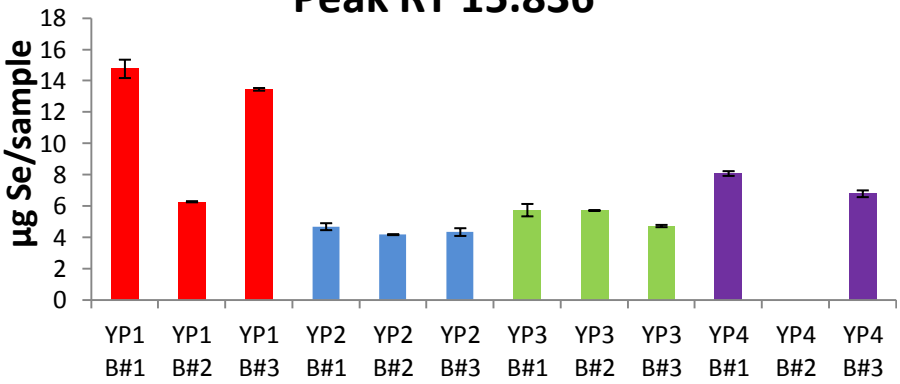
YP4 B#1
YP4 B#2
YP4 B#3

(m)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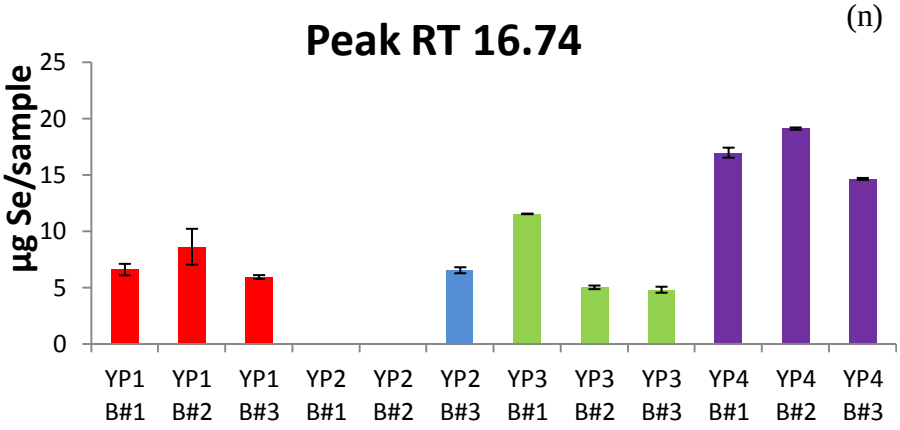
YP4 B#1
YP4 B#2
YP4 B#3

(n)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YP4 B#1
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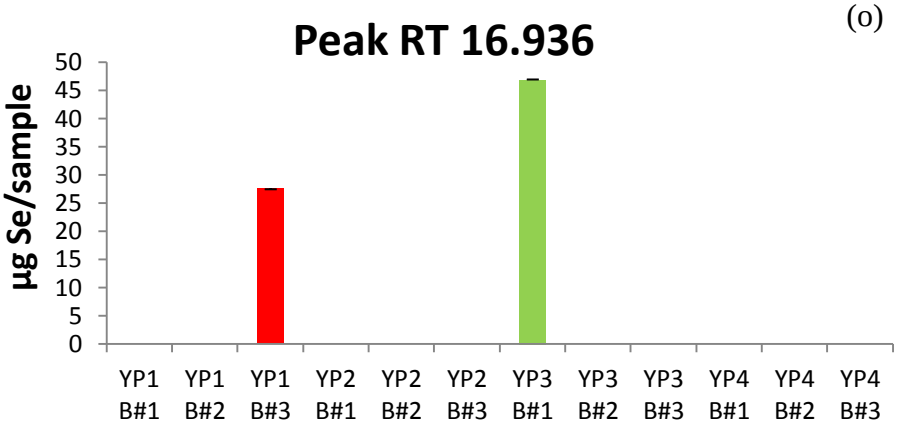
(o)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YP2 B#1
YP2 B#2
YP2 B#3
YP3 B#1
YP3 B#2
YP3 B#3
YP4 B#1
YP4 B#2
YP4 B#3

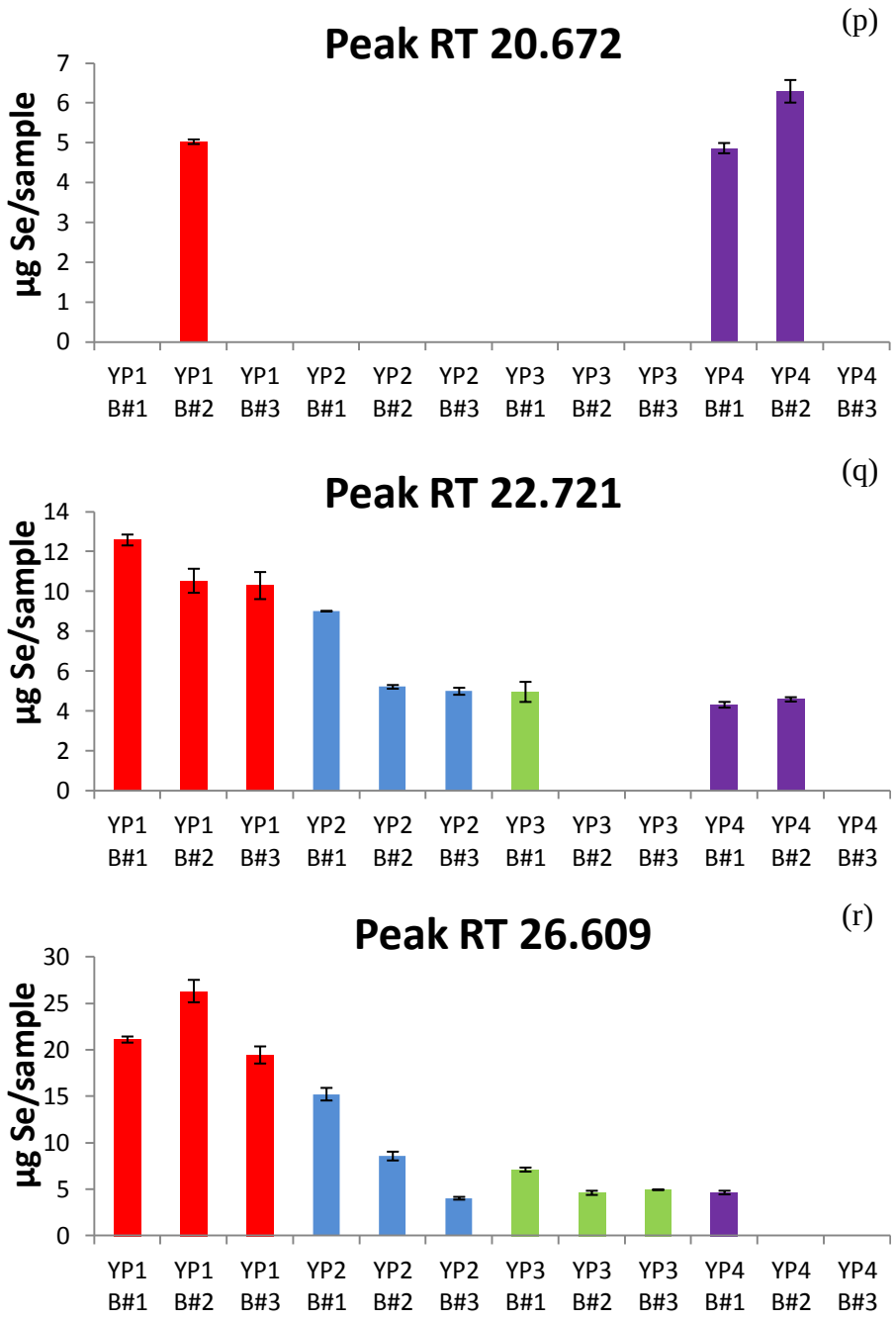


Figure A1 (a) - (r): Unknown selenocompounds found in yeast products (YP1-4) by HPLC-ICP-MS.

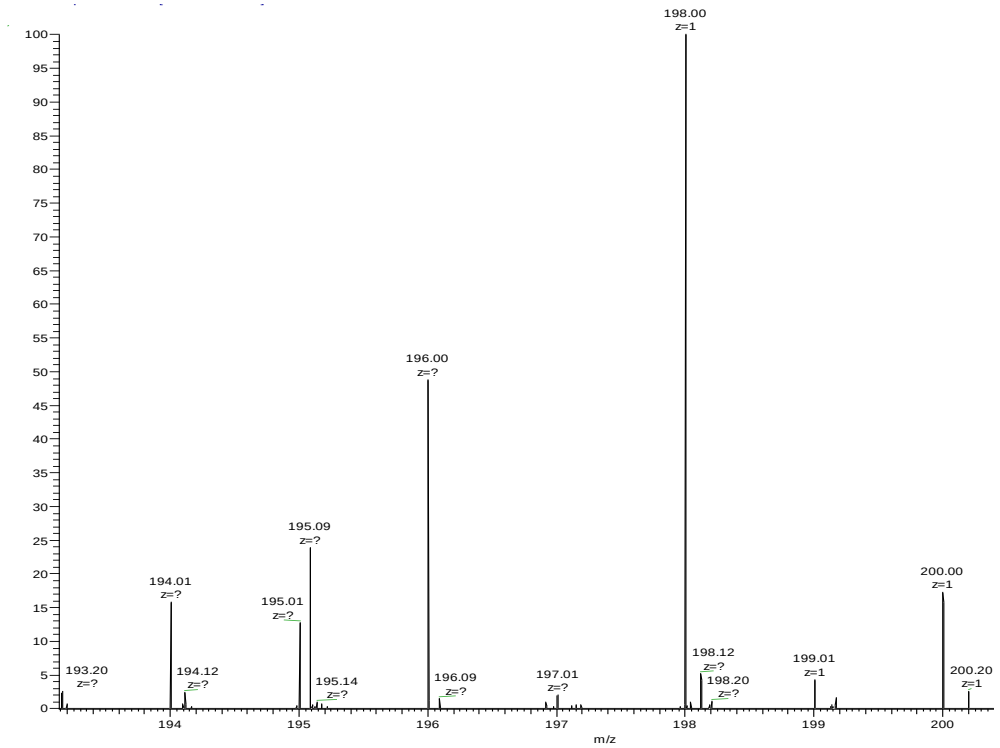


Figure A2: Mass spectrum of selenomethionine after direct infusion by ESI-LTQ-Orbitrap.

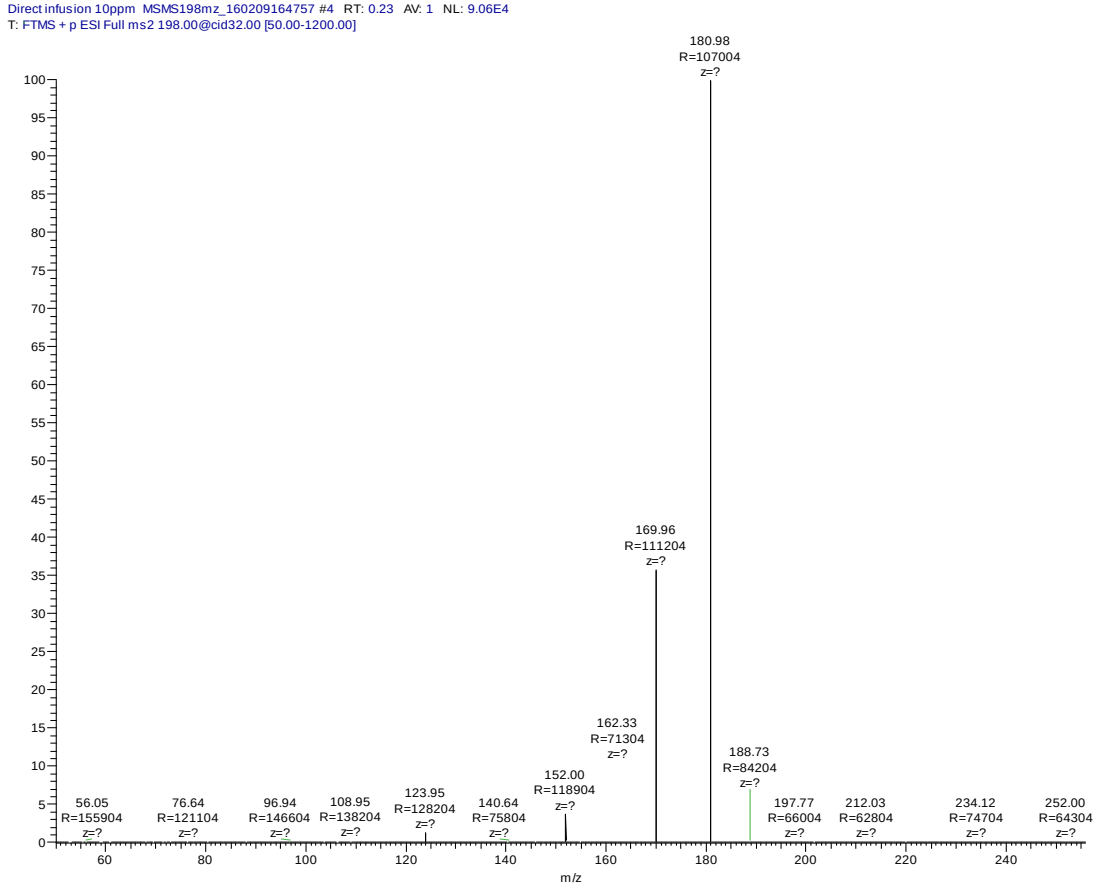


Figure A3: MS^2 analysis of selenomethionine.

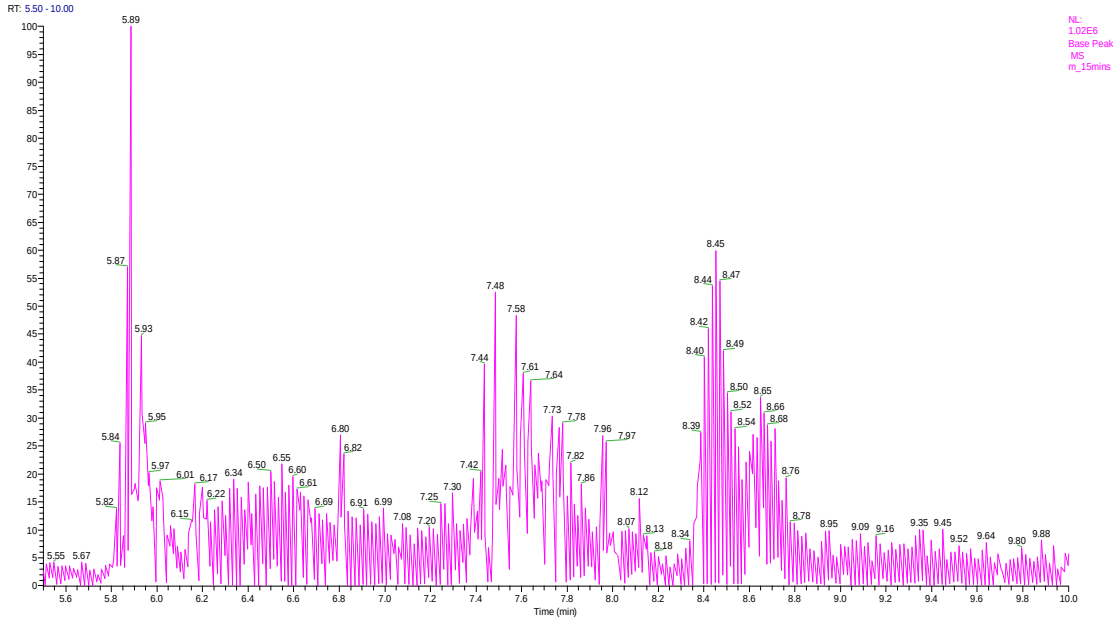


Figure A4: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

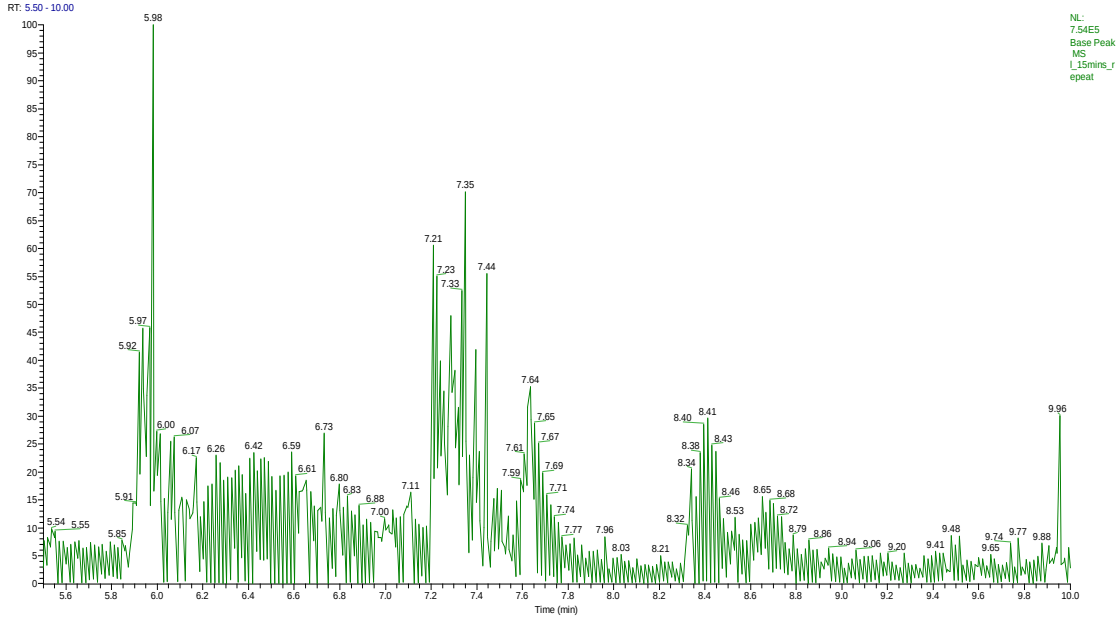


Figure A5: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

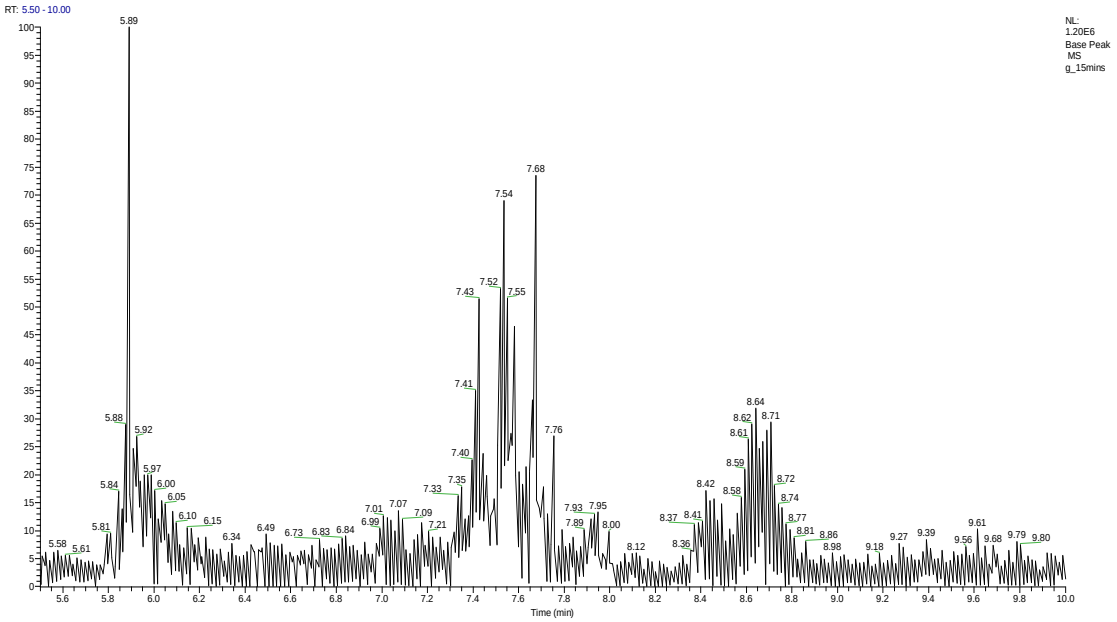


Figure A6: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

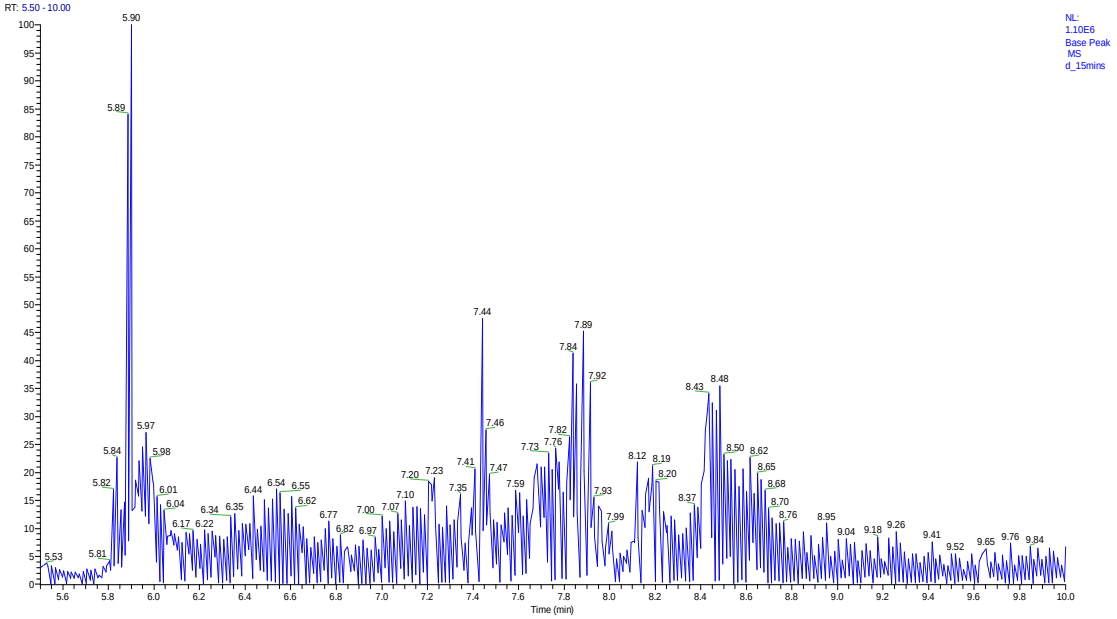


Figure A7: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

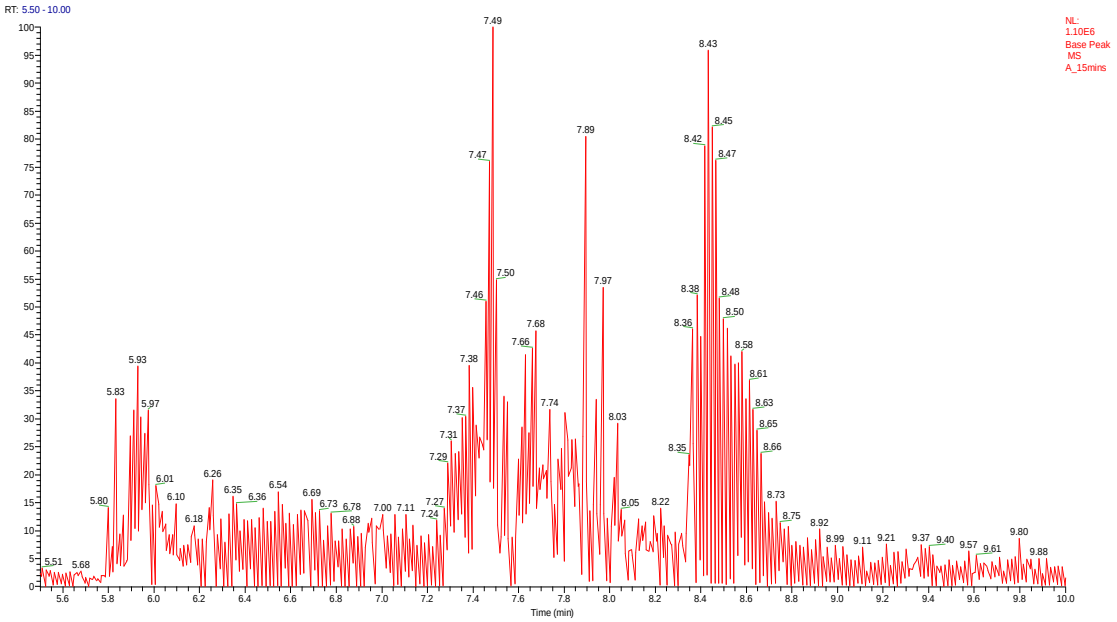


Figure A8: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

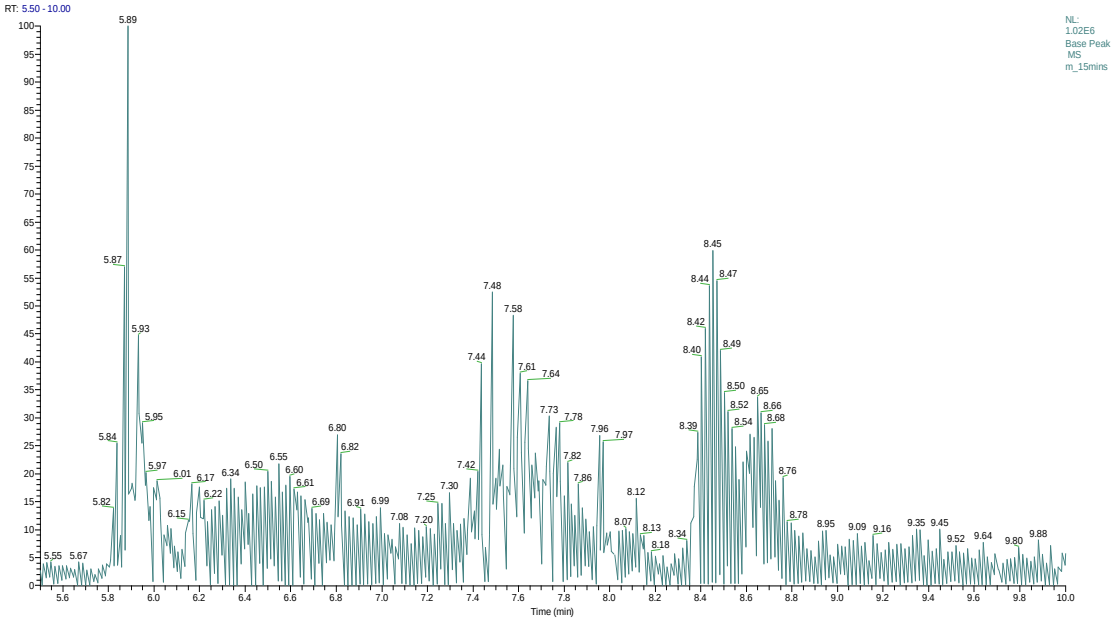


Figure A9: LC-ESI-OrbitrapXL-MS chromatogram of the yeast product between 5.5 and 10 minutes.

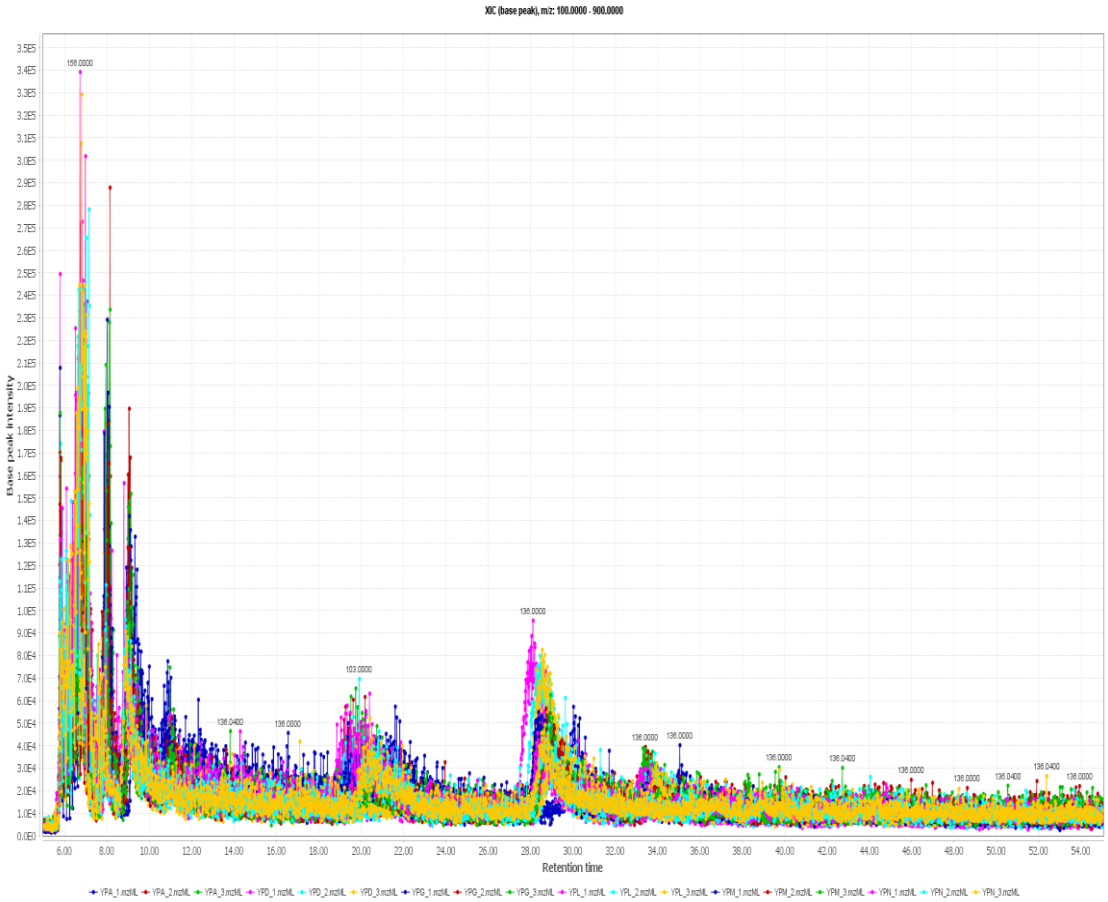


Figure A10: Overlay of LC-ESI-LTQ-OrbitrapXL chromatograms from 5 commercial selenised yeast products (0 - 60 mins).

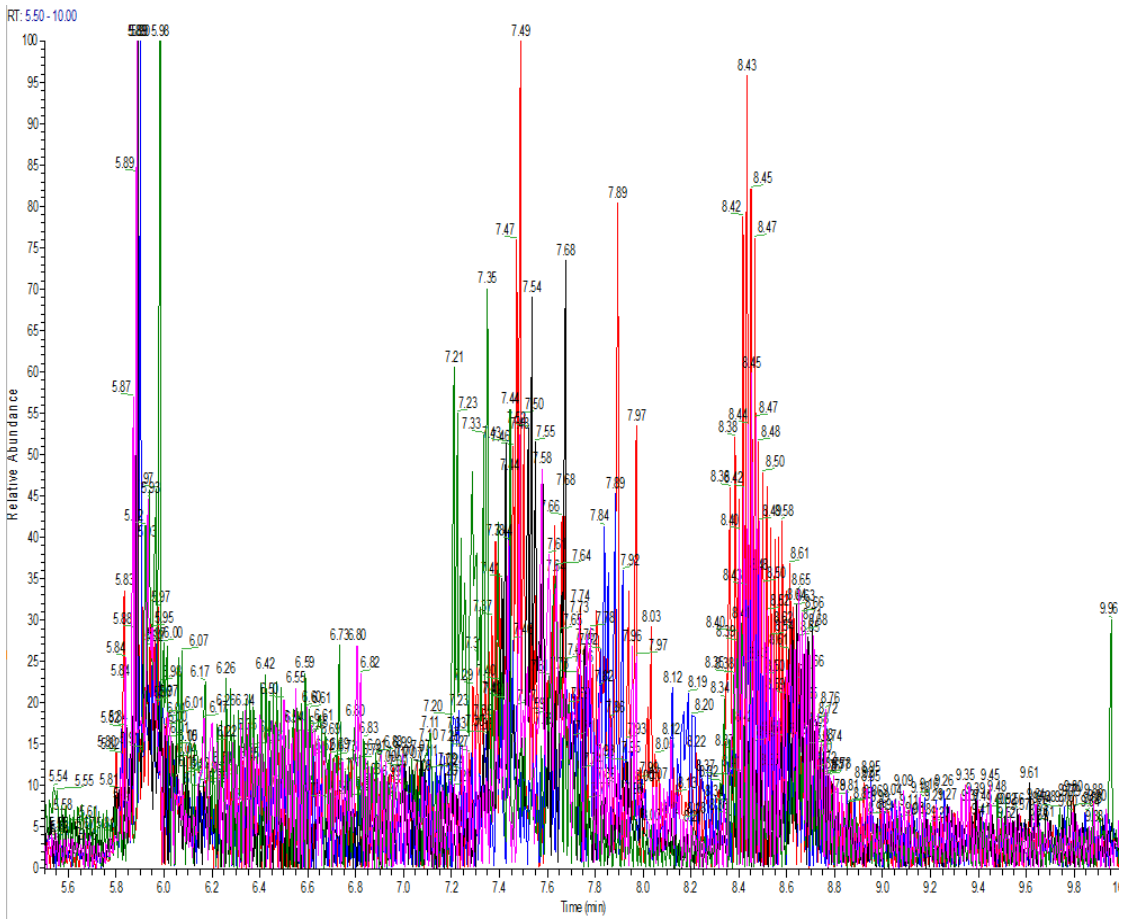


Figure A11: Overlay of LC-ESI-LTQ-OrbitrapXL chromatograms from 5 commercial selenised yeast products (5 - 10 mins).

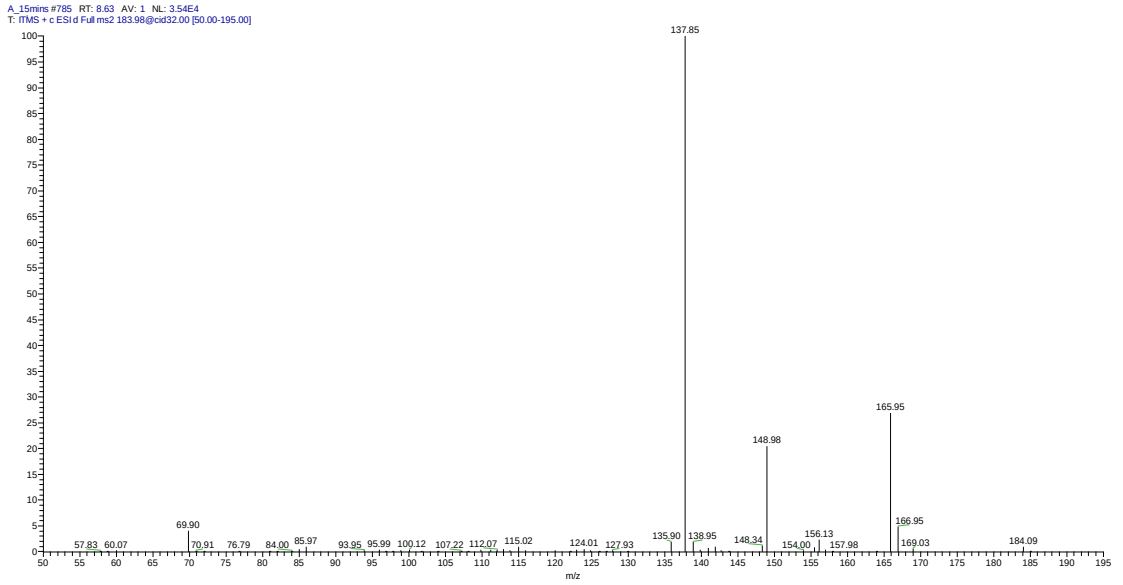


Figure A12: MS^2 of parent ion, *m/z* 181.92.

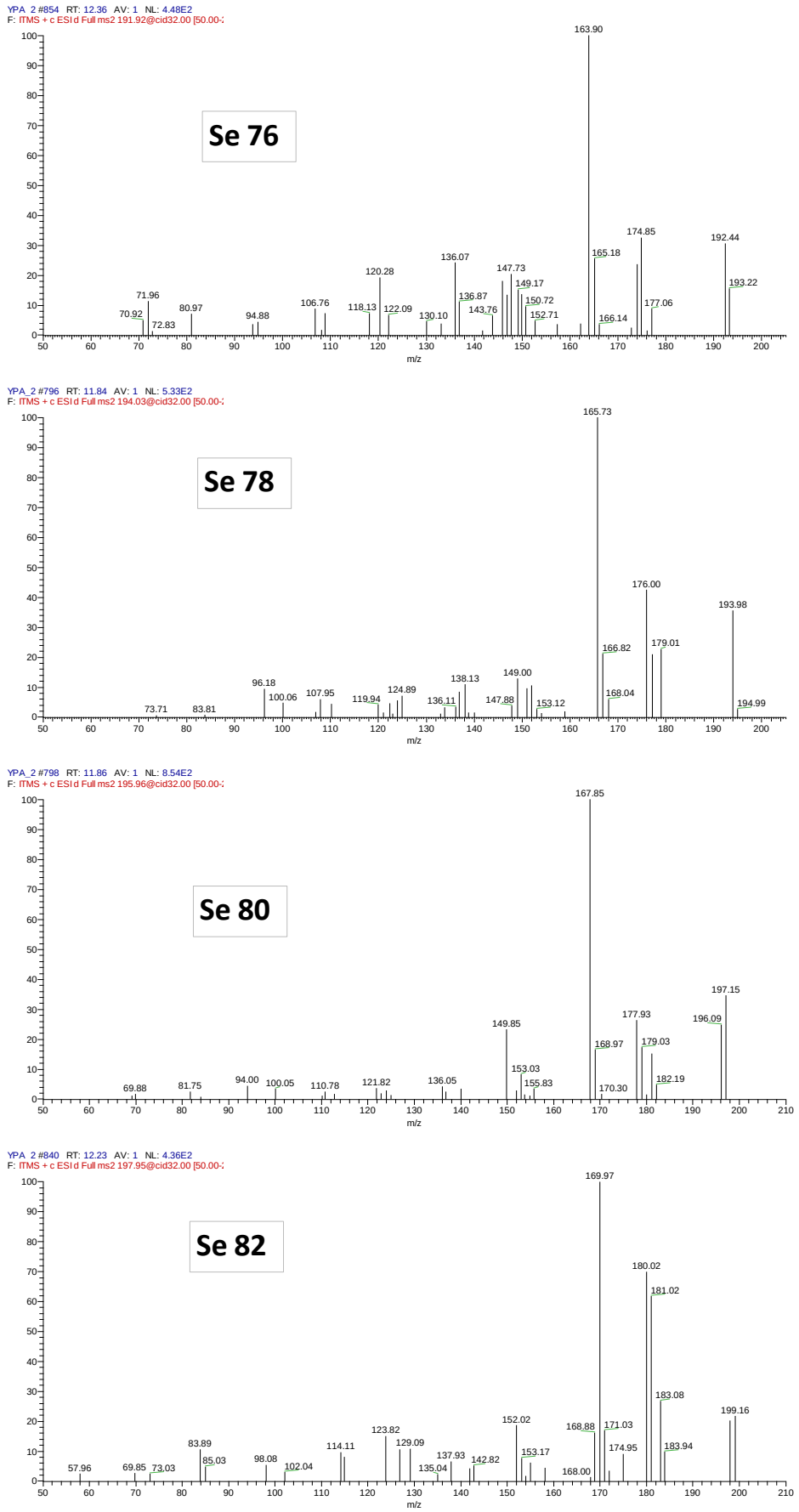


Figure A13: MS^2 spectra of proposed methyl-dehydrohomocysteine ion, *m/z* 195.92.

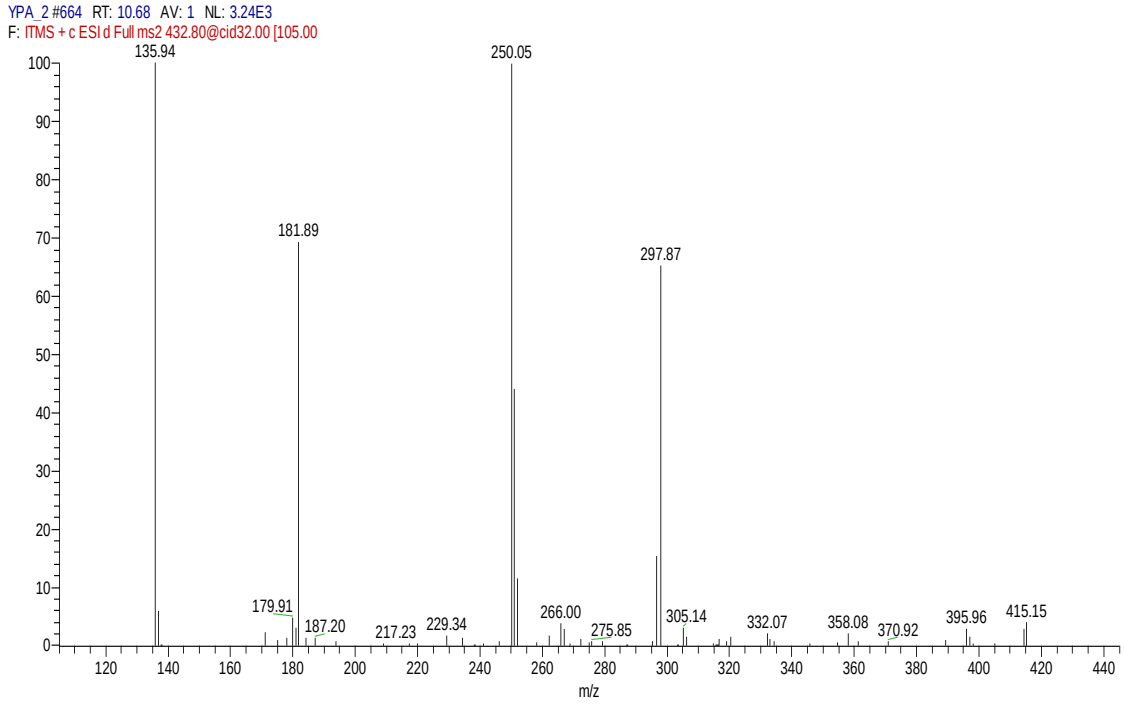


Figure A14: LC-ESI-OrbitrapXL-MS mass spectra of *m/z* 432.80.

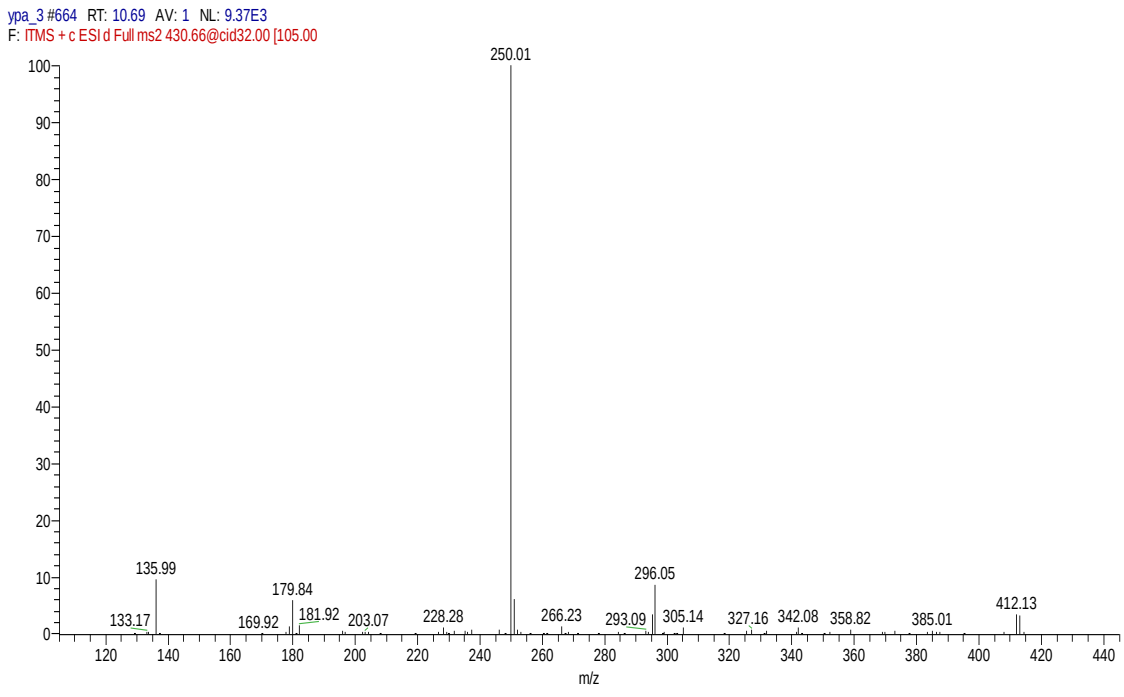


Figure A15: LC-ESI-OrbitrapXL-MS mass spectra of *m/z* 430.66.

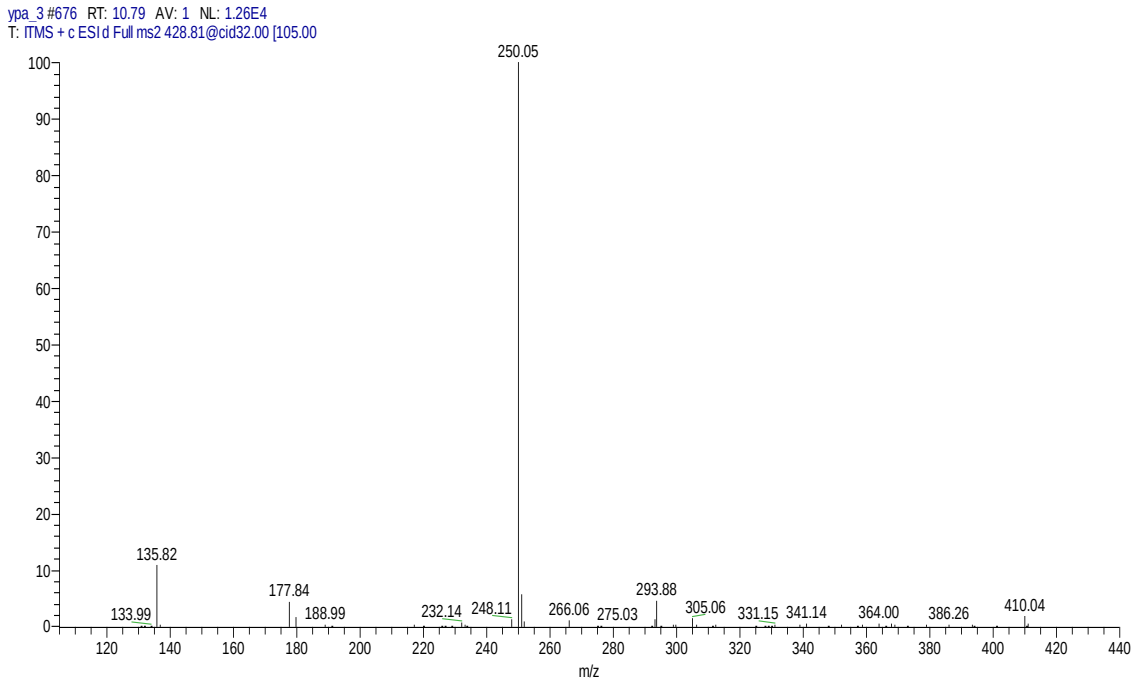


Figure A16: LC-ESI-OrbitrapXL-MS mass spectra of *m/z* 428.81.
The selenium isotopic pattern in Chapter 5, Figure 5.5, was verified by looking at the shift of *m/z* 181.89 and 297.87 from Figure 14A to 16A. As the overall mass-to-charge ratio decreases for the parent ion, indicating the transition from ^{80}Se to ^{78}Se to ^{76}Se, these two ions also change by the same amount. Indicating the presence of selenium. As mentioned in chapter 5, as the mass-to-charge parent ion decreases, a similar decrease was witnessed in the MS^2 spectra which was consistent with the presence of a selenium atom.

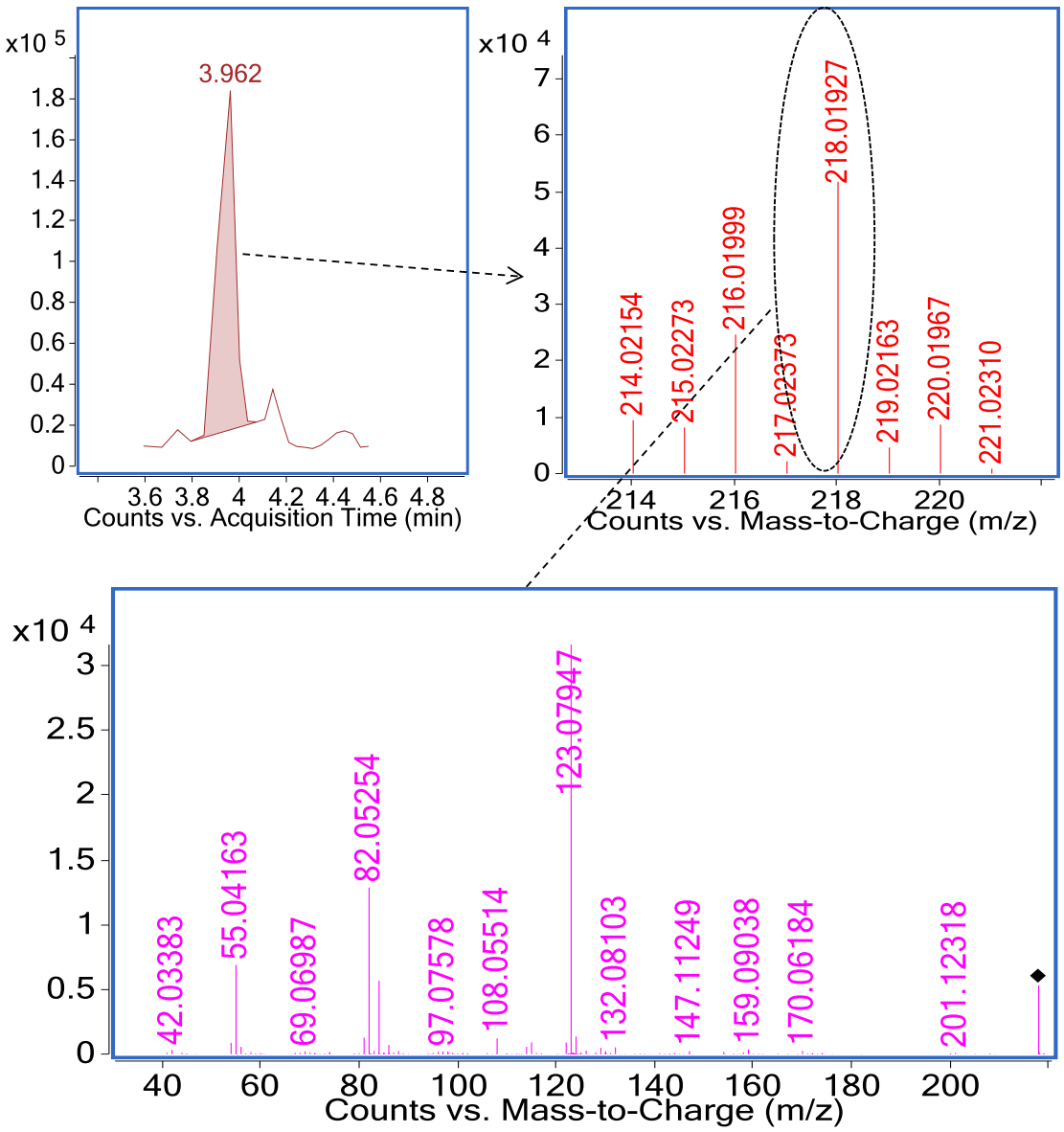
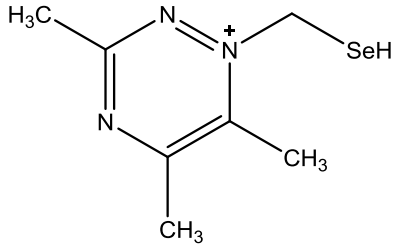


Figure A17: Chromatogram of *m/z* 218 selenocompound followed by MS[1] and MS[2] analysis.

Table A1: Elemental composition and proposed structure of *m/z* 218 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
218.01927	$C_7H_{12}N_3Se^+$	218.01964	-0.78	3.96	$C_6H_9N_3^+$ (loss of CH_3Se)	123.07947 (3.01)
					$C_5H_6N_3^+$	108.05514 (-4.44)
					$C_5H_9N_2^+$	97.07578 (-2.47)
					$C_4H_6N_2^+$	82.05254 (-0.12)
					$C_3H_5N^+$	55.04163 (-0.37)

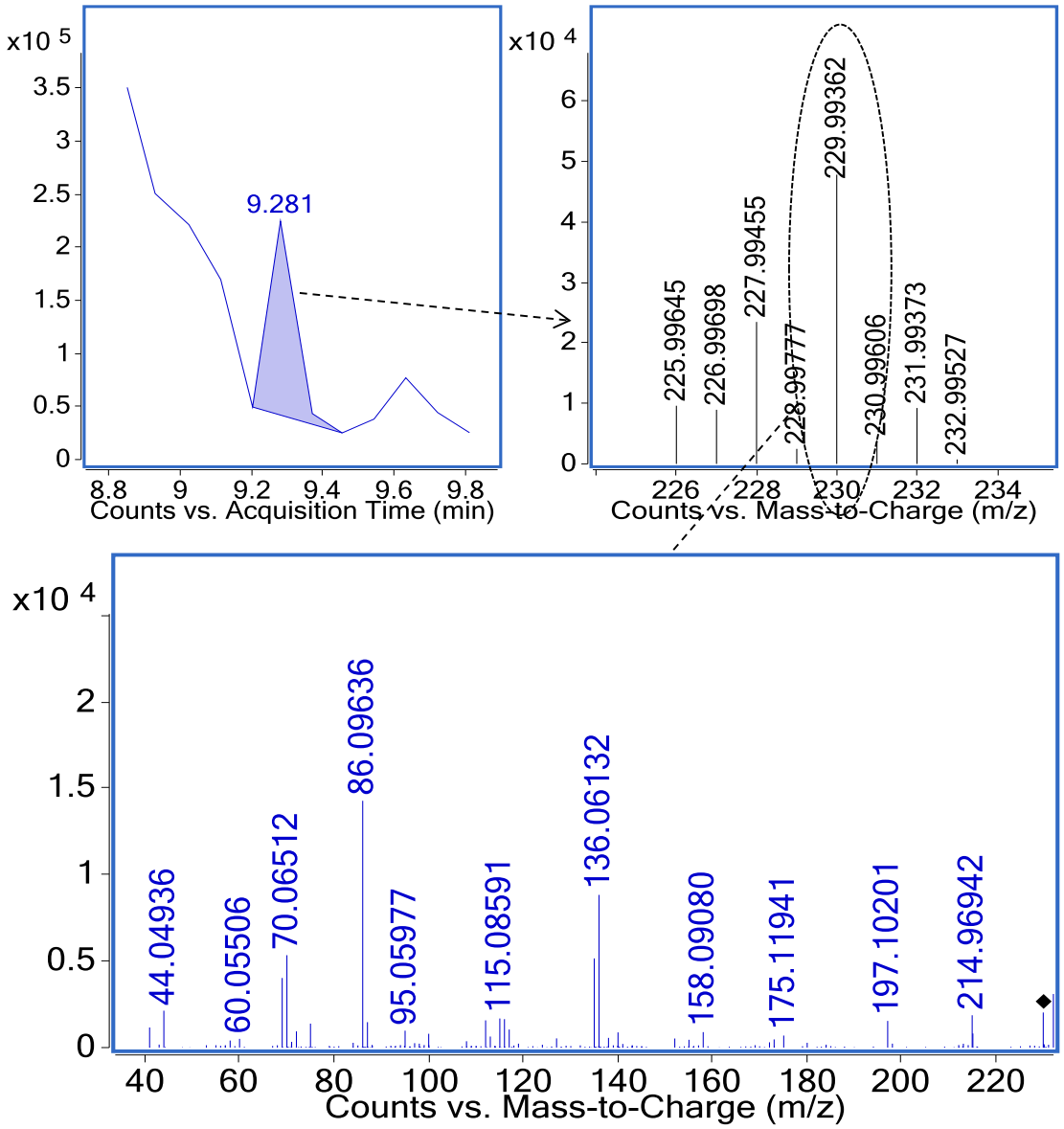
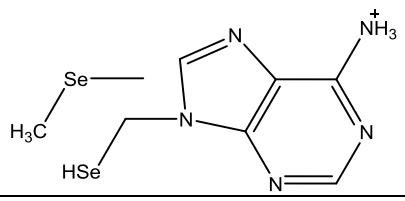


Figure A18: Chromatogram of *m/z* 230 selenocompound followed by MS[1] and MS[2] analysis.

Table A2: Elemental composition and proposed structure of *m/z* 230 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
229.99362	$C_6H_8N_5Se^+$	229.99394	-1.39	9.28	$C_5H_6N_5^+$ (loss of CH_2Se)	136.06132 (-3.31)
					$C_4H_8N^+$	70.06512 (-0.08)
					$C_2H_6N^+$	44.04936 (-2.62)



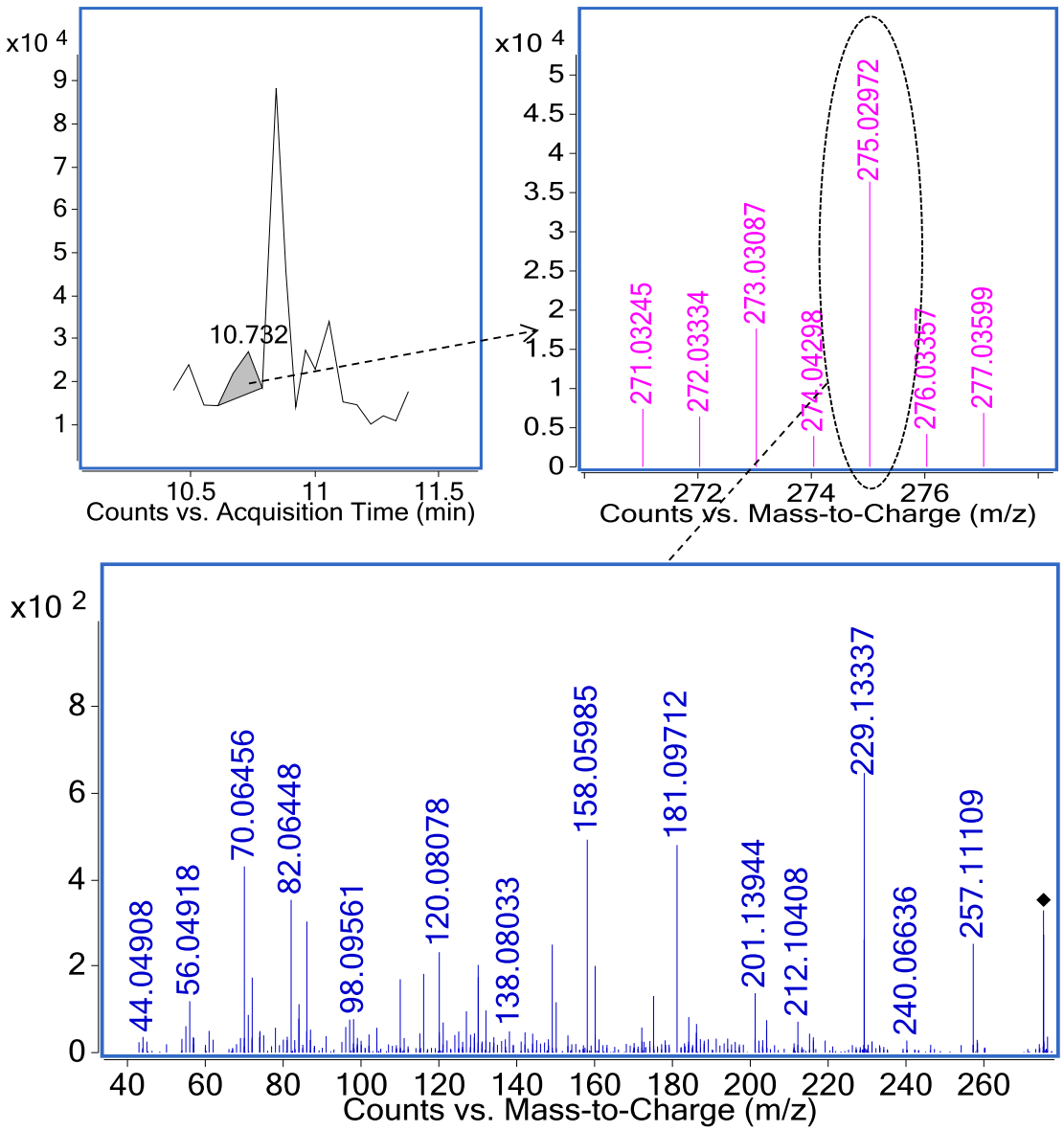
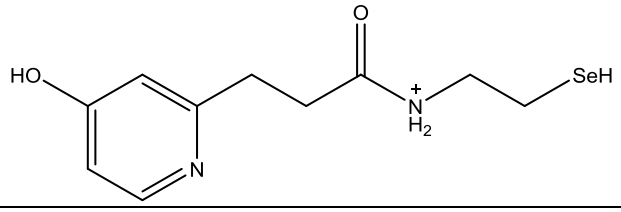


Figure A19: Chromatogram of *m/z* 275 selenocompound followed by MS[1] and MS[2] analysis.

Table A3: Elemental composition and proposed structure of *m/z* 275 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
275.02972	$C_{10}H_{15}N_2O_2Se^+$	275.02933	1.41	10.73	$C_9H_{13}N_2O_2^+$ (loss of CH_2Se)	181.09712 (-0.17)
					$C_{10}H_8NO^+$	158.05985 (-1.20)
					$C_8H_9N_2O^+$	149.07163 (4.63)
					$C_8H_{10}N^+$	120.08078 (0.00)
					$C_4H_8N^+$	70.06511 (-0.22)



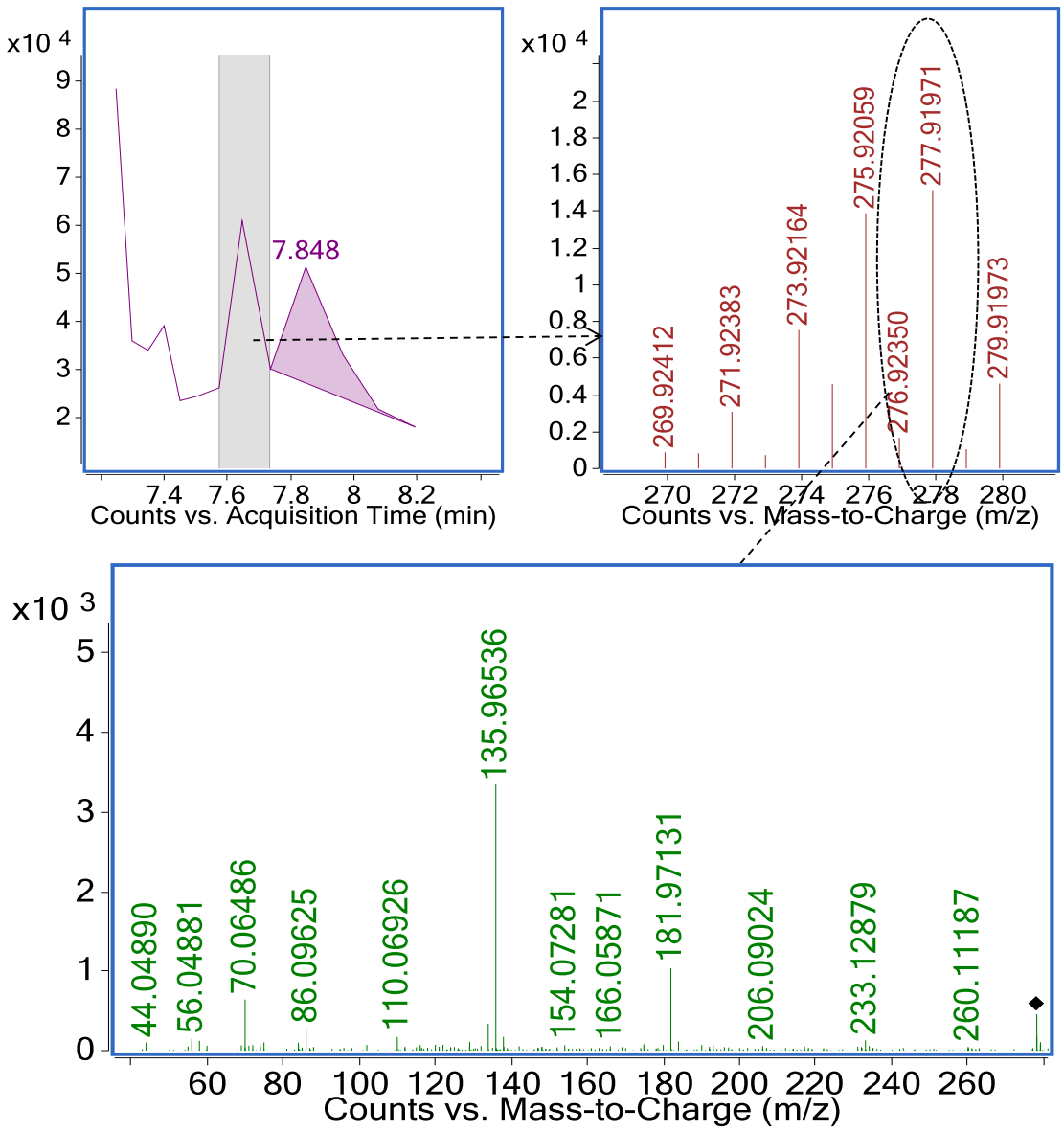
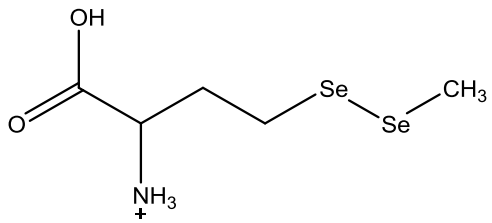


Figure A20: Chromatogram of *m/z* 278 selenocompound followed by MS[1] and MS[2] analysis.

Table A4: Elemental composition and proposed structure of *m/z* 278 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
277.91971	$C_5H_{12}NO_2Se_2^+$	277.91930	1.48	7.65	$C_4H_8NO_2Se^+$ (SehomoCys fragment, loss of CH_4Se)	181.97131 (-0.93)
					$C_3H_6NSe^+$ (loss of formic acid from SehomoCys)	135.96536 (-4.70)
					$C_5H_{12}N^+$	86.09625 (-2.04)
					$C_4H_8N^+$	70.06486 (-3.79)



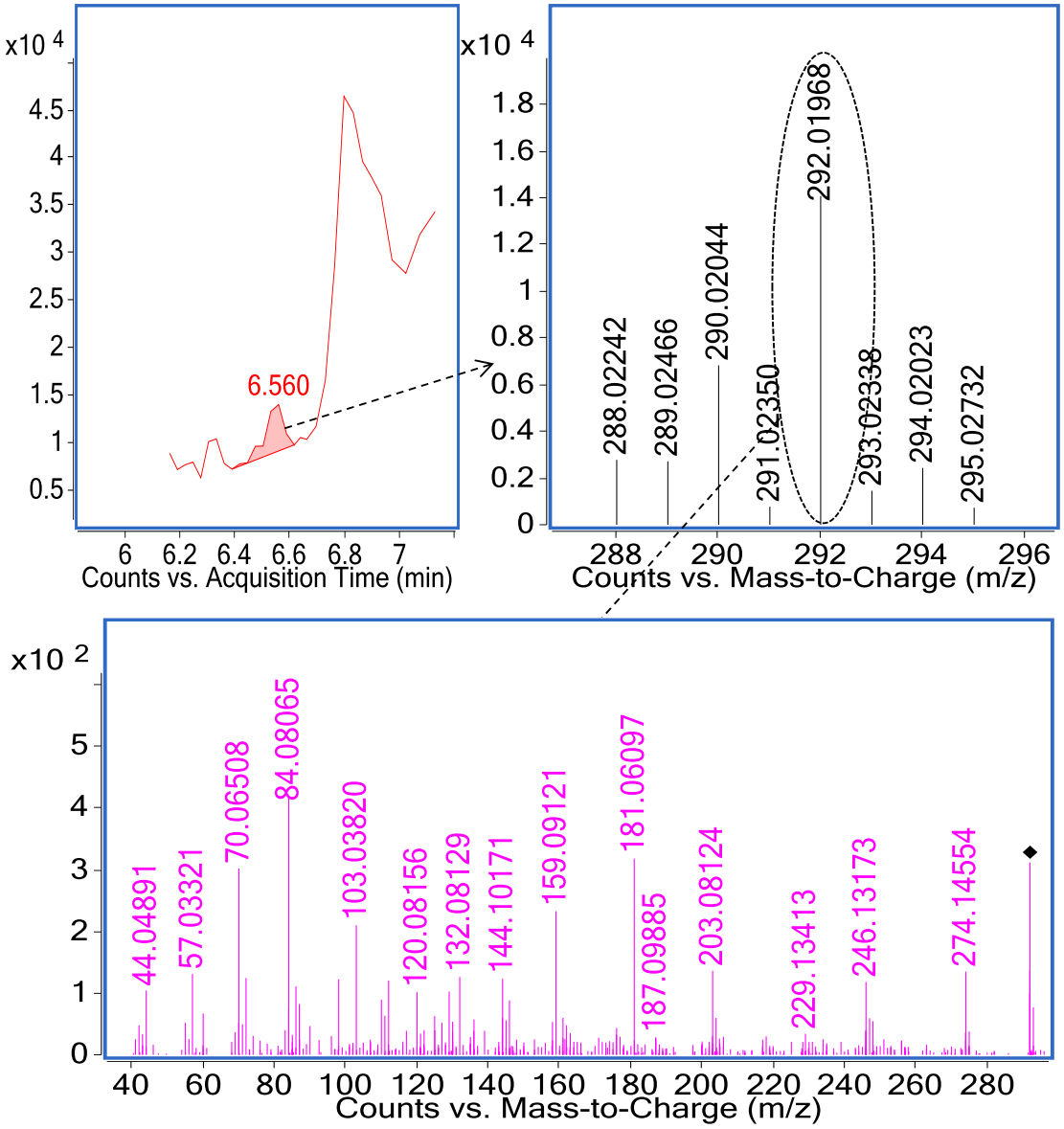


Figure A21: Chromatogram of *m/z* 292 selenocompound followed by MS[1] and MS[2] analysis.

Table A5: Elemental composition and proposed structure of *m/z* 292 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
292.01968	$C_9H_{14}N_3O_3Se^+$	292.02004	-1.23	6.56	$C_8H_9N_2O_3^+$ (loss of CH_5NSe)	181.06097 (1.11)
					$C_7H_{14}NO_2^+$	144.10171 (1.39)
					$C_9H_{10}N^+$	132.08129 (-3.89)
					$C_7H_{14}N^+$	112.11202 (-0.50)
					$C_2H_5N_3O_2^+$ (loss of C_7H_9OSe)	103.03820 (5.53)
					$C_5H_{10}N^+$	84.08065 (-1.50)
					$C_4H_8N^+$	70.06483 (-4.22)
					$C_3H_5O^+$	57.03321 (-4.91)

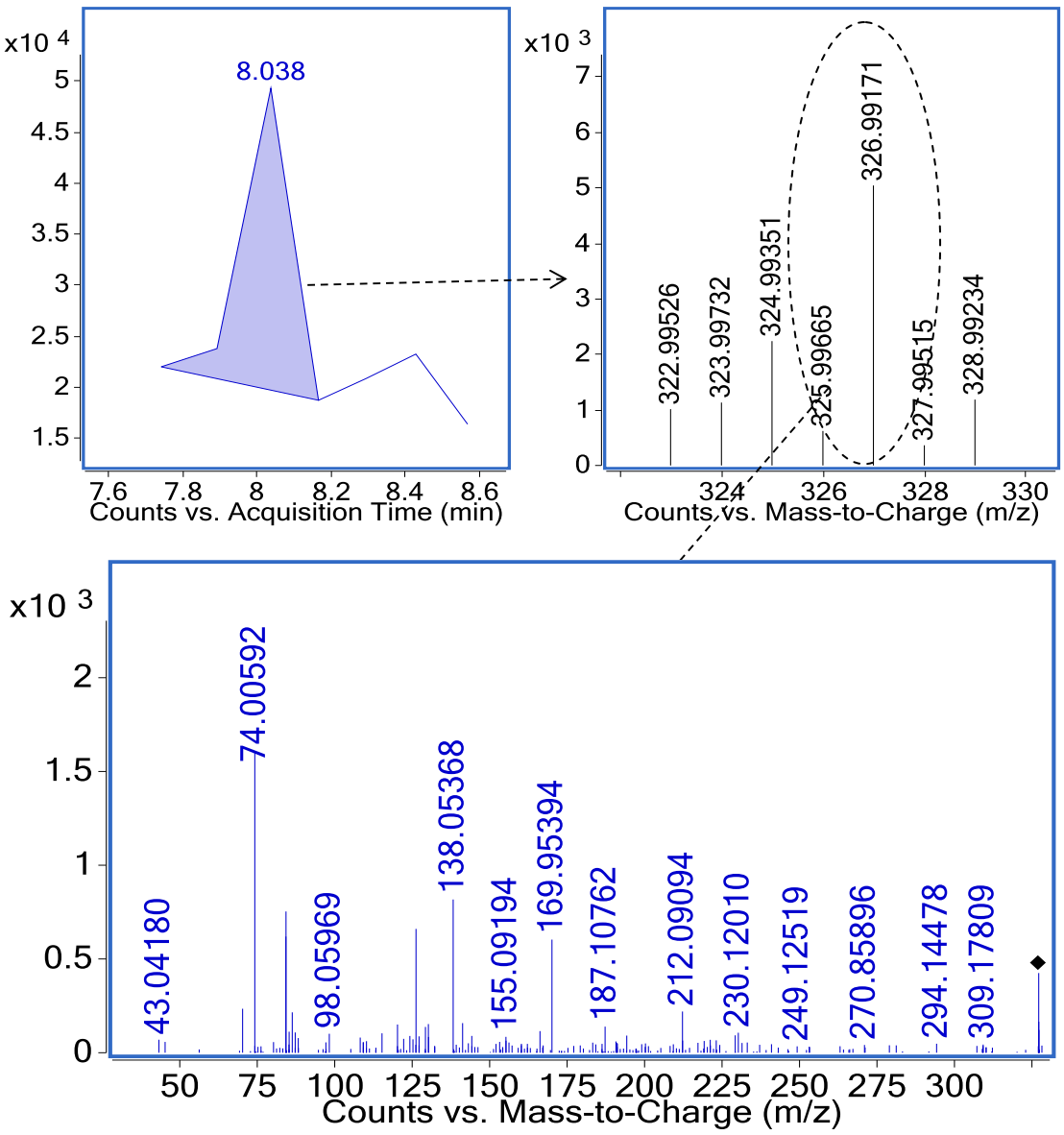
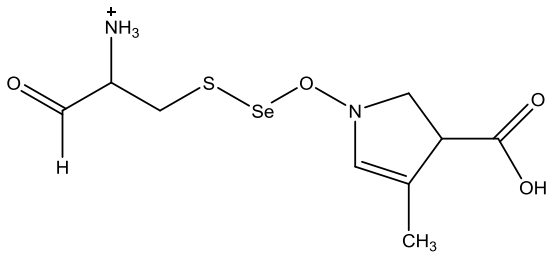


Figure A22: Chromatogram of *m/z* 327 selenocompound followed by MS[1] and MS[2] analysis.

Table A6: Elemental composition and proposed structure of *m/z* 327 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
326.99171	$C_9H_{15}N_2O_4SSe^+$	326.99123	1.47	8.04	$C_3H_8NSSe^+$ (loss of $C_6H_7NO_4$)	169.95368 (-0.24)
					$C_6H_8NO_2^+$	126.05511 (1.23)
					$C_5H_8NO^+$	98.05969 (-3.57)
					$C_5H_{10}N^+$	84.08022 (-6.61)
					$C_2H_4NS^+$	74.00592 (0.32)



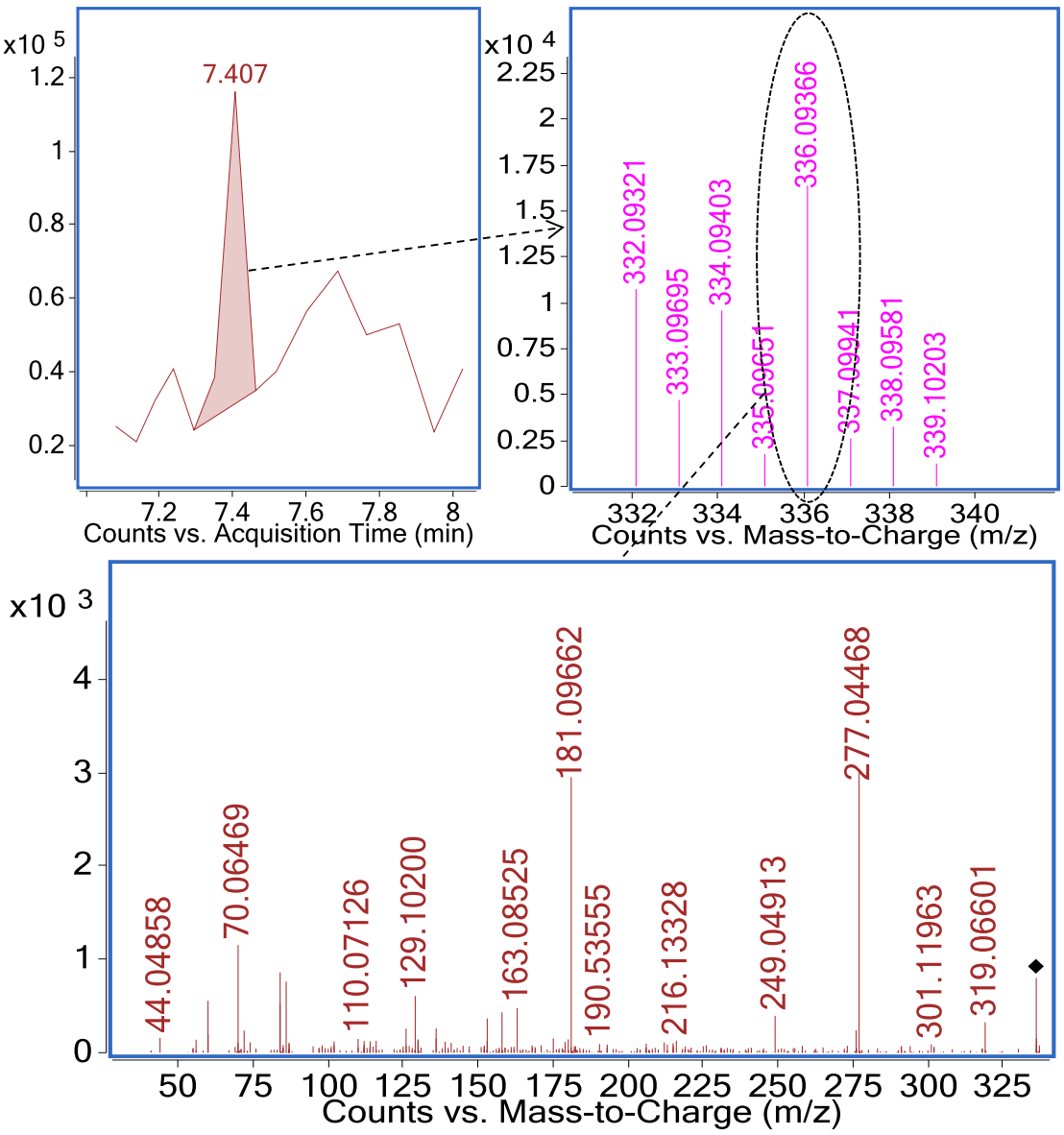
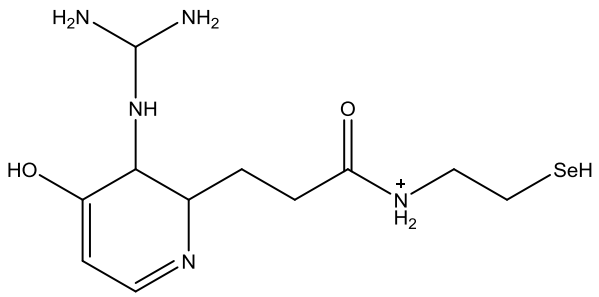


Figure A23: Chromatogram of *m/z* 336 selenocompound followed by MS[1] and MS[2] analysis.

Table A7: Elemental composition and proposed structure of *m/z* 336 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
336.09366	$C_{11}H_{22}N_5O_2Se^+$	336.09332	1.01	7.41	$C_{10}H_{17}N_2O_2Se^+$	277.04468 (-1.08)
					$C_7H_{15}N_5Se^+$	249.04913 (1.64)
					$C_9H_{13}N_2O_2^+$	181.09662 (-2.93)
					$C_6H_{13}N_2O^+$	129.10200 (-1.86)
					$C_5H_{12}N^+$	86.09644 (-0.17)
					$C_5H_{10}N^+$	84.08051 (-3.16)
					$C_4H_8N^+$	70.06469 (-6.22)

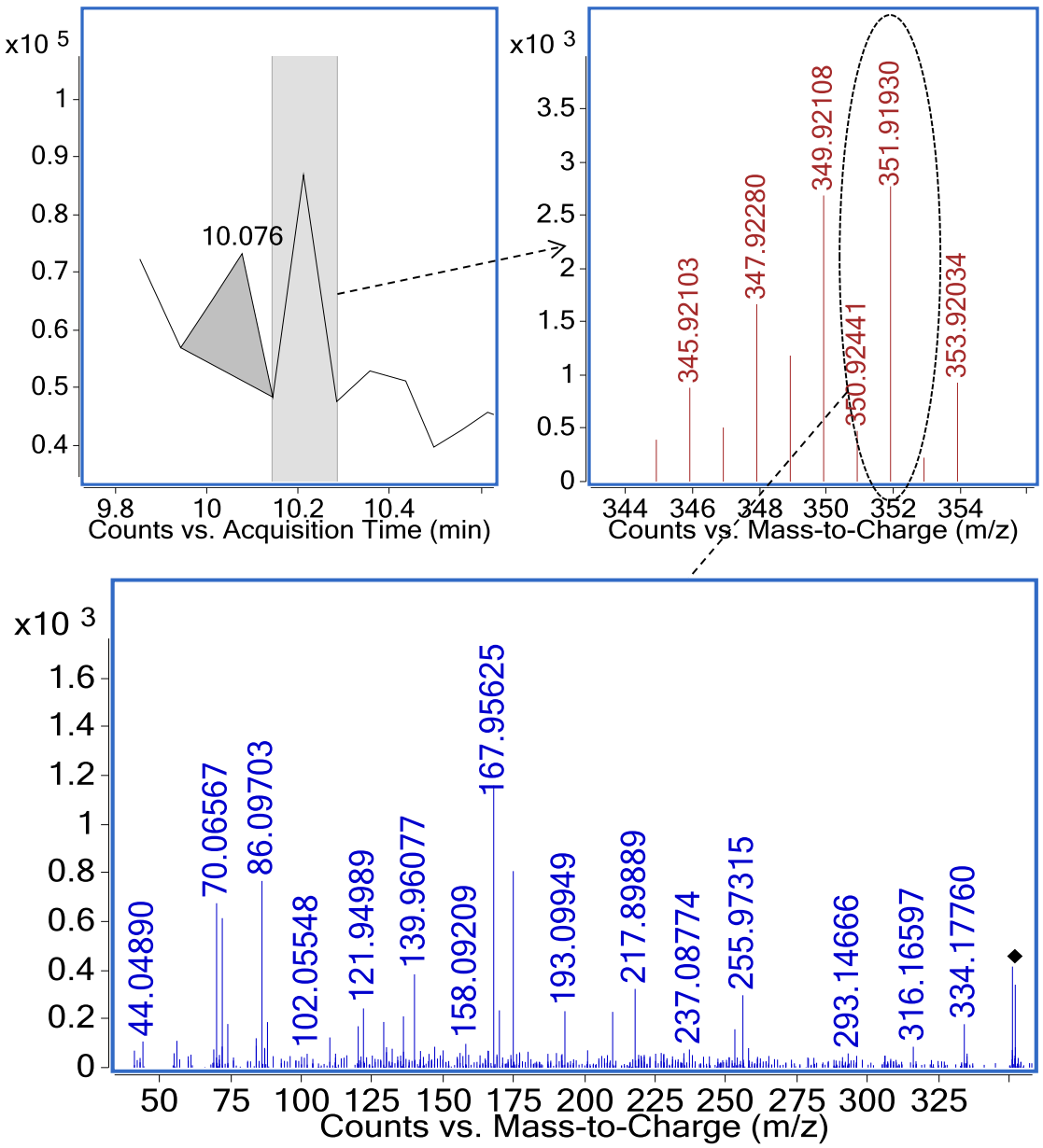
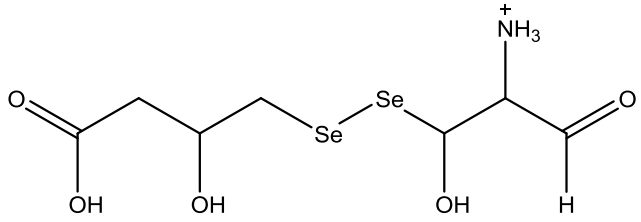


Figure A24: Chromatogram of *m/z* 352 selenocompound followed by MS[1] and MS[2] analysis.

Table A8: Elemental composition and proposed structure of *m/z* 352 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
351.91930	$C_7H_{14}NO_5Se_2^+$	351.91969	-1.11	10.21	$C_6H_{10}NO_5Se^+$ (loss of CH_4Se)	255.97315 (5.00)
					$C_3H_6NO_2Se^+$ (loss of $C_4H_8O_3Se$)	167.95625 (2.50)
					$C_2H_6NOSe^+$	139.96077 (-1.00)
					$C_2H_4NSe^+$	121.94989 (-3.77)
					$C_5H_{12}N^+$	86.09703 (6.97)
					$C_4H_{10}N^+$	72.08134 (7.76)
					$C_4H_8N^+$	70.06567 (7.77)



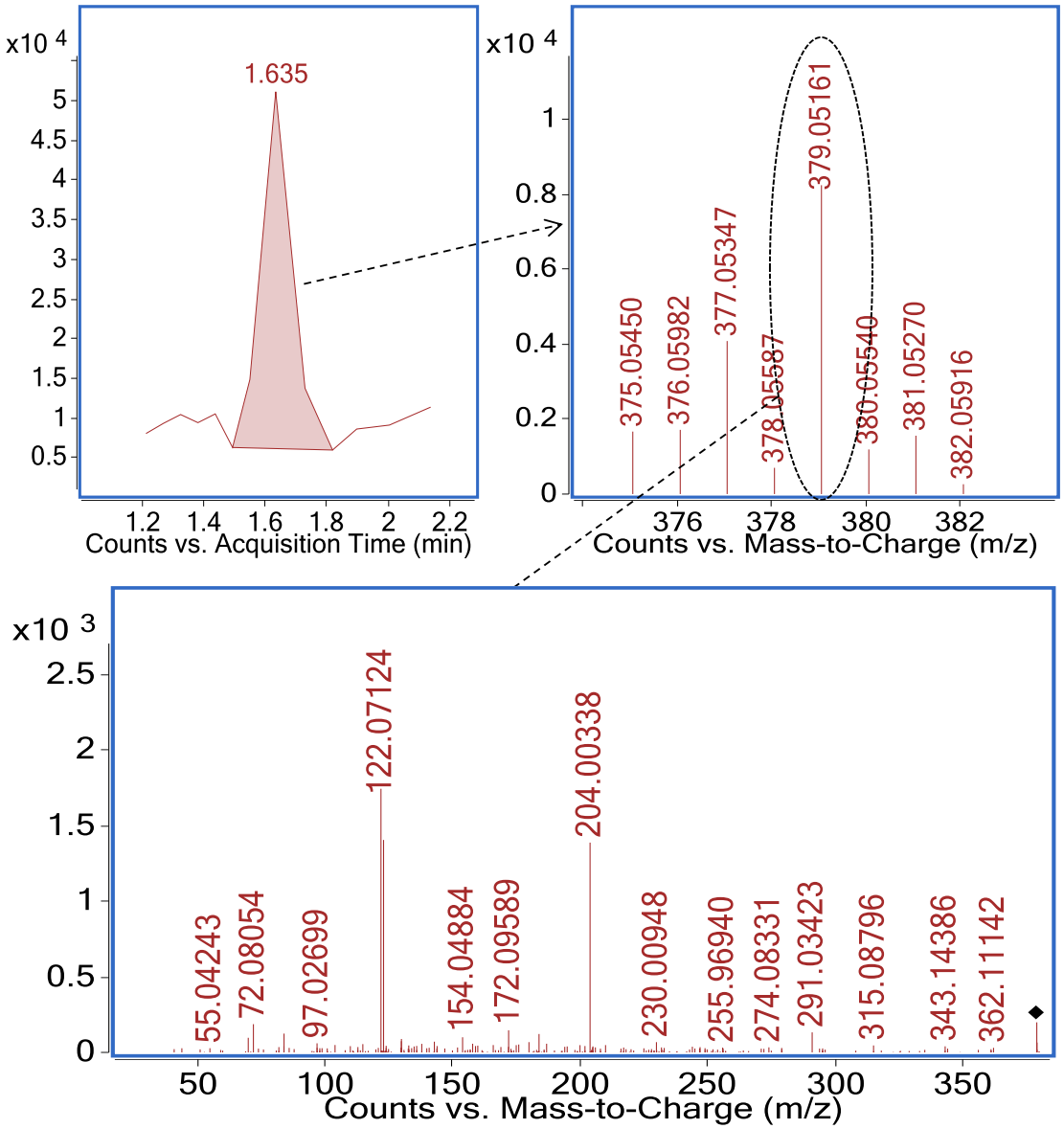
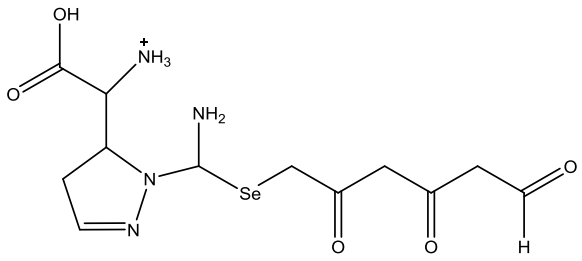


Figure A25: Chromatogram of *m/z* 379 selenocompound followed by MS[1] and MS[2] analysis.

Table A9: Elemental composition and proposed structure of *m/z* 379 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
379.05161	$C_{12}H_{19}N_4O_5Se^+$	379.05152	0.24	1.64	$C_9H_{15}N_4O_2Se^+$	291.03423 (-4.26)
					$C_6H_{10}N_3Se^+$	204.00338 (-0.34)
					$C_6H_{12}N_4O_2^+$	172.09589 (-2.40)
					$C_6H_9N_3^+$	123.07924 (-1.15)
					$C_6H_8N_3^+$	122.07124 (-0.28)
					$C_4H_{10}N^+$	72.08054 (-3.27)

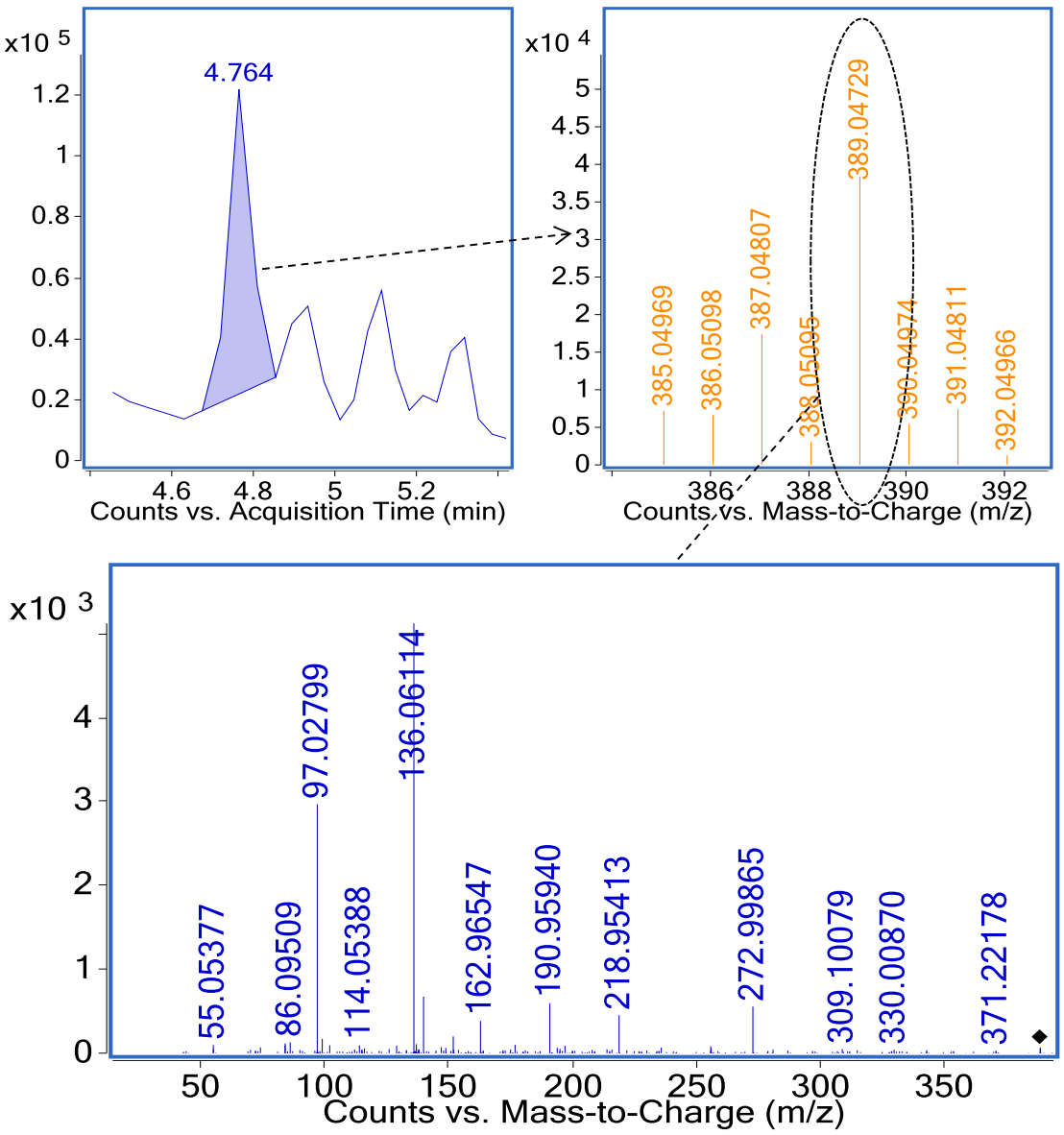
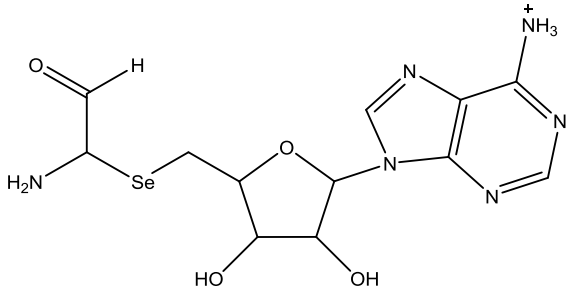


Figure A26: Chromatogram of *m/z* 389 selenocompound followed by MS[1] and MS[2] analysis.

Table A10: Elemental composition and proposed structure of *m/z* 389 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
389.04729	$C_{12}H_{17}N_6O_4Se^+$	389.04710	0.49	4.76	$C_7H_9N_6OSe^+$	272.99865 (-4.07)
					$C_5H_5N_3O_2Se^+$	218.95413 (-0.09)
					$C_4H_5N_3OSe^+$	190.95940 (0.89)
					$C_5H_7OSe^+$	162.96547 (-1.17)
					$C_5H_6N_5^+$ (adenine frag)	136.06114 (-4.63)
					$C_5H_5O_2^+$(dehydroxyribo-furanose frag)	97.02799 (-4.33)



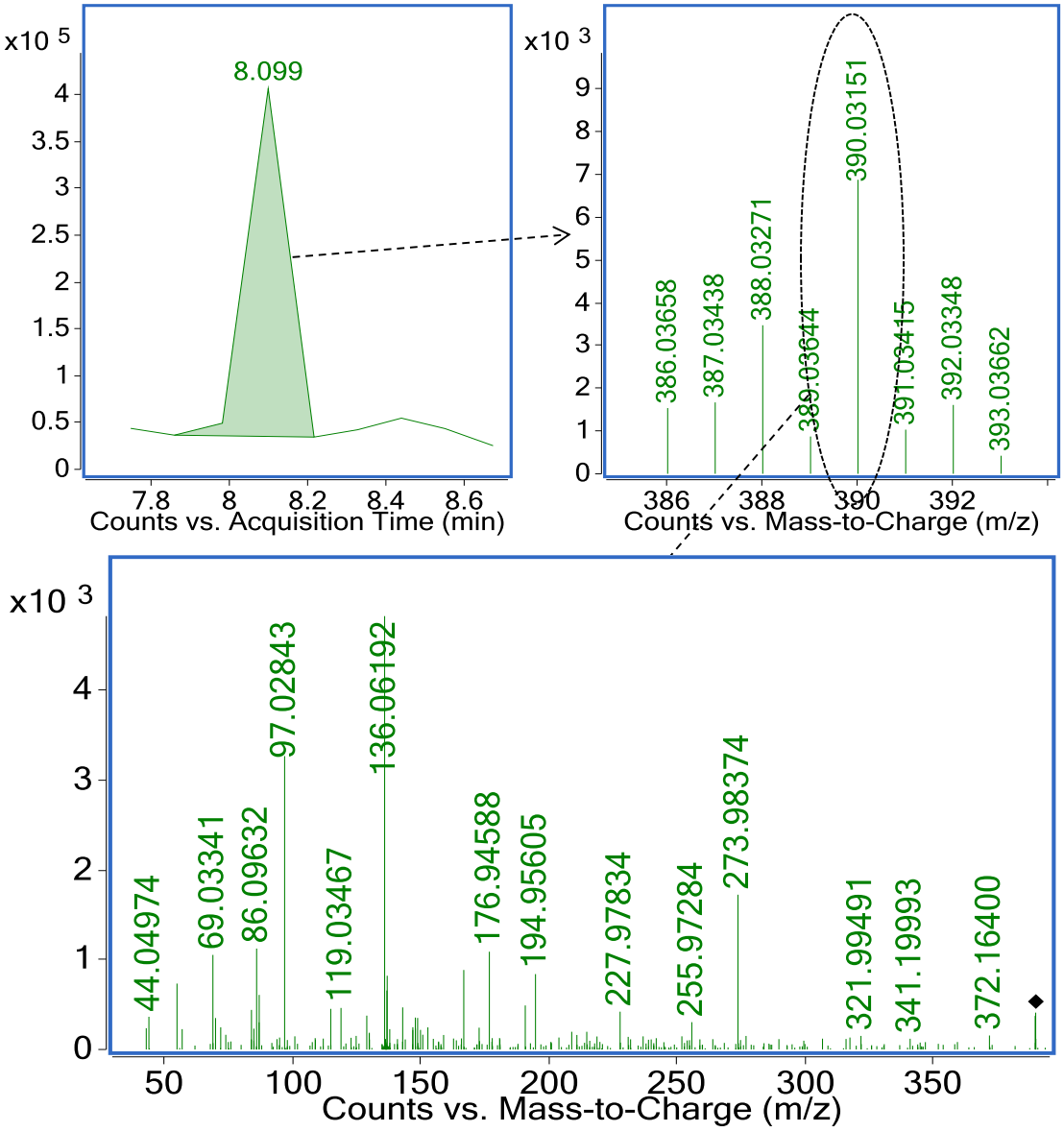
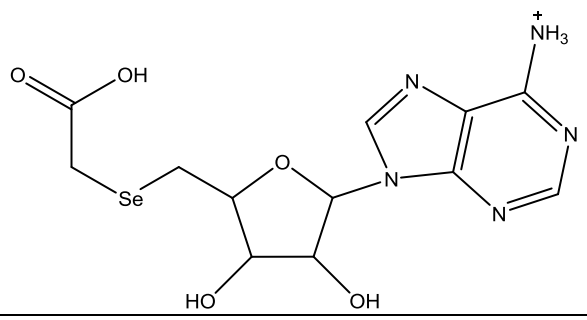


Figure A27: Chromatogram of *m/z* 390 selenocompound followed by MS[1] and MS[2] analysis.

Table A11: Elemental composition and proposed structure of *m/z* 390 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
390.03151	$C_{12}H_{16}N_5O_5Se^+$	390.03112	1.00	8.09	$C_7H_8N_5O_2Se^+$ (loss of $C_5H_8O_3$)	273.98374 (-0.11)
					$C_5H_5O_2Se^+$	176.94588 (5.37)
					$C_5H_6N_5^+$ (adenine frag)	136.06192 (-1.09)
					$C_5H_3N_4^+$	119.03467 (-4.62)
					$C_5H_5O_2^+$(dehydroxyribo-furanose frag)	97.02843 (-0.21)
					$C_5H_{12}N^+$	86.09632 (-1.23)
					$C_4H_5O^+$ (loss of CO from dehydroxyribo-furanose)	69.03341 (-1.18)



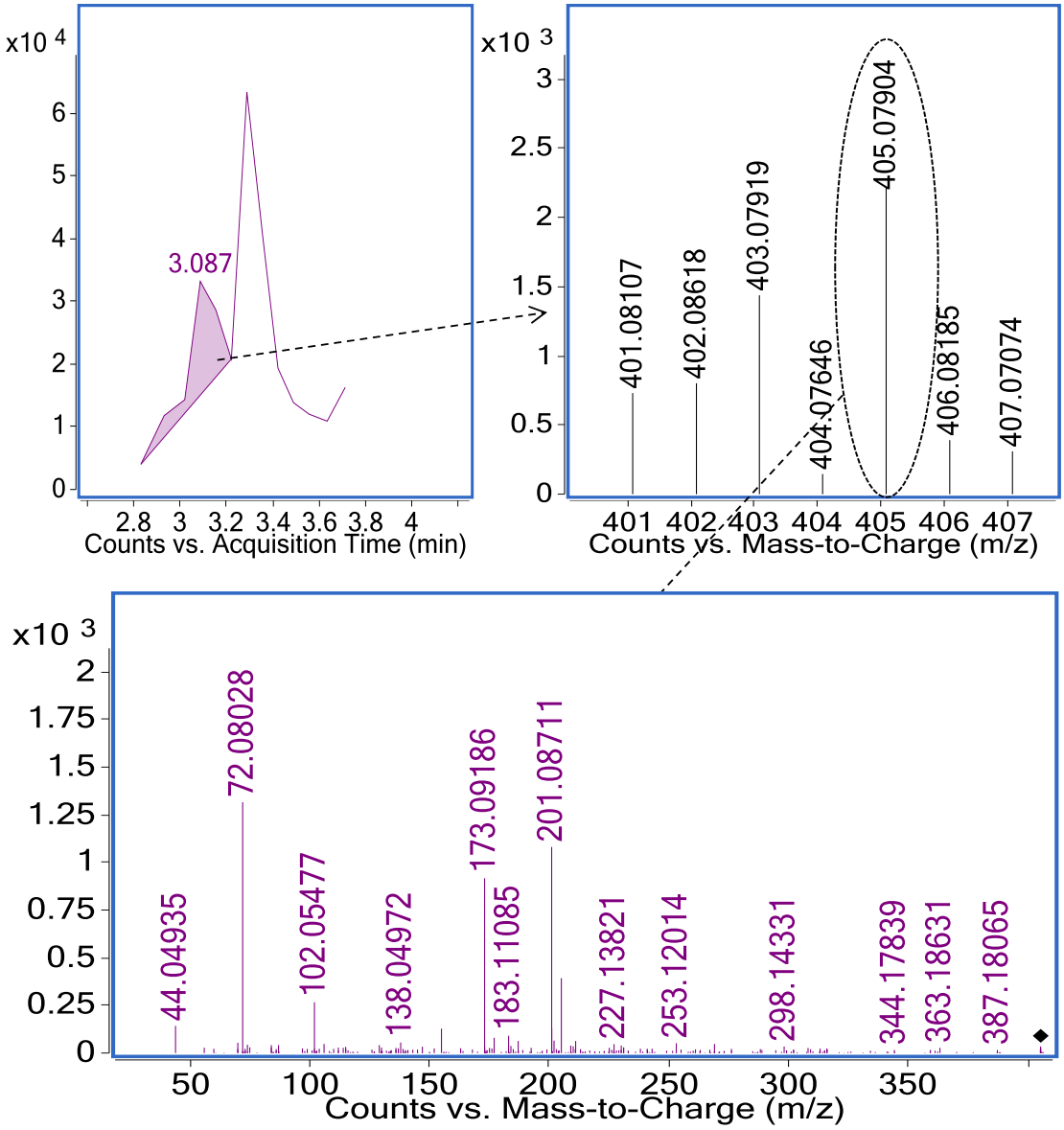
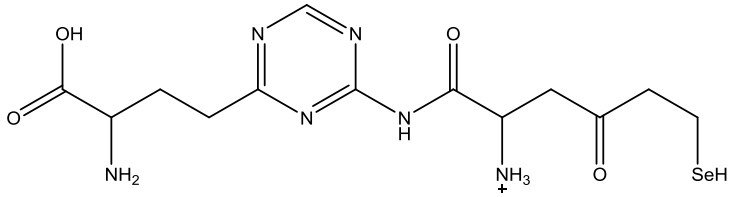


Figure A28: Chromatogram of *m/z* 405 selenocompound followed by MS[1] and MS[2] analysis.

Table A12: Elemental composition and proposed structure of *m/z* 405 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
405.07904	$C_{13}H_{21}N_6O_4Se^+$	405.07974	1.58	3.09	$C_8H_{13}N_2O_4^+$	201.08711 (0.65)
					$C_7H_{13}N_2O_3^+$	173.09186 (-1.21)
					$C_5H_9N_5O^+$	155.08035 (1.23)
					$C_4H_8NO_2^+$ (heteroatom-free SehomoCys frag)	102.05477 (-1.76)
					$C_4H_{10}N^+$	72.08028 (-6.88)



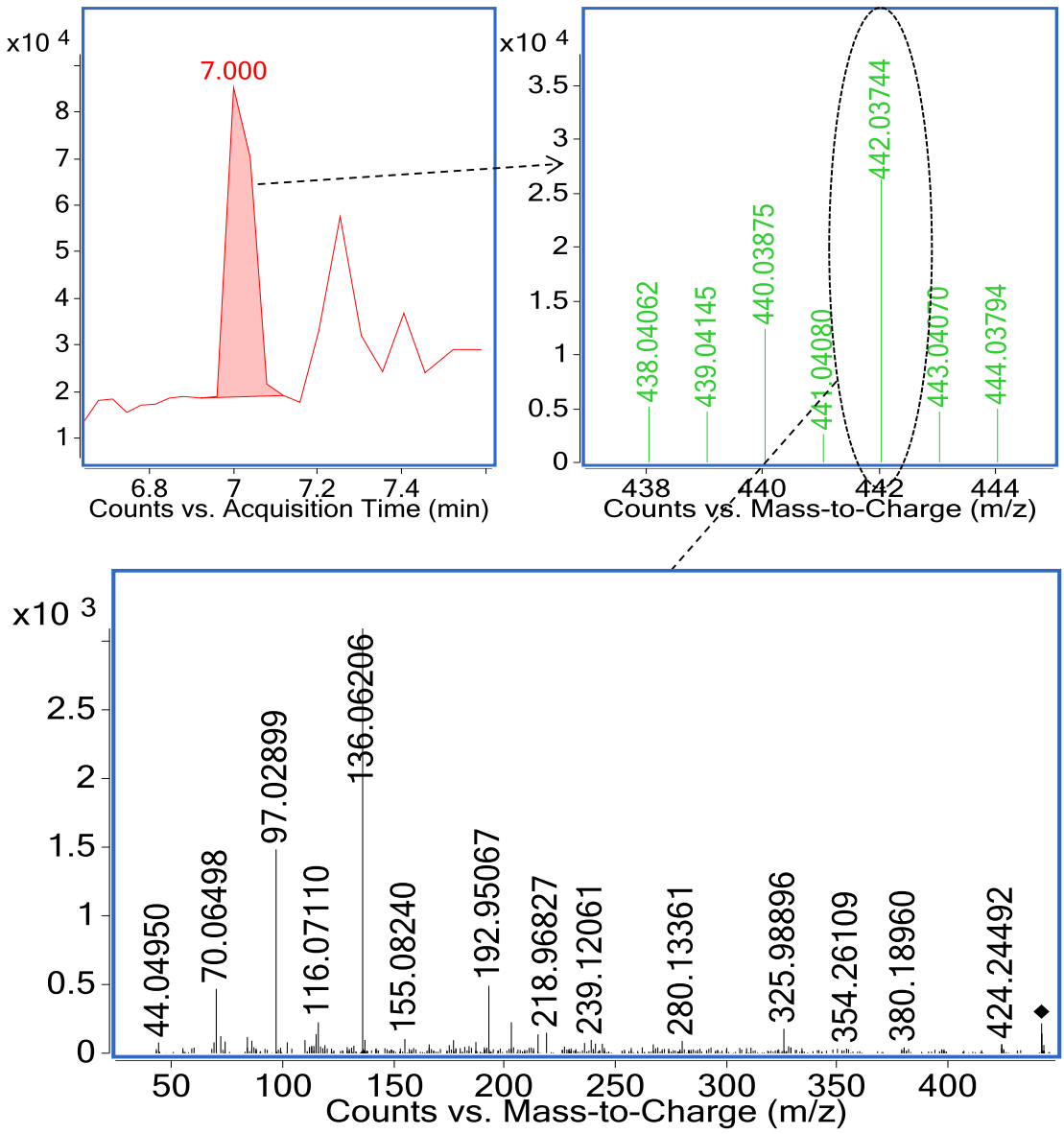
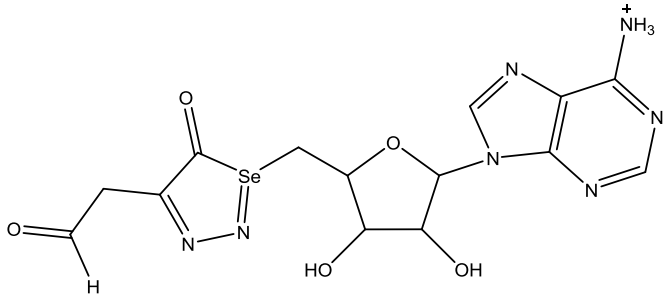


Figure A29: Chromatogram of *m/z* 442 selenocompound followed by MS[1] and MS[2] analysis.

Table A13: Elemental composition and proposed structure of *m/z* 442 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
442.03744	$C_{14}H_{16}N_7O_5Se^+$	442.03726	0.41	7.00	$C_9H_8N_7O_2Se^+$	325.98896 (-2.94)
					$C_4H_5N_2O_2Se^+$	192.95067 (-2.12)
					$C_5H_6N_5^+$ (adenine frag)	136.06206 (-2.12)
					$C_5H_5O_2^+$(dehydroxyribo-furanose frag)	97.02899 (6.02)
					$C_4H_8N^+$	70.06498 (-2.08)
					$C_2H_6N^+$	44.04950 (-0.55)



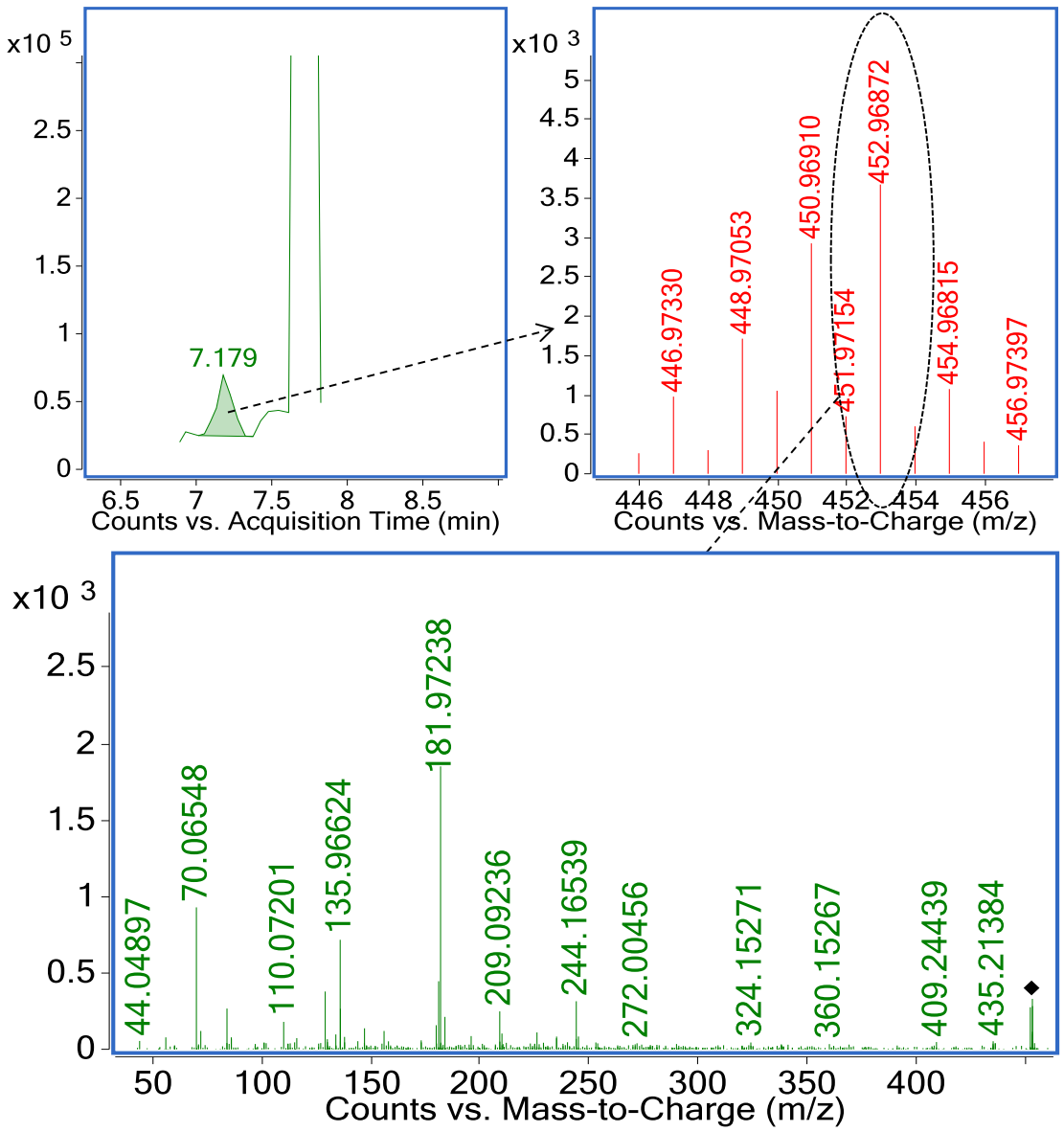
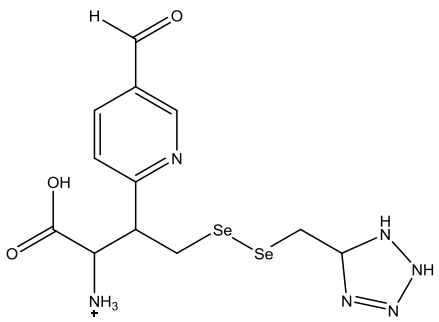


Figure A30: Chromatogram of *m/z* 453 selenocompound followed by MS[1] and MS[2] analysis.

Table A14: Elemental composition and proposed structure of *m/z* 453 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
452.96872	$C_{12}H_{17}N_6O_3Se_2^+$	452.96871	0.02	7.18	$C_{10}H_{13}N_2O_3^+$ (loss of $C_2H_4N_4Se_2$)	209.09236 (1.39)
					$C_4H_8NO_2Se^+$ (SehomoCys)	181.97238 (4.95)
					$C_3H_6NSe^+$ (loss of formic acid from SehomoCys)	135.96624 (1.77)
					$C_6H_{13}N_2O^+$	129.10266 (3.26)
					$C_5H_{10}N^+$	84.08079 (0.17)
					$C_4H_8N^+$	70.06548 (5.06)
					$C_3H_6N^+$ (loss of Se and formic acid from SehomoCys residue)	56.04982 (6.14)



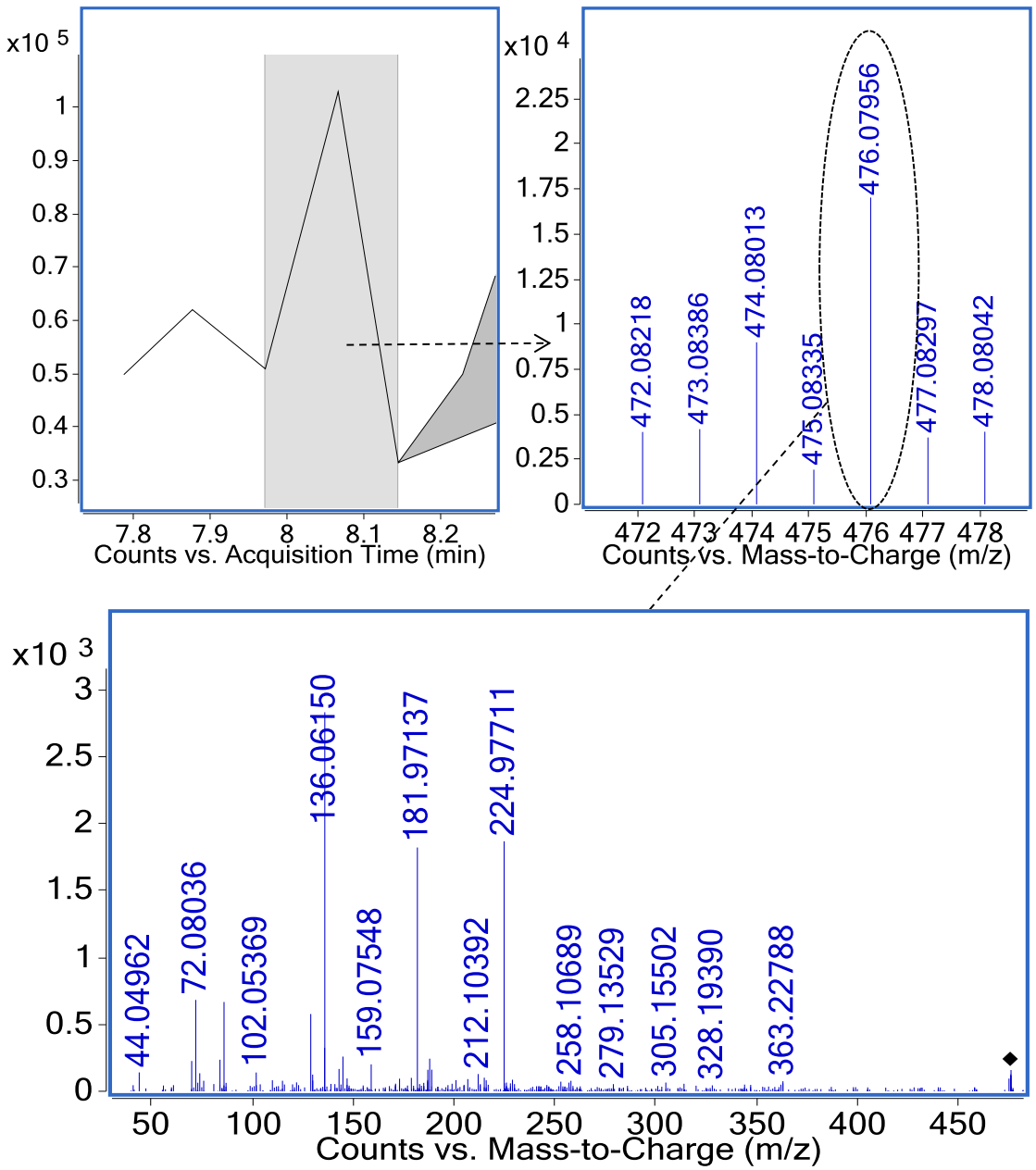
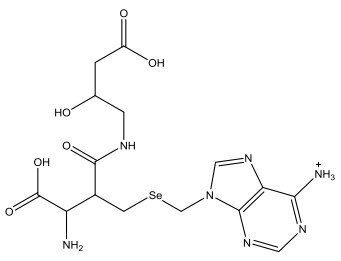


Figure A31: Chromatogram of *m/z* 476 selenocompound followed by MS[1] and MS[2] analysis.

Table A15: Elemental composition and proposed structure of *m/z* 476 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
476.07956	$C_{15}H_{22}N_7O_6Se^+$	476.07913	0.90	8.06	$C_5H_9N_2O_3Se^+$	224.97711 (-0.80)
					$C_4H_8NO_2Se^+$ (SehomoCys frag)	181.97137 (-0.60)
					$C_5H_6N_5^+$ (adenine frag)	136.06150 (-1.98)
					$C_5H_{12}N^+$	86.09693 (5.86)



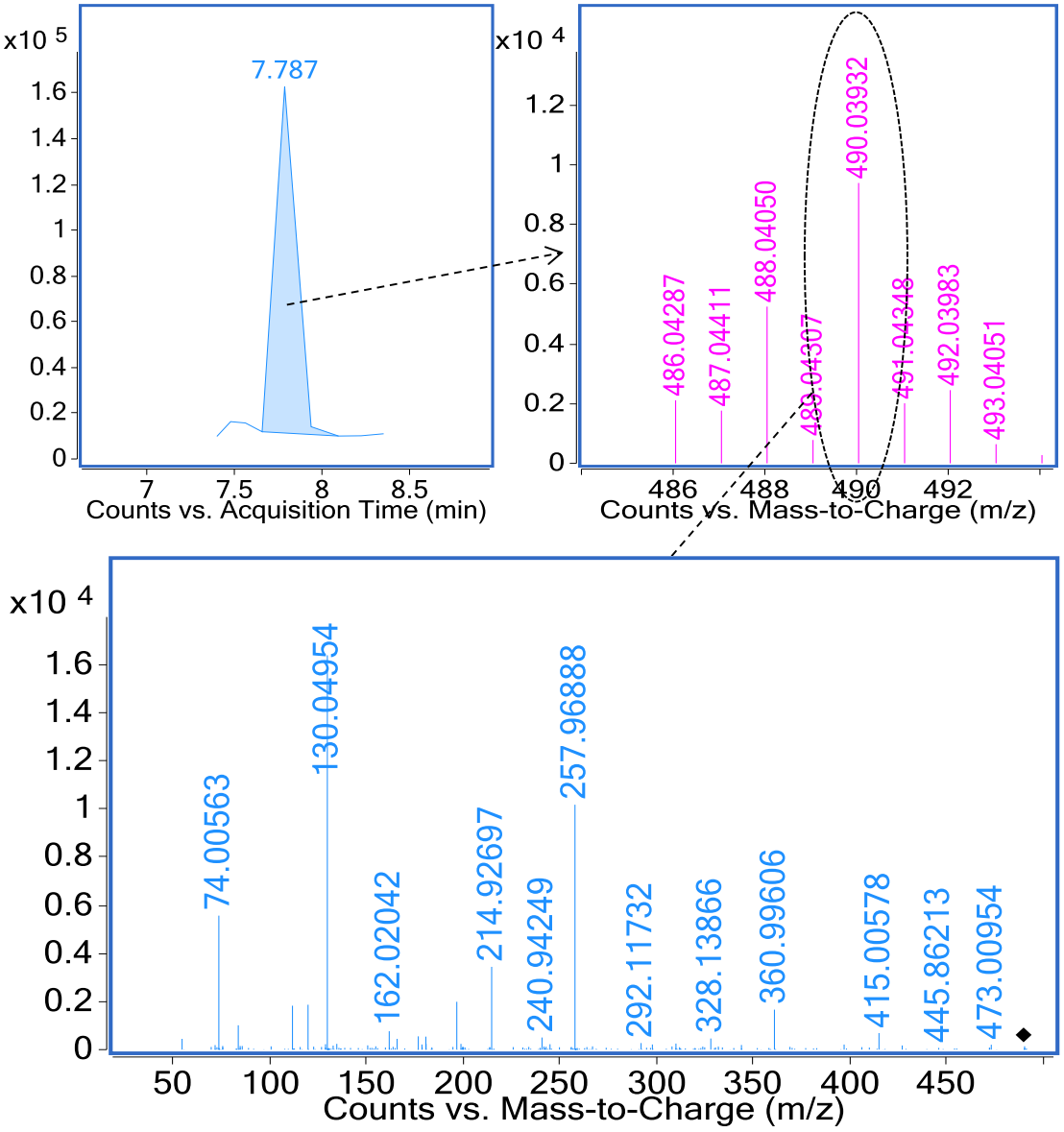


Figure A32: Chromatogram of *m/z* 490 selenocompound followed by MS[1] and MS[2] analysis.

Table A16: Elemental composition and proposed structure of *m/z* 490 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
490.03932	$C_{14}H_{24}N_3O_9SSe^+$	490.03930	0.04	7.79	$C_9H_{17}N_2O_6SSe^+$ (loss of $C_5H_7NO_3$)	360.99606 (-1.80)
					$C_6H_{12}NO_3SSe^+$ (loss of $C_3H_5NO_3$)	257.96888 (-3.41)
					$C_4H_7O_3SSe^+$ (loss of C_2H_5N)	214.92697 (-2.75)
					$C_5H_8NO_3^+$ (γ-Glu frag)	130.04954 (-2.54)
					$C_5H_6NO_2^+$ (loss of H_2O from γ-Glu frag)	112.03957 (2.41)
					$C_2H_4NS^+$	74.00563 (-3.65)

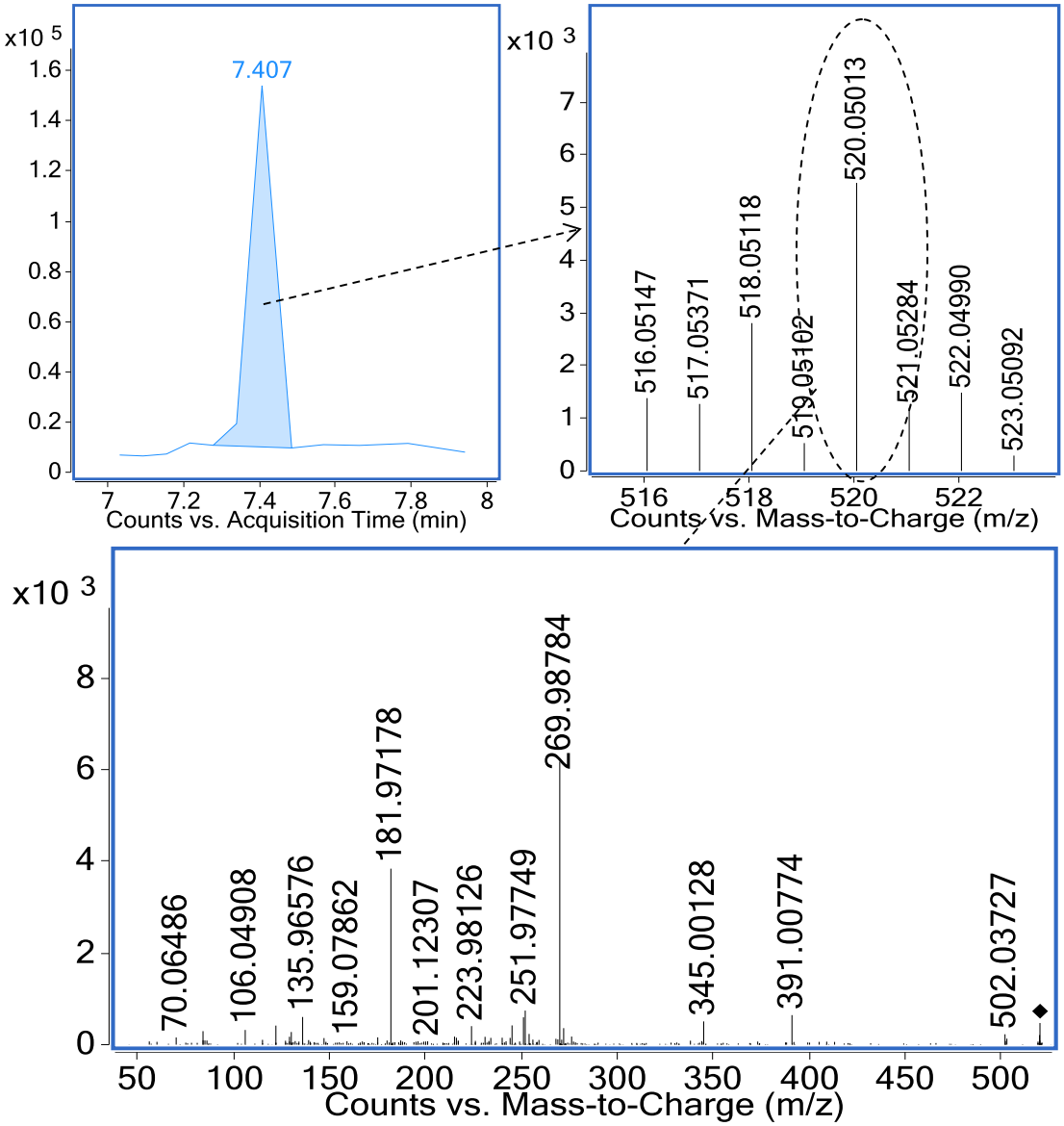
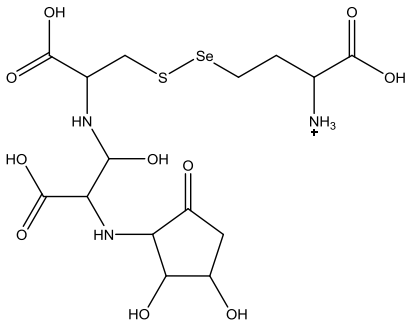
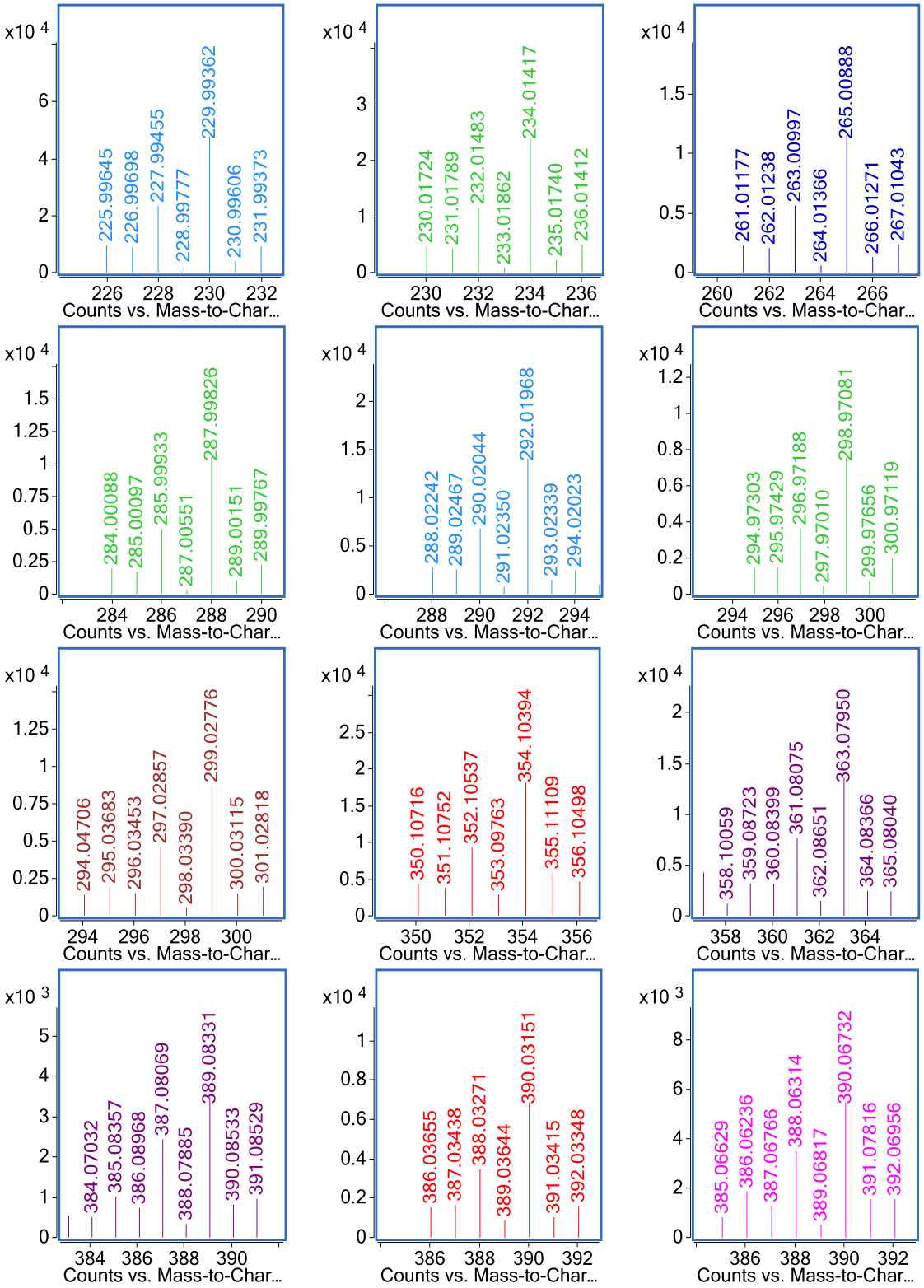


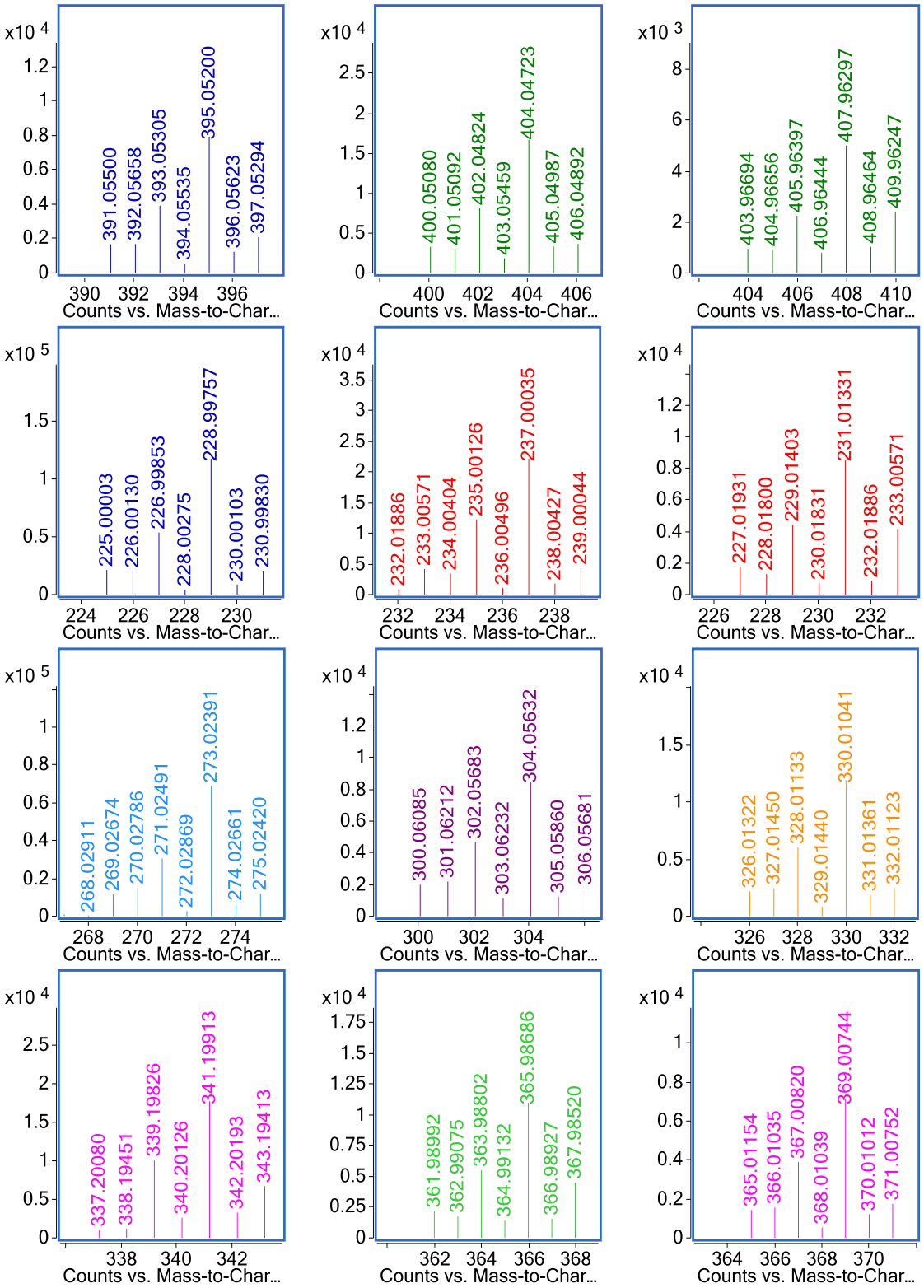
Figure A33: Chromatogram of *m/z* 520 selenocompound followed by MS[1] and MS[2] analysis.

Table A17: Elemental composition and proposed structure of *m/z* 520 based on exact mass, MS[2] and selenium isotopic pattern.

Experimental *m/z*	Elemental Composition	Theoretical *m/z*	Δ ppm	RT (min)	MS[2] Element Composition	*m/z* (Δppm)
520.05013	$C_{15}H_{26}N_3O_{10}SSe^+$	520.04986	0.52	7.42	$C_{10}H_{19}N_2O_7SSe^+$ (loss of $C_5H_7NO_3$)	391.00774 (1.20)
					$C_9H_{17}N_2O_5SSe^+$ (loss of formic acid, N-acetylcysteine-SehomoCys frag)	345.00128 (-1.48)
					$C_7H_{12}NO_5Se^+$	269.98784 (1.19)
					$C_4H_8NO_2Se^+$ (SehomoCys frag)	181.97178 (1.65)
					$C_3H_6NSe^+$ (loss of formic acid from SehomoCys frag)	135.96576 (-1.77)
					$C_3H_8NO_3^+$	106.04908 (-7.45)

Selenium compounds (single and double selenium isotopic patterns) detected in water-soluble extracts of four commercial yeast products.

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226.99853
228.00275
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230.00103
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Counts vs. Mass-to-Char...
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234.00404
235.00126
236.00496
237.00035
238.00427
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229.01403
230.01831
231.01331
232.01886
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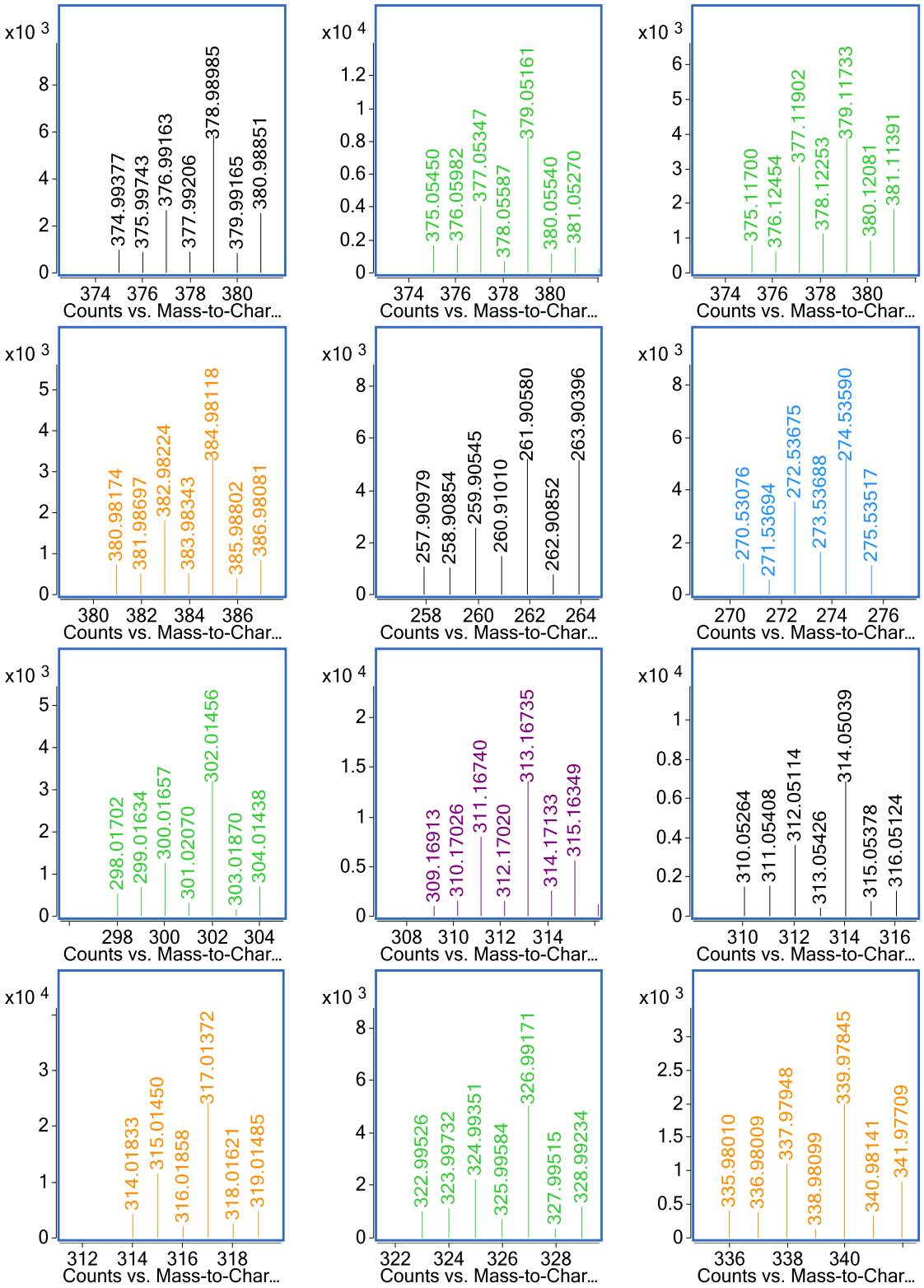
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Counts vs. Mass-to-Char...
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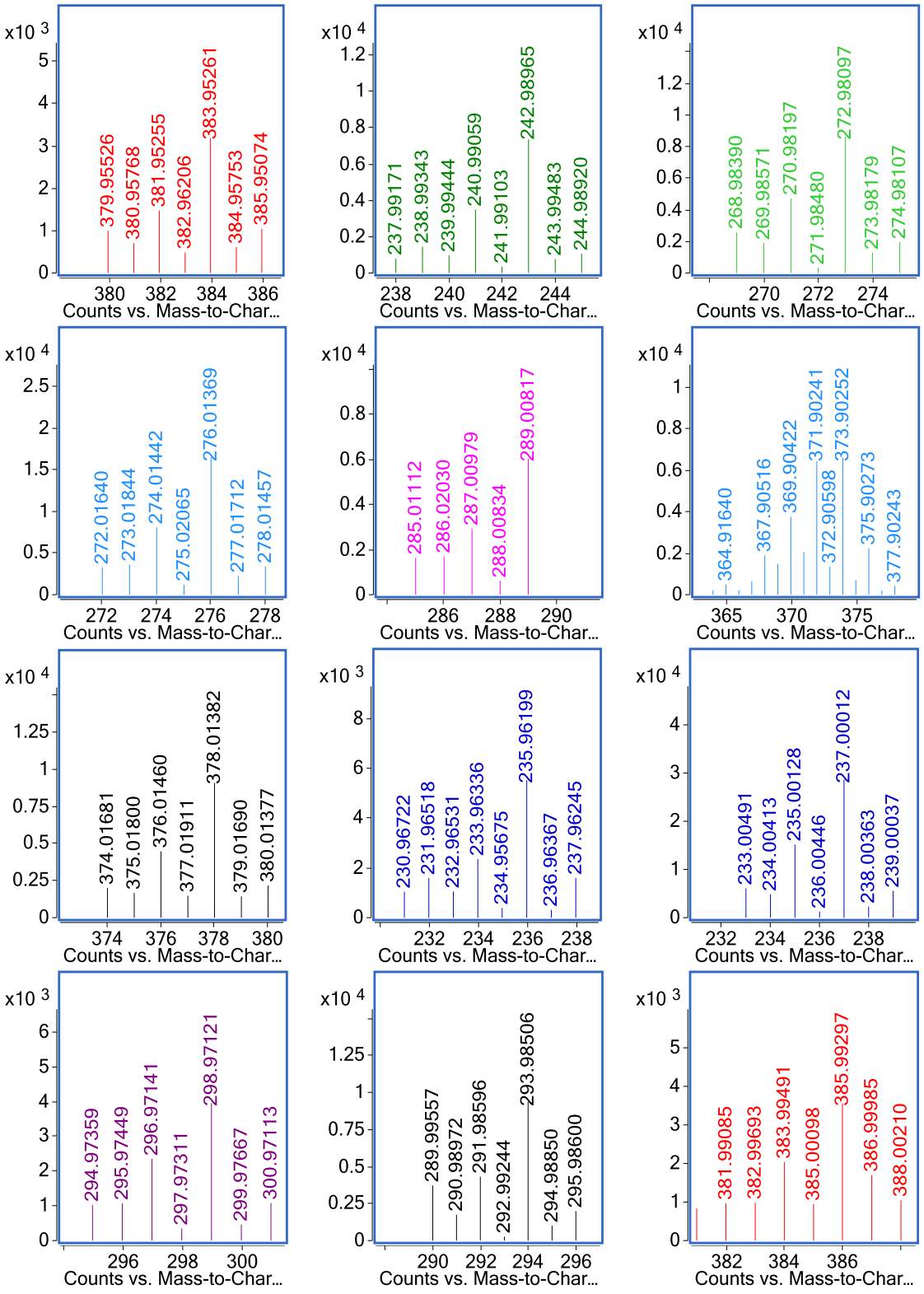
Counts vs. Mass-to-Char...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Counts vs. Mass-to-Char...
x10 4
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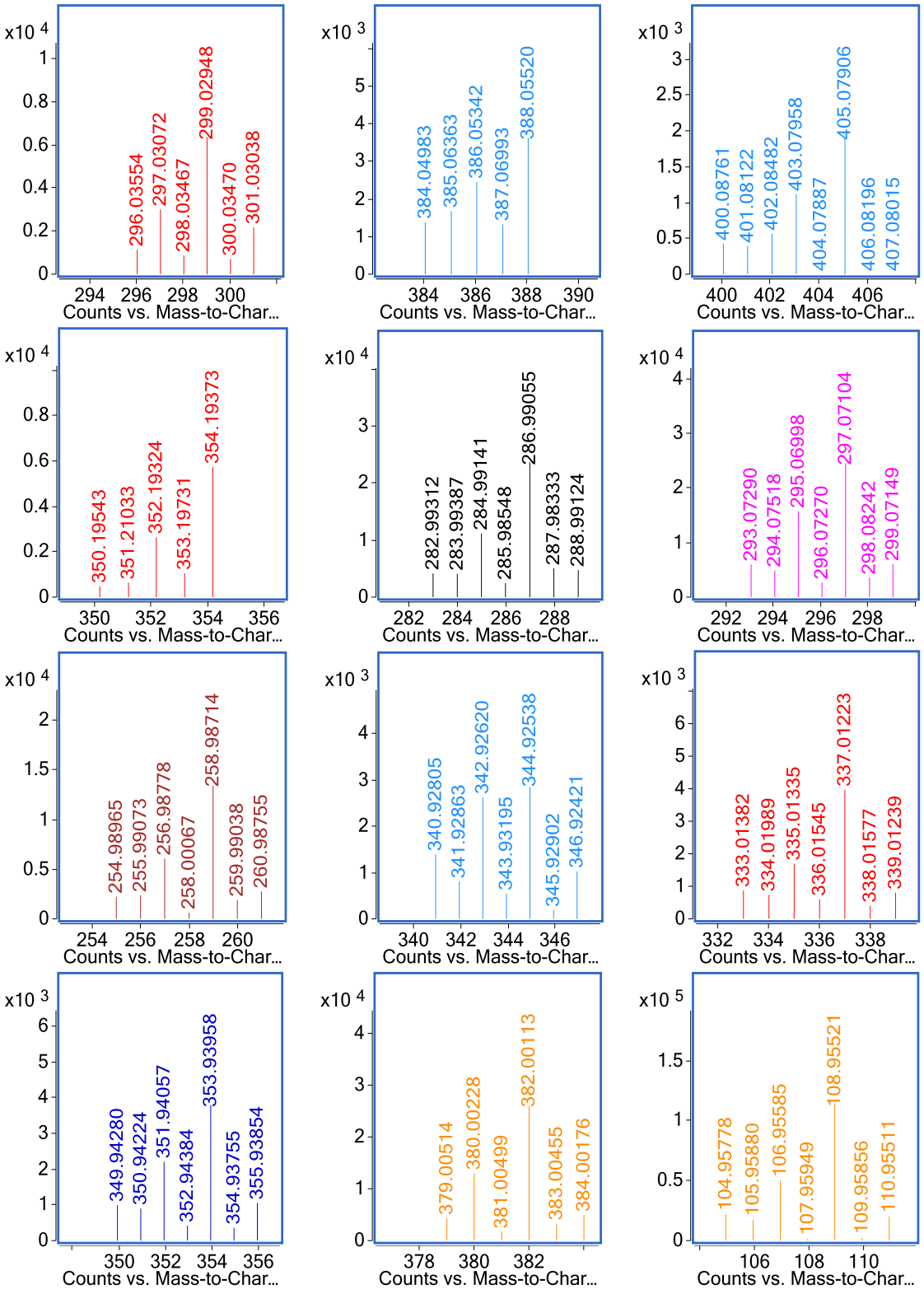
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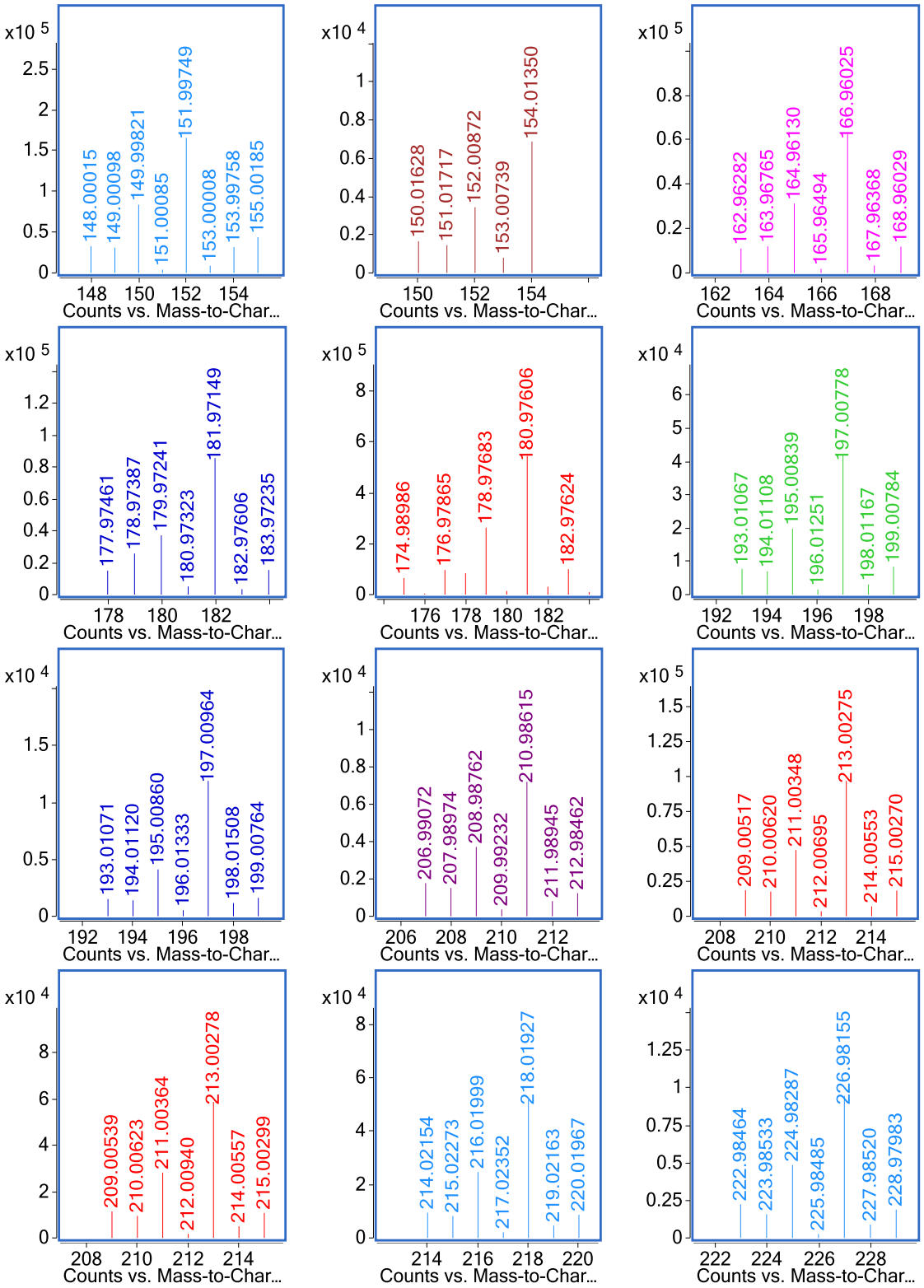
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162 164 166 168
Counts vs. Mass-to-Char...
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183.97235
178 180 182
Counts vs. Mass-to-Char...
x10 5
174.98986
176.97865
178.97683
180.97606
182.97624
176 178 180 182
Counts vs. Mass-to-Char...
x10 4
193.01067
194.01108
195.00839
196.01251
197.00778
198.01167
199.00784
192 194 196 198
Counts vs. Mass-to-Char...
x10 4
193.01071
194.01120
195.00860
196.01333
197.00964
198.01508
199.00764
192 194 196 198
Counts vs. Mass-to-Char...
x10 4
206.99072
207.98974
208.98762
209.99232
210.98615
211.98945
212.98462
206 208 210 212
Counts vs. Mass-to-Char...
x10 5
209.00517
210.00620
211.00348
212.00695
213.00275
214.00553
215.00270
208 210 212 214
Counts vs. Mass-to-Char...
x10 4
209.00539
210.00623
211.00364
212.00940
213.00278
214.00557
215.00299
208 210 212 214
Counts vs. Mass-to-Char...
x10 4
214.02154
215.02273
216.01999
217.02352
218.01927
219.02163
220.01967
214 216 218 220
Counts vs. Mass-to-Char...
x10 4
222.98464
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226.98155
227.98520
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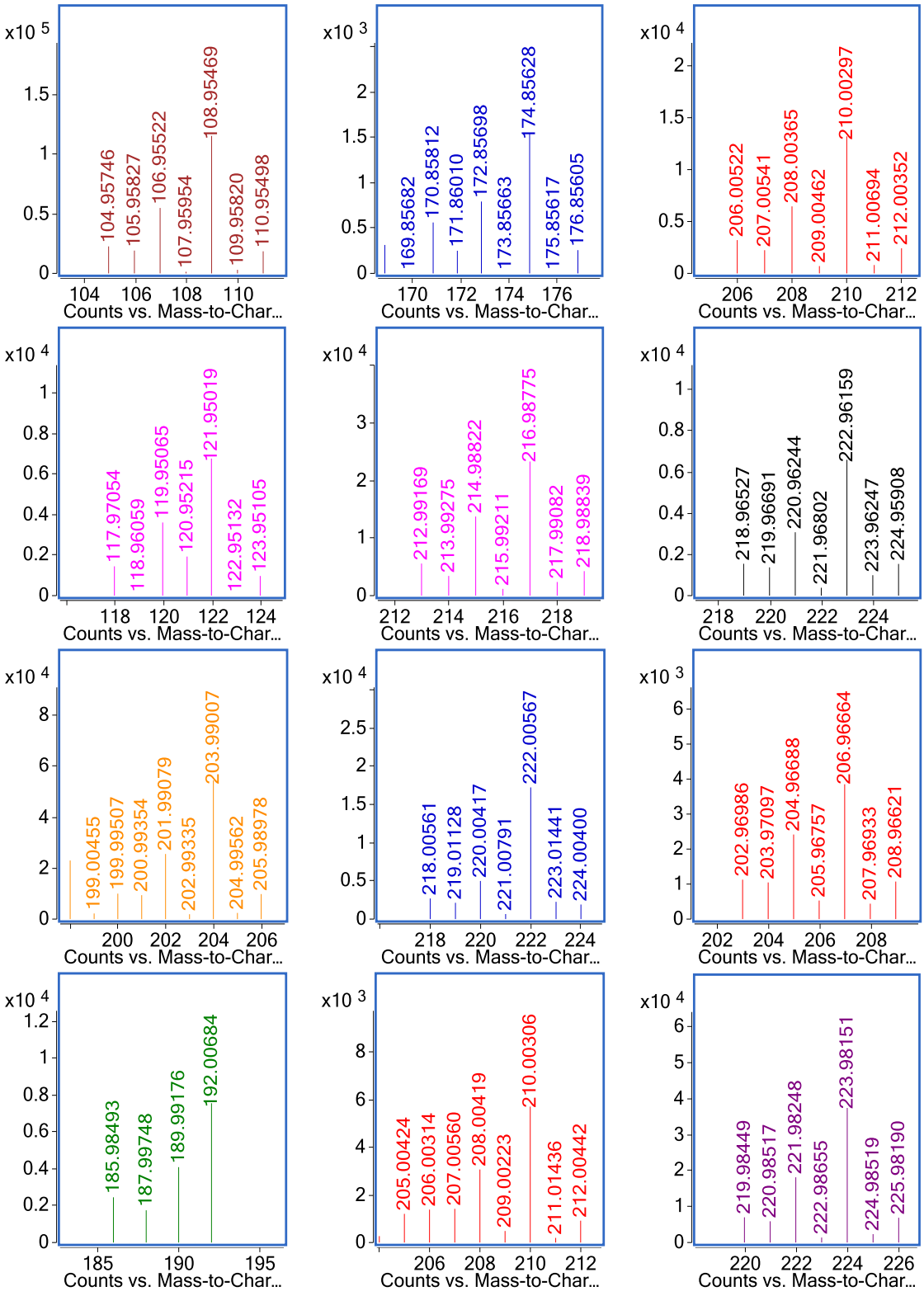
222 224 226 228
Counts vs. Mass-to-Char...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Counts vs. Mass-to-Char...
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Counts vs. Mass-to-Char...
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120.95215
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Counts vs. Mass-to-Char...
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Counts vs. Mass-to-Char...
x10 4
218.96527
219.96691
220.96244
221.96802
222.96159
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Counts vs. Mass-to-Char...
x10 4
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199.99507
200.99354
201.99079
202.99335
203.99007
204.99562
205.98978
Counts vs. Mass-to-Char...
x10 4
218.00561
219.01128
220.00417
221.00791
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223.01441
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Counts vs. Mass-to-Char...
x10 3
202.96986
203.97097
204.96688
205.96757
206.96664
207.96933
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Counts vs. Mass-to-Char...
x10 4
185.98493
187.99748
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192.00684
Counts vs. Mass-to-Char...
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205.00424
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208.00419
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Counts vs. Mass-to-Char...
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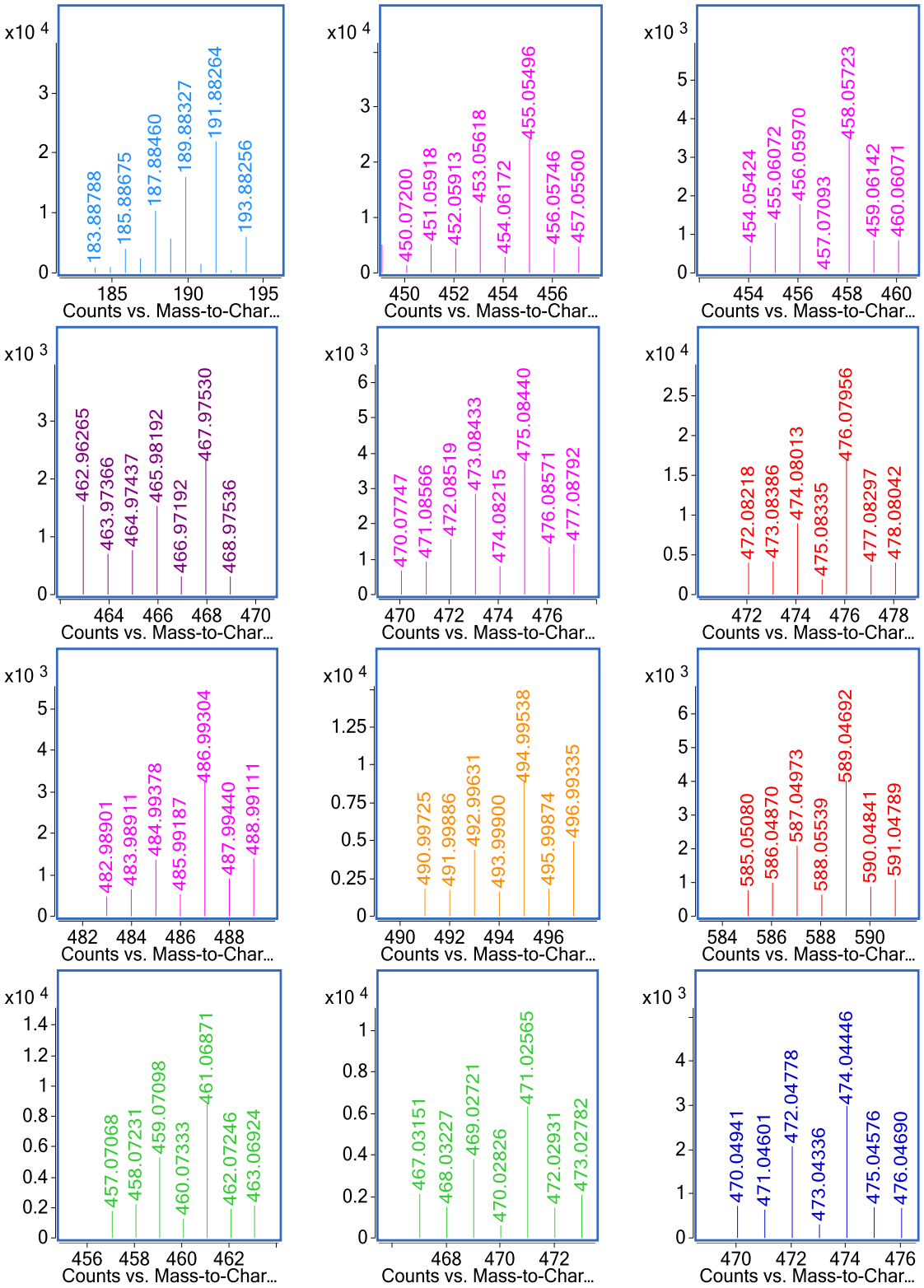
Counts vs. Mass-to-Char...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Counts vs. Mass-to-Char...
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451.05918
452.05913
453.05618
454.06172
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452
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Counts vs. Mass-to-Char...
x10 3
454.05424
455.06072
456.05970
457.07093
458.05723
459.06142
460.06071
454
456
458
460
Counts vs. Mass-to-Char...
x10 3
462.96265
463.97366
464.97437
465.98192
466.97192
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468.97536
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468
470
Counts vs. Mass-to-Char...
x10 3
470.07747
471.08566
472.08519
473.08433
474.08215
475.08440
476.08571
477.08792
470
472
474
476
Counts vs. Mass-to-Char...
x10 4
472.08218
473.08386
474.08013
475.08335
476.07956
477.08297
478.08042
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474
476
478
Counts vs. Mass-to-Char...
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482.98901
483.98911
484.99378
485.99187
486.99304
487.99440
488.99111
482
484
486
488
Counts vs. Mass-to-Char...
x10 4
490.99725
491.99886
492.99631
493.99900
494.99538
495.99874
496.99335
490
492
494
496
Counts vs. Mass-to-Char...
x10 3
585.05080
586.04870
587.04973
588.05539
589.04692
590.04841
591.04789
584
586
588
590
Counts vs. Mass-to-Char...
x10 4
457.07068
458.07231
459.07098
460.07333
461.06871
462.07246
463.06924
456
458
460
462
Counts vs. Mass-to-Char...
x10 4
467.03151
468.03227
469.02721
470.02826
471.02565
472.02931
473.02782
468
470
472
Counts vs. Mass-to-Char...
x10 3
470.04941
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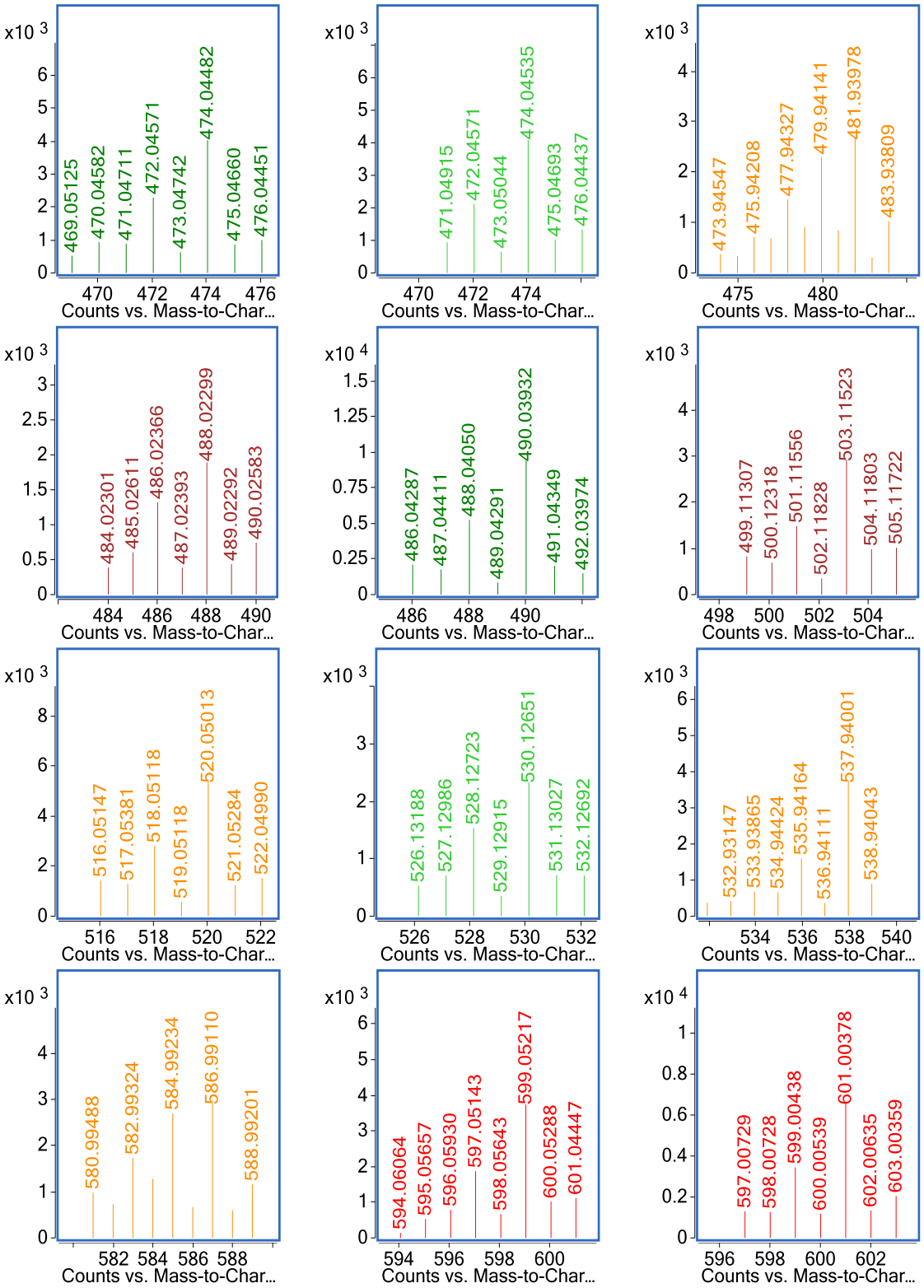
472
474
476
Counts vs. Mass-to-Char...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472.04571
473.05044
474.04535
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Counts vs. Mass-to-Char...
x10 3
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Counts vs. Mass-to-Char...
x10 3
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485.02611
486.02366
487.02393
488.02299
489.02292
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Counts vs. Mass-to-Char...
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488.04050
489.04291
490.03932
491.04349
492.03974
Counts vs. Mass-to-Char...
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499.11307
500.12318
501.11556
502.11828
503.11523
504.11803
505.11722
Counts vs. Mass-to-Char...
x10 3
516.05147
517.05381
518.05118
519.05118
520.05013
521.05284
522.04990
Counts vs. Mass-to-Char...
x10 3
526.13188
527.12986
528.12723
529.12915
530.12651
531.13027
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Counts vs. Mass-to-Char...
x10 3
532.93147
533.93865
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536.94111
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Counts vs. Mass-to-Char...
x10 3
580.99488
582.99324
584.99234
586.99110
588.99201
Counts vs. Mass-to-Char...
x10 3
594.06064
595.05657
596.05930
597.05143
598.05643
599.05217
600.05288
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Counts vs. Mass-to-Char...
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602.00635
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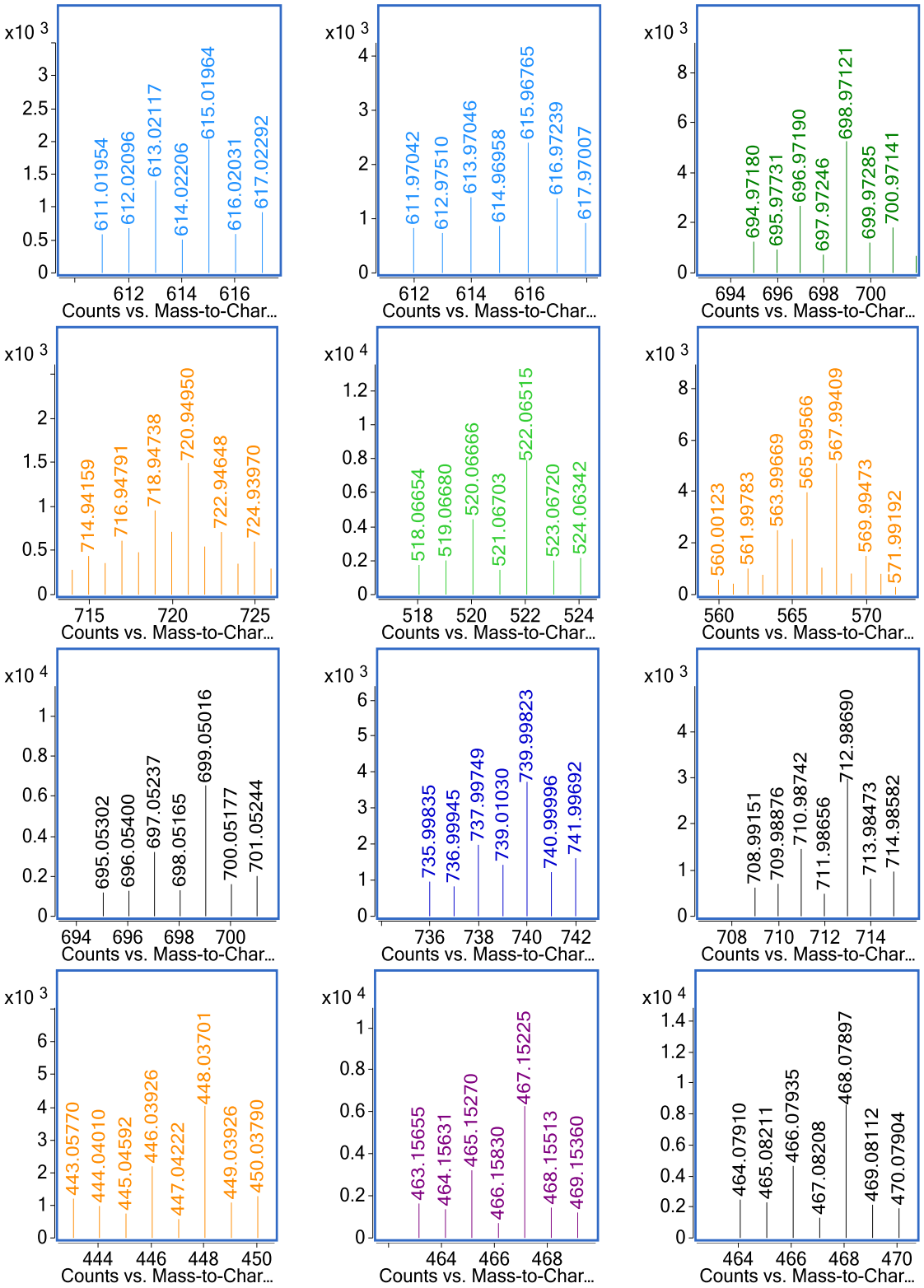
Counts vs. Mass-to-Char...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613.97046
614.96958
615.96765
616.97239
617.97007
Counts vs. Mass-to-Char...
x10 3
694.97180
695.97731
696.97190
697.97246
698.97121
699.97285
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Counts vs. Mass-to-Char...
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714.94159
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718.94738
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722.94648
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Counts vs. Mass-to-Char...
x10 4
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520.06666
521.06703
522.06515
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Counts vs. Mass-to-Char...
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563.99669
565.99566
567.99409
569.99473
571.99192
Counts vs. Mass-to-Char...
x10 4
695.05302
696.05400
697.05237
698.05165
699.05016
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701.05244
Counts vs. Mass-to-Char...
x10 3
735.99835
736.99945
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739.01030
739.99823
740.99996
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Counts vs. Mass-to-Char...
x10 3
708.99151
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710.98742
711.98656
712.98690
713.98473
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Counts vs. Mass-to-Char...
x10 3
443.05770
444.04010
445.04592
446.03926
447.04222
448.03701
449.03926
450.03790
Counts vs. Mass-to-Char...
x10 4
463.15655
464.15631
465.15270
466.15830
467.15225
468.15513
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Counts vs. Mass-to-Char...
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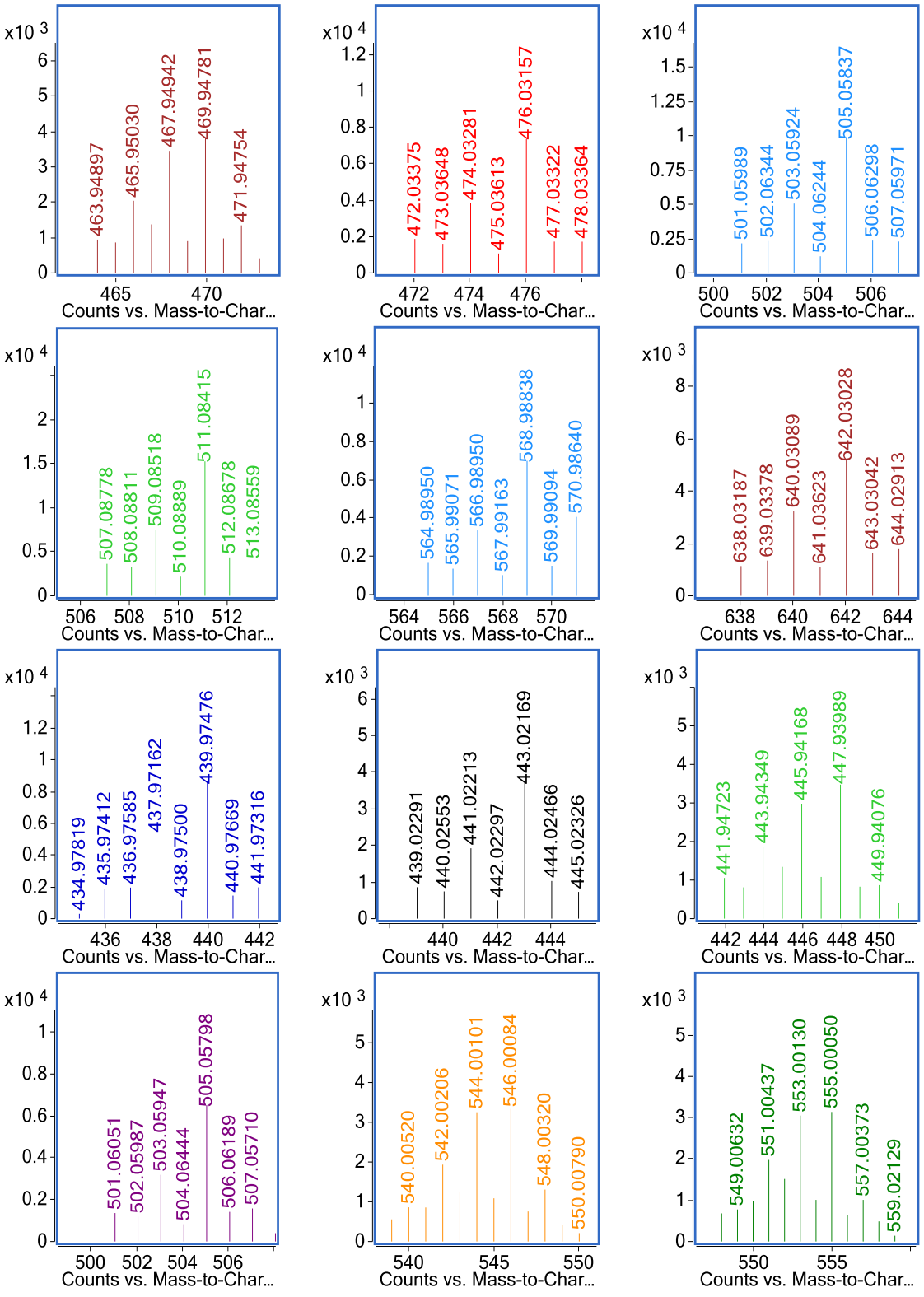
Counts vs. Mass-to-Char...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Counts vs. Mass-to-Char...
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472.03375
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475.03613
476.03157
477.03322
478.03364
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474
476
Counts vs. Mass-to-Char...
x10 4
501.05989
502.06344
503.05924
504.06244
505.05837
506.06298
507.05971
500
502
504
506
Counts vs. Mass-to-Char...
x10 4
507.08778
508.08811
509.08518
510.08889
511.08415
512.08678
513.08559
506
508
510
512
Counts vs. Mass-to-Char...
x10 4
564.98950
565.99071
566.98950
567.99163
568.98838
569.99094
570.98640
564
566
568
570
Counts vs. Mass-to-Char...
x10 3
638.03187
639.03378
640.03089
641.03623
642.03028
643.03042
644.02913
638
640
642
644
Counts vs. Mass-to-Char...
x10 4
434.97819
435.97412
436.97585
437.97162
438.97500
439.97476
440.97669
441.97316
436
438
440
442
Counts vs. Mass-to-Char...
x10 3
439.02291
440.02553
441.02213
442.02297
443.02169
444.02466
445.02326
440
442
444
Counts vs. Mass-to-Char...
x10 3
441.94723
443.94349
445.94168
447.93989
449.94076
442
444
446
448
450
Counts vs. Mass-to-Char...
x10 4
501.06051
502.05987
503.05947
504.06444
505.05798
506.06189
507.05710
500
502
504
506
Counts vs. Mass-to-Char...
x10 3
540.00520
542.00206
544.00101
546.00084
548.00320
550.00790
540
545
550
Counts vs. Mass-to-Char...
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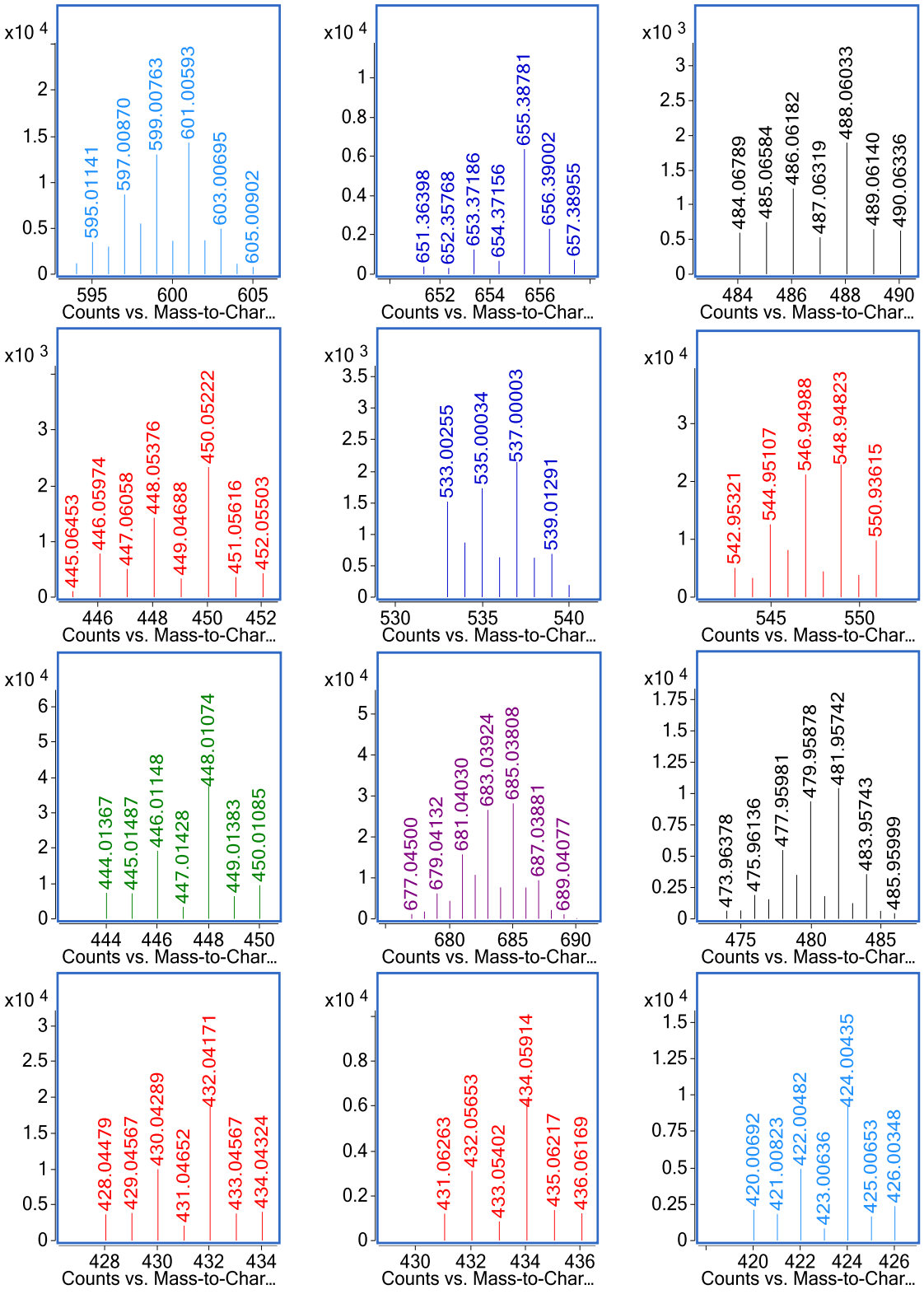
555
Counts vs. Mass-to-Char...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605
Counts vs. Mass-to-Char...
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651.36398
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654.37156
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657.38955
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654
656
Counts vs. Mass-to-Char...
x10 3
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486.06182
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488.06033
489.06140
490.06336
484
486
488
490
Counts vs. Mass-to-Char...
x10 3
445.06453
446.05974
447.06058
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451.05616
452.05503
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448
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452
Counts vs. Mass-to-Char...
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537.00003
539.01291
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535
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Counts vs. Mass-to-Char...
x10 4
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548.94823
550.93615
545
550
Counts vs. Mass-to-Char...
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447.01428
448.01074
449.01383
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444
446
448
450
Counts vs. Mass-to-Char...
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677.04500
679.04132
681.04030
683.03924
685.03808
687.03881
689.04077
680
685
690
Counts vs. Mass-to-Char...
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473.96378
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477.95981
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475
480
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Counts vs. Mass-to-Char...
x10 4
428.04479
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430.04289
431.04652
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433.04567
434.04324
428
430
432
434
Counts vs. Mass-to-Char...
x10 4
431.06263
432.05653
433.05402
434.05914
435.06217
436.06169
430
432
434
436
Counts vs. Mass-to-Char...
x10 4
420.00692
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422
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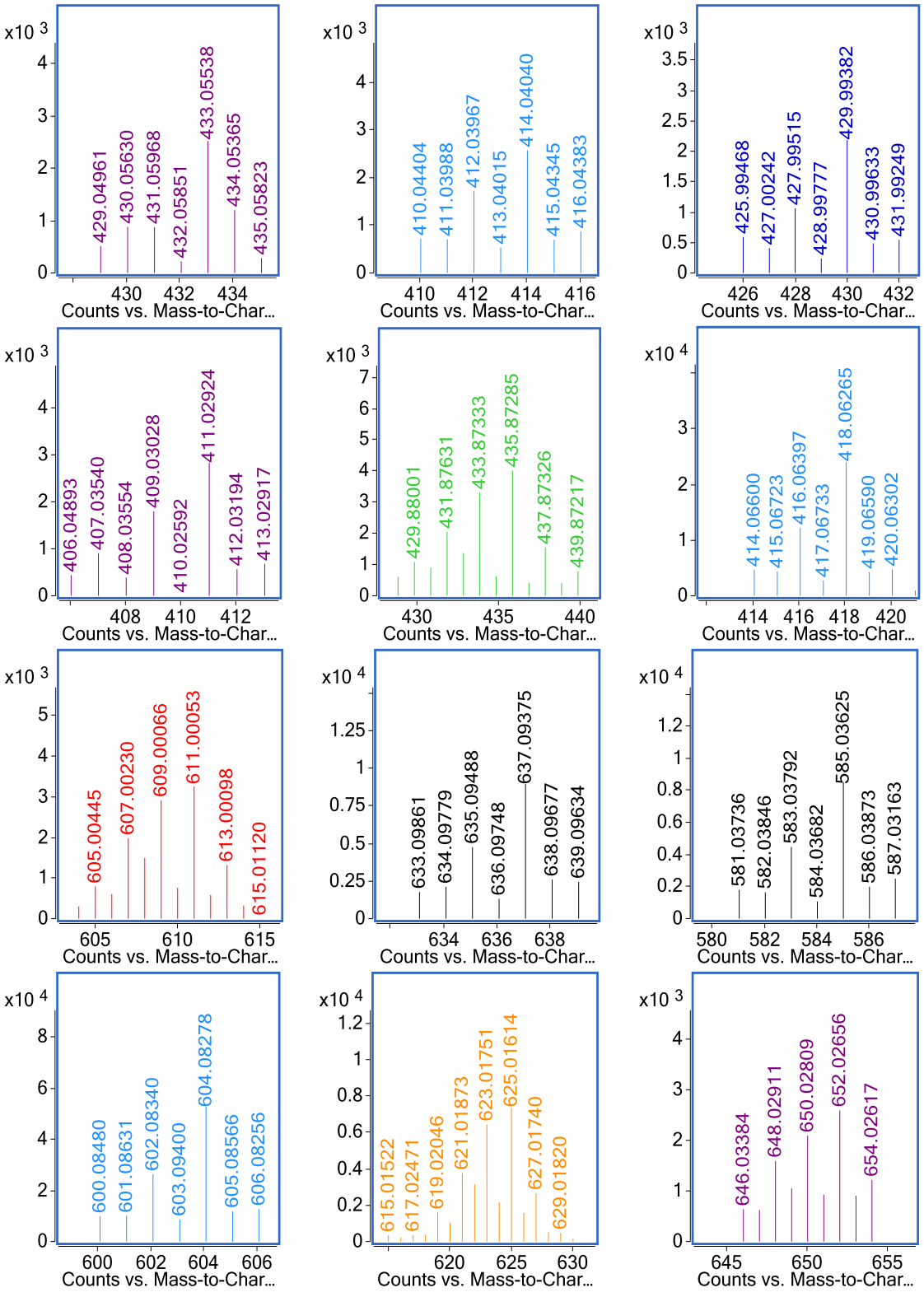
Counts vs. Mass-to-Char...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412.03967
413.04015
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Counts vs. Mass-to-Char...
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Counts vs. Mass-to-Char...
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408.03554
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Counts vs. Mass-to-Char...
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609.00066
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613.00098
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Counts vs. Mass-to-Char...
x10 4
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635.09488
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Counts vs. Mass-to-Char...
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581.03736
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586.03873
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Counts vs. Mass-to-Char...
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600.08480
601.08631
602.08340
603.09400
604.08278
605.08566
606.08256
Counts vs. Mass-to-Char...
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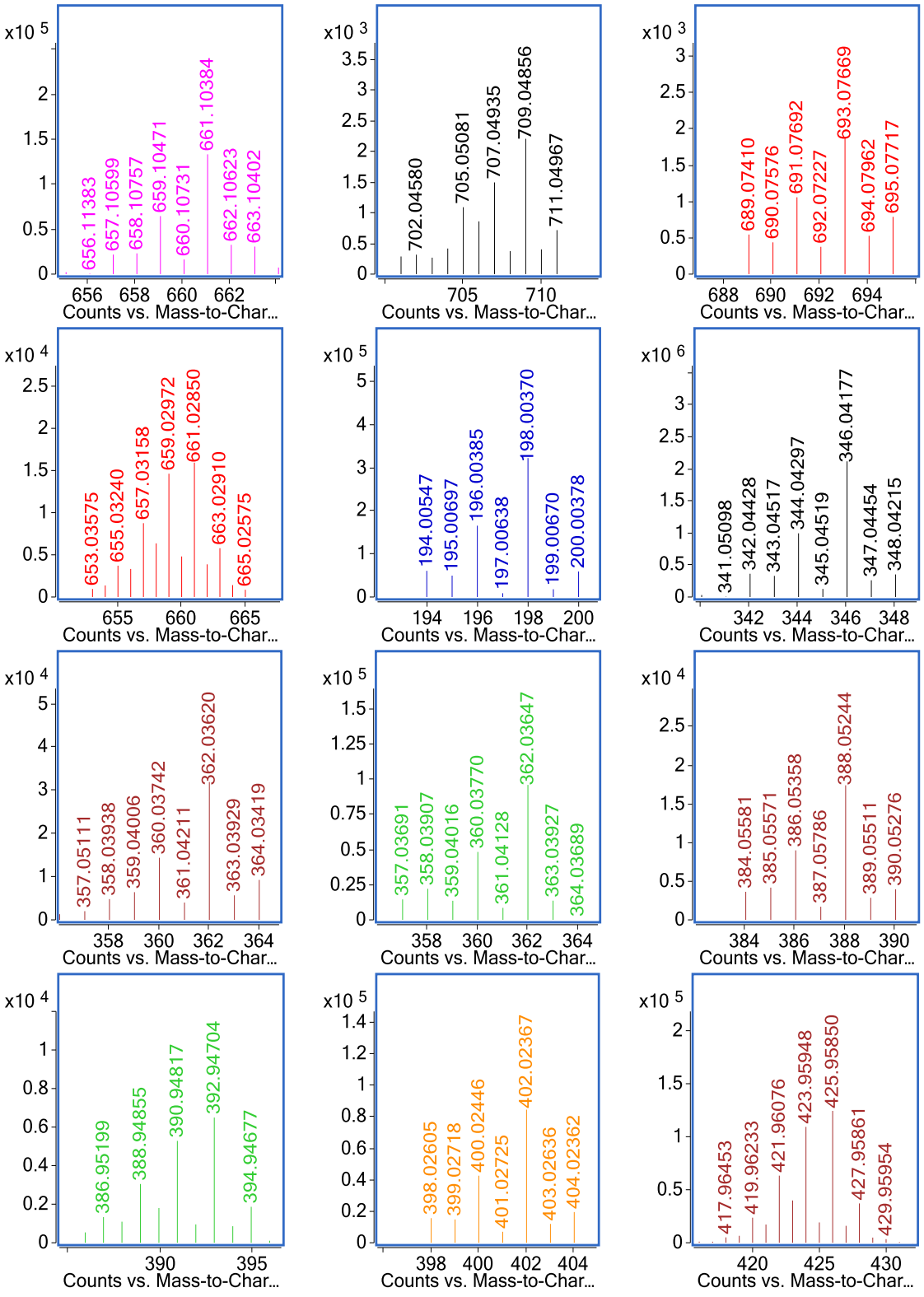
Counts vs. Mass-to-Char...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Counts vs. Mass-to-Char...
x10 3
702.04580
705.05081
707.04935
709.04856
711.04967
705 710
Counts vs. Mass-to-Char...
x10 3
689.07410
690.07576
691.07692
692.07227
693.07669
694.07962
695.07717
688 690 692 694
Counts vs. Mass-to-Char...
x10 4
653.03575
655.03240
657.03158
659.02972
661.02850
663.02910
665.02575
655 660 665
Counts vs. Mass-to-Char...
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196.00385
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199.00670
200.00378
194 196 198 200
Counts vs. Mass-to-Char...
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344.04297
345.04519
346.04177
347.04454
348.04215
342 344 346 348
Counts vs. Mass-to-Char...
x10 4
357.05111
358.03938
359.04006
360.03742
361.04211
362.03620
363.03929
364.03419
358 360 362 364
Counts vs. Mass-to-Char...
x10 5
357.03691
358.03907
359.04016
360.03770
361.04128
362.03647
363.03927
364.03689
358 360 362 364
Counts vs. Mass-to-Char...
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384.05581
385.05571
386.05358
387.05786
388.05244
389.05511
390.05276
384 386 388 390
Counts vs. Mass-to-Char...
x10 4
386.95199
388.94855
390.94817
392.94704
394.94677
390 395
Counts vs. Mass-to-Char...
x10 5
398.02605
399.02718
400.02446
401.02725
402.02367
403.02636
404.02362
398 400 402 404
Counts vs. Mass-to-Char...
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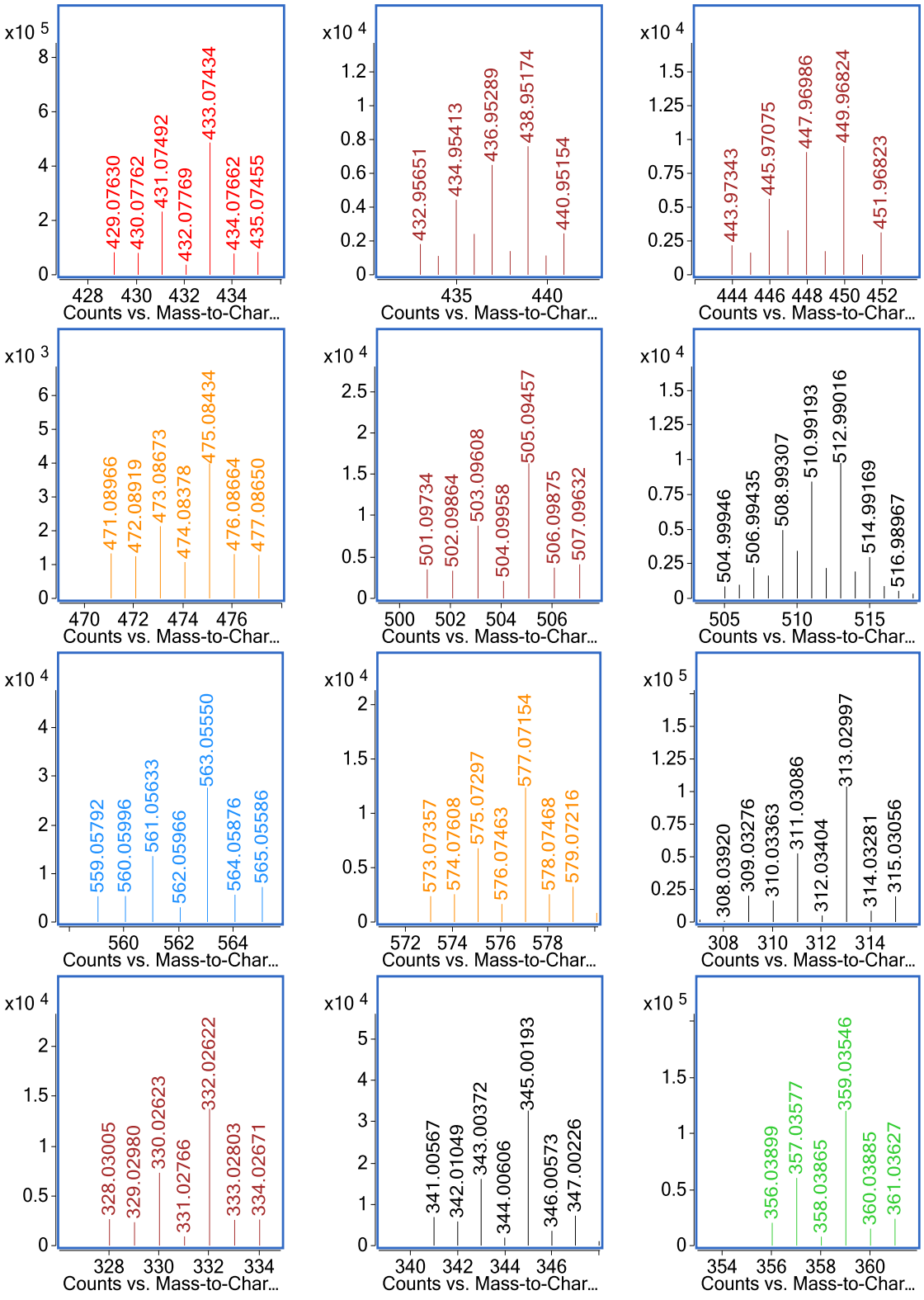
Counts vs. Mass-to-Char...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477.08650
Counts vs. Mass-to-Char...
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Counts vs. Mass-to-Char...
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510.99193
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Counts vs. Mass-to-Char...
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561.05633
562.05966
563.05550
564.05876
565.05586
Counts vs. Mass-to-Char...
x10 4
573.07357
574.07608
575.07297
576.07463
577.07154
578.07468
579.07216
Counts vs. Mass-to-Char...
x10 5
308.03920
309.03276
310.03363
311.03086
312.03404
313.02997
314.03281
315.03056
Counts vs. Mass-to-Char...
x10 4
328.03005
329.02980
330.02623
331.02766
332.02622
333.02803
334.02671
Counts vs. Mass-to-Char...
x10 4
341.00567
342.01049
343.00372
344.00606
345.00193
346.00573
347.00226
Counts vs. Mass-to-Char...
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358.03865
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360.03885
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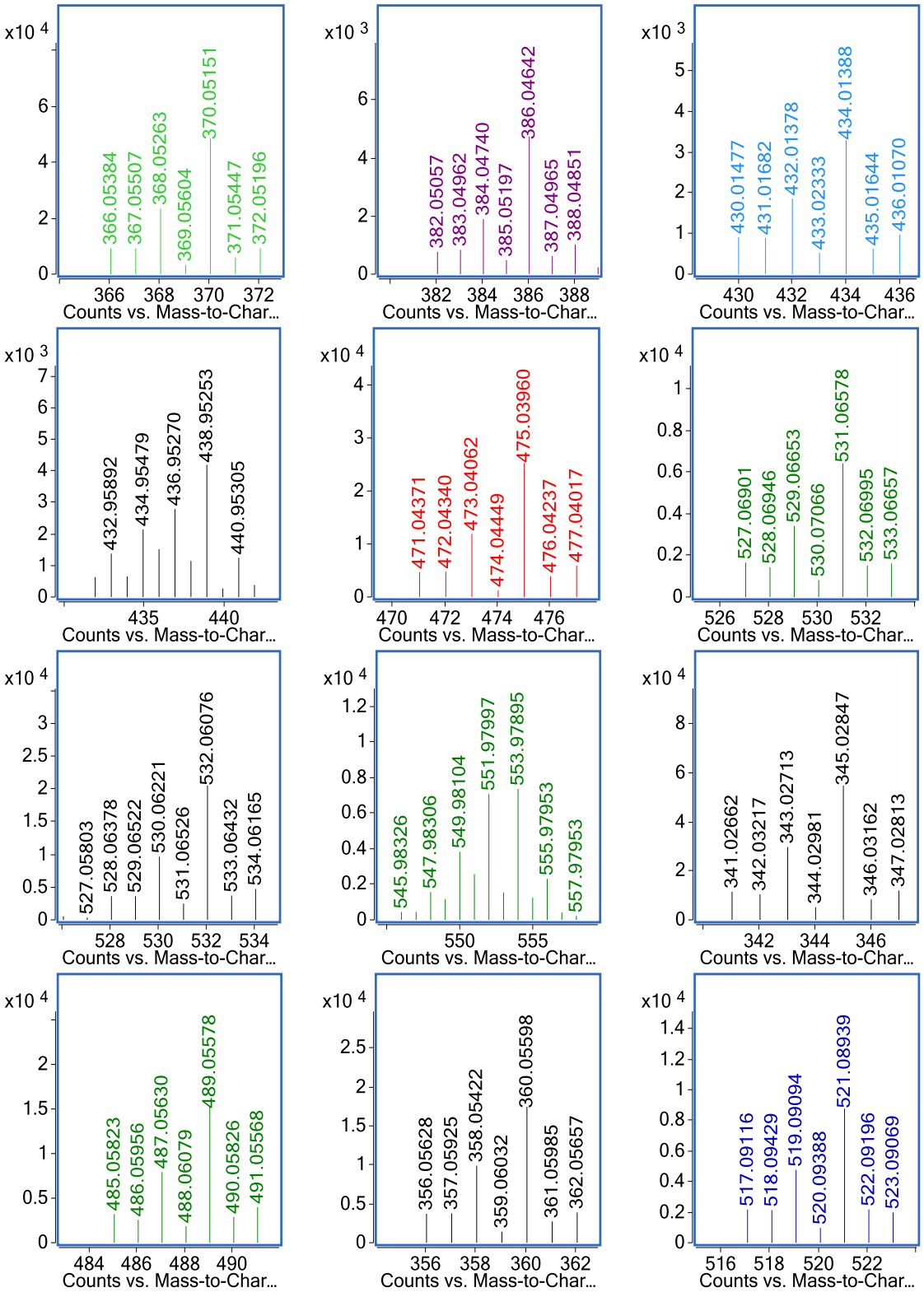
Counts vs. Mass-to-Char...


x10 4
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370.05151
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372.05196
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384.04740
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386.04642
387.04965
388.04851
382 384 386 388
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432.01378
433.02333
434.01388
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430 432 434 436
Counts vs. Mass-to-Char...
x10 3
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434.95479
436.95270
438.95253
440.95305
435 440
Counts vs. Mass-to-Char...
x10 4
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472.04340
473.04062
474.04449
475.03960
476.04237
477.04017
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Counts vs. Mass-to-Char...
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528.06946
529.06653
530.07066
531.06578
532.06995
533.06657
526 528 530 532
Counts vs. Mass-to-Char...
x10 4
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528.06378
529.06522
530.06221
531.06526
532.06076
533.06432
534.06165
528 530 532 534
Counts vs. Mass-to-Char...
x10 4
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547.98306
549.98104
551.97997
553.97895
555.97953
557.97953
550 555
Counts vs. Mass-to-Char...
x10 4
341.02662
342.03217
343.02713
344.02981
345.02847
346.03162
347.02813
342 344 346
Counts vs. Mass-to-Char...
x10 4
485.05823
486.05956
487.05630
488.06079
489.05578
490.05826
491.05568
484 486 488 490
Counts vs. Mass-to-Char...
x10 4
356.05628
357.05925
358.05422
359.06032
360.05598
361.05985
362.05657
356 358 360 362
Counts vs. Mass-to-Char...
x10 4
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519.09094
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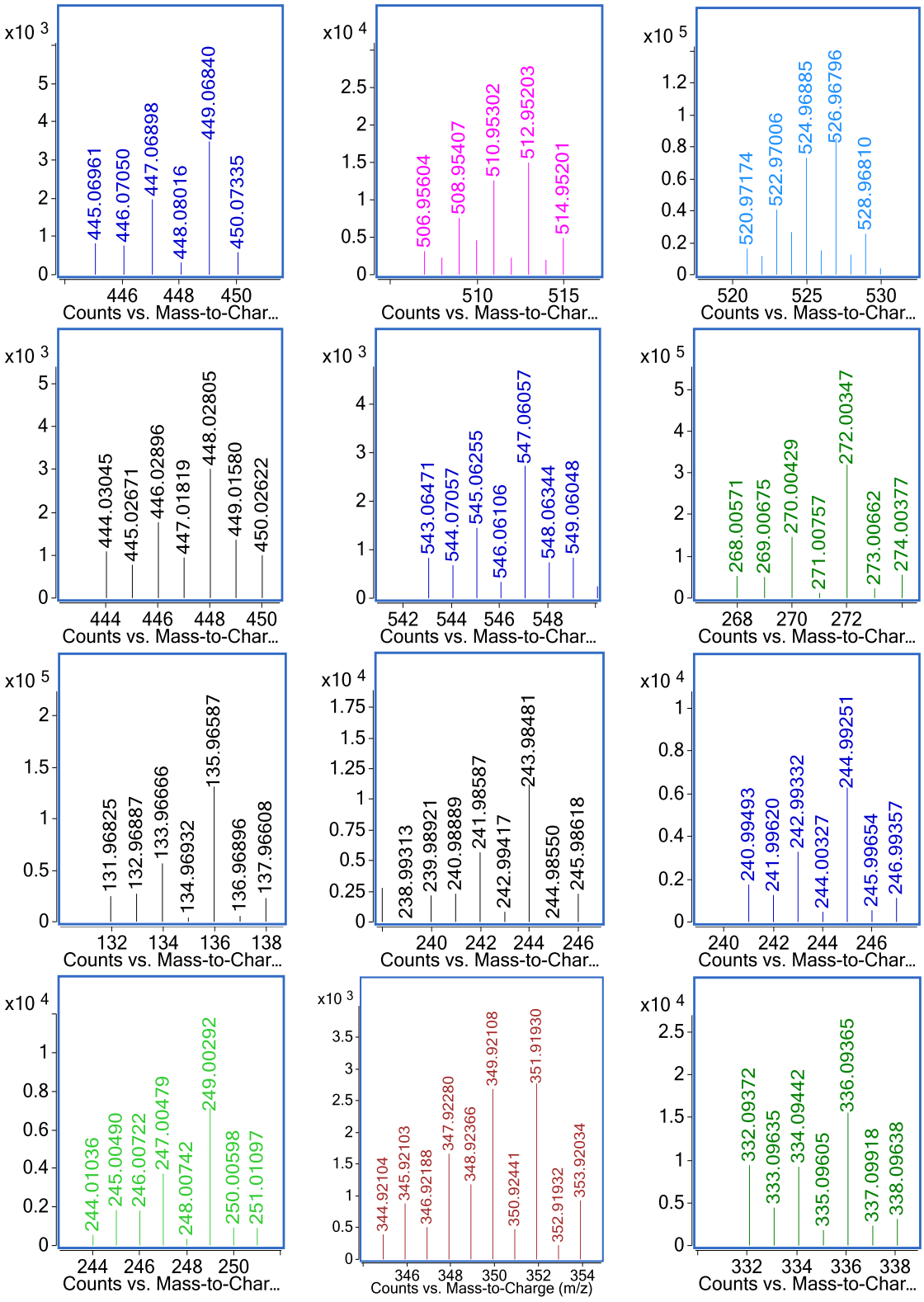
521.08939
522.09196
523.09069
516 518 520 522
Counts vs. Mass-to-Char...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447.06898
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Counts vs. Mass-to-Char...
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508.95407
510.95302
512.95203
514.95201
Counts vs. Mass-to-Char...
x10 5
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522.97006
524.96885
526.96796
528.96810
Counts vs. Mass-to-Char...
x10 3
444.03045
445.02671
446.02896
447.01819
448.02805
449.01580
450.02622
Counts vs. Mass-to-Char...
x10 3
543.06471
544.07057
545.06255
546.06106
547.06057
548.06344
549.06048
Counts vs. Mass-to-Char...
x10 5
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270.00429
271.00757
272.00347
273.00662
274.00377
Counts vs. Mass-to-Char...
x10 5
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132.96887
133.96666
134.96932
135.96587
136.96896
137.96608
Counts vs. Mass-to-Char...
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239.98921
240.98889
241.98587
242.99417
243.98481
244.98550
245.98618
Counts vs. Mass-to-Char...
x10 4
240.99493
241.99620
242.99332
244.00327
244.99251
245.99654
246.99357
Counts vs. Mass-to-Char...
x10 4
244.01036
245.00490
246.00722
247.00479
248.00742
249.00292
250.00598
251.01097
Counts vs. Mass-to-Char...
x10 3
344.92104
345.92103
346.92188
347.92280
348.92366
349.92108
350.92441
351.91930
352.91932
353.92034
Counts vs. Mass-to-Charge (m/z)
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333.09635
334.09442
335.09605
336.09365
337.09918
338.09638
Counts vs. Mass-to-Char...

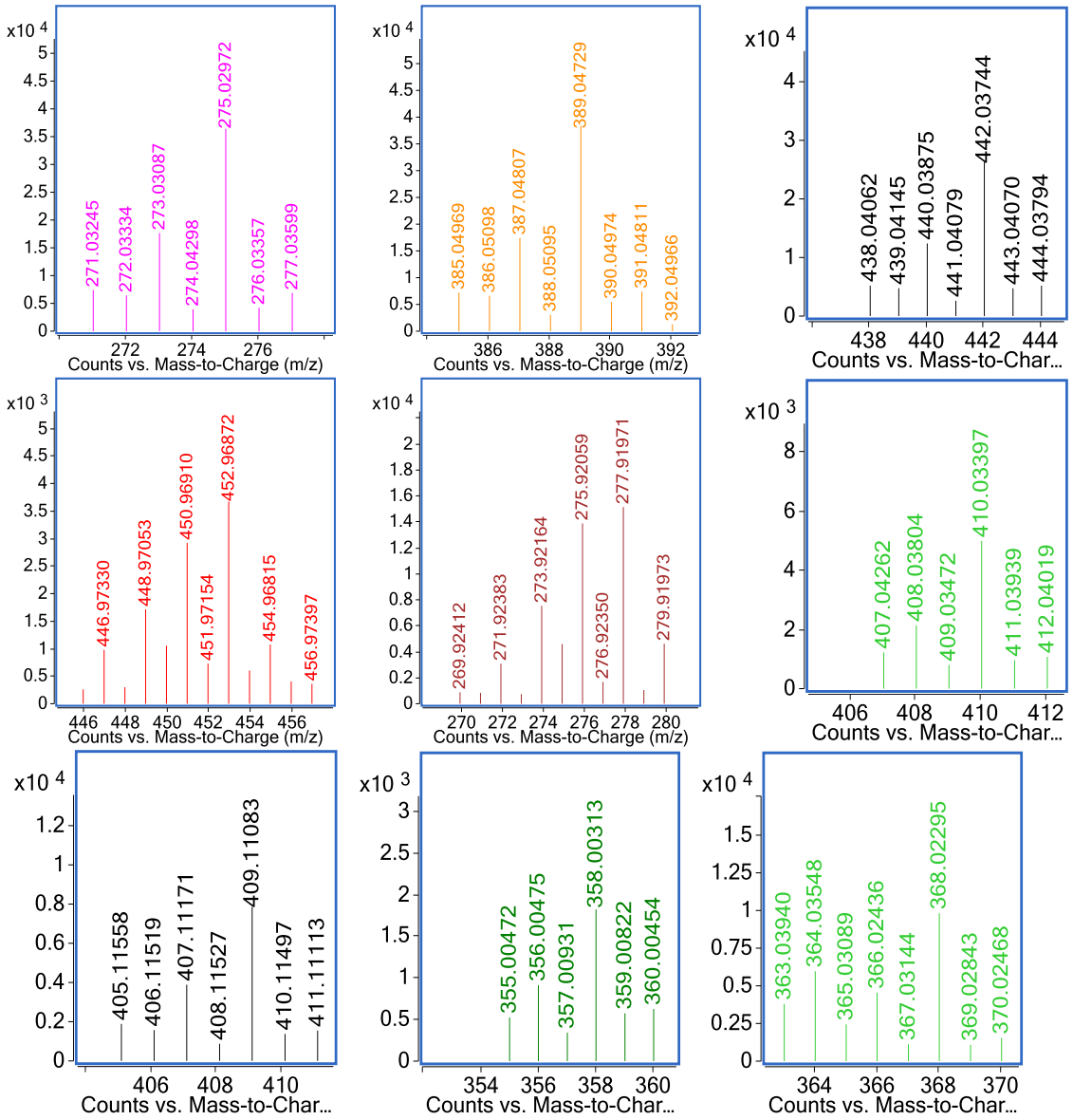

Figure A34: Selenium isotopic patterns for all selenocompounds detected by LC-ESI-QTOF-MS

Biochemical profiling of commercially available selenium-enriched yeast

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Abstract: Selenium enriched yeast is traditionally characterised by its total selenium content or by its dominant seleno-species, selenomethionine. Different yeast strains can have the same selenomethionine and total selenium content and by traditional methods of characterisation these yeasts are identified as the same. However, clear differences were seen in 15 minute water extracts of selenium enriched yeast. This assay determined the selenomethionine as well as other seleno-species present in a water extract,. Further enzymatic digestion of the yeast water extract identified how much protein or peptide bound selenomethionine was present. The water soluble, peptide bound selenomethionine was found to vary from product to product and for various lots of the same products. An entire selenium mass balance was carried out to account for the selenium in each fraction. The lowest mean total selenium mass balance recovery was ~98% and the highest was ~102.5%.

Objective

To show the differences between multiple selenized yeast products using a water extraction and analysis by LC ICP-MS.

Instrumentation/ Conditions/ Materials

- Agilent Technologies 1260 infinity series LC system
- Agilent Zorbax RX-C8 4.6 x 250 mm 5 µm
- Agilent Technologies 7700x series ICP-MS[1, 2]
- Heinemann 130W ultrasonic-homogenizer[3, 4]
- A CEM Discover microwave[3]

- Mobile phase - water: methanol: TFA (97.9:2.0:0.1)
- Flow rate - 1 mL/minute
- Column temperature - 30°C

- Seleno-L-methionine (SeMet) standard
- Protease XIV
- Trizma Base primary
- Certified reference material - SELM-1[5] (NRC, Canada)

Procedure

Free water soluble selenomethionine analysis:

- ~0.5g of selenium enriched yeast samples were extracted with 5 mL deionised H_2O.
- Samples were mixed for 15 mins at 300 rpm on a shaker
- Centrifuged at 8500 rpm for 5 minutes.

Total water soluble selenomethionine analysis:

- 500µL of the water extract was added to 13.33mg of protease.
- Samples were ultrasonicated for 25 seconds at 80% amplitude
- The probe washed with 250 µL enzyme solution (6.667mg Protease XIV /250 µL
- Samples were extracted in the CEM microwave for 15 minutes at 30 watts.
- Extracted samples were centrifuged at 14,000 rpm for 3 minutes[3]

Analysis:

The samples was filtered, diluted and analysed by LC-ICPMS.

Results

Total Se as Selenomethionine

Table 1 – Batch wise analysis of total selenium as selenomethionine content of the different yeast products

Samples	Se as SeMet [ppm]	S.D	R.S.D
Yeast Product 1 Batch #1	878	27	3.0
Yeast Product 1 Batch #2	1102	19	1.7
Yeast Product 1 Batch #3	1189	35	2.9
Yeast Product 2 Batch #1	1013	14	1.4
Yeast Product 2 Batch #2	1091	4	0.4
Yeast Product 2 Batch #3	1305	14	1.0
Yeast Product 3 Batch #1	1162	8	0.7
Yeast Product 3 Batch #2	874	23	2.6
Yeast Product 3 Batch #3	1012	18	1.8
Yeast Product 4 Batch #1	1404	4	0.3
Yeast Product 4 Batch #2	1264	16	1.3
Yeast Product 4 Batch #3	1229	4	0.3

Results expressed as Mean ± S.D (n=3)

Mass Balance of water extract and yeast pellet

Table 2 – Total selenium mass balance in the water extract and the subsequent pellet

	% Recovery of Total Se For mass balance	S.D
Yeast Product 1 Batch #1	98.3	2.2
Yeast Product 1 Batch #2	99.2	1.2
Yeast Product 1 Batch #3	101.8	2.3
Yeast Product 2 Batch #1	97.9	2.8
Yeast Product 2 Batch #2	101.2	0.9
Yeast Product 2 Batch #3	101.8	2.2
Yeast Product 3 Batch #1	100.0	2.4
Yeast Product 3 Batch #2	101.2	0.5
Yeast Product 3 Batch #3	102.5	1.4
Yeast Product 4 Batch #1	101.0	2.3
Yeast Product 4 Batch #2	100.8	2.4
Yeast Product 4 Batch #3	101.7	1.4

Results expressed as Mean % ± S.D (n=3)

Selenomethionine Distribution

- Free, peptide bound and total water soluble selenomethionine varied significantly between the yeast products ($P < 0.01$).
- Significant variation between three batches of each yeast product for free, peptide bound and total water soluble selenomethionine ($P<0.01$) except for yeast product 2 which didn't have significant variation between batches of its bound and total water soluble selenomethionine ($P=0.1$ and 0.012 respectively).

Free selenium as SeMet in the water extract was quantified by direct analysis of this supernatant. Total selenium as SeMet in the water extract was calculated by enzymatically extracting this supernatant prior to analysis.

The enzymatic extraction of the supernatant was essential to determine the total SeMet present in the water extract. These results suggest that there was more SeMet present in peptides of the yeast water extract that were not detected until an enzymatic extraction was carried out.

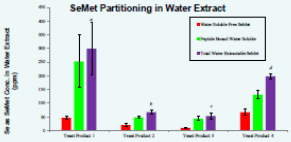

Figure 1: The partitioning of SeMet into water soluble free SeMet, peptide bound water soluble SeMet and total water extractable SeMet of 4 yeast products. [a, b, c, d] - average values that lack a common superscript letter, differ in significance ($p \leq 0.01$) (n=9).

Unknown Selenium Compounds

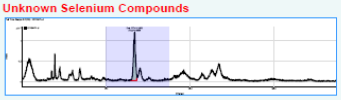

Figure 2: Selenium speciation chromatogram of water extracted selenized yeast.

- Selenium enriched yeast is traditionally characterised by its dominant seleno-species, SeMet. To further distinguish between these yeast samples other selenium peaks in the chromatograms were investigated.
- Retention times and a Compound Independent Calibration (CIC) were used to estimate the concentration of unknown selenium compounds and to highlight further differences (see Fig 2).

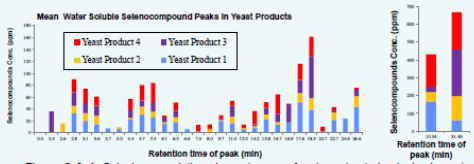

Figure 3 & 4: Selenium speciation chromatogram of water extracted selenized yeast (n=3).

3: Conclusions and future work

- All yeast products varied from each other either by SeMet or unknown selenocompound content.
- Enzymatic extraction method was essential to determine the true value of SeMet present in the water extract as the larger water soluble SeMet containing peptides were broken down thus liberating the selenoamino acid for detection.
- Batch to batch variation graphs showed yeast product 1 to have a much higher total SeMet standard deviation over its three batches. This may be due to age or fermentation process changes.
- Regardless of yeast product 1 inter batch variations, the free SeMet is still consistent between the three batches. This relatively consistent trait was evident throughout all the yeast products.
- Free SeMet was determined by water extraction, thus raising the question about the final stages of the yeast fermentation process (i.e. washing/spray drying steps etc). Multiple washing steps could reduce the free SeMet available for detection and may need further investigation.
- The mass balance recovered 98% - 102.5% of total selenium.
- Future work will involve identification of unknown selenocompounds in selenized yeast products.

Acknowledgements

The authors would like to thank the Irish Research Council for research funding under the Employment Based Postgraduate Program 2012.

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Figure A35: Poster of selenomethionine fractionation of commercially-available selenized yeast.

Presented at the BNASS/TraceSpec Conference, 31st August - 4 September 2014, University of Aberdeen.

Direct enantioseparation of selenomethionine in water soluble fractions of commercially available selenium-enriched yeast by HPLC-ICP-MS

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Abstract

Chiral speciation can be separated into two sections; indirect or direct enantioseparation[1]. Indirect methods utilise derivatisation steps or chiral inducing reagents (CIR) to produce diastereoisomers which can then be separated by various techniques [2]. Direct methods utilise chiral chromatography columns. There were only three commercially available columns used in publications up to 2014; Cyclobond 1 β-CD, Daicel Crownpak CR(±) and the Chirobiotic T[3, 4, 5]. The Sigma Chirobiotic T column was selected for the present study due to its previously documented resolution of D and L methionine. This direct enantioseparation method was developed to provide resolution and quantification of D and L-Selenomethionine (SeMet). Once the analytical method was optimised for the standards, it was then applied to water soluble yeast extracts. The SeMet standards recovered ~96 % of the theoretical concentration by HPLC-ICP-MS. The total selenium of the D,L and L-SeMet standards was also confirmed by ICP-MS prior to enantioseparation, 101 and 99.7 % recovery respectively. The water soluble D and L SeMet concentration varied between yeast products and batches.

Objectives

•To develop a direct enantioseparation method for determination of D and L selenomethionine
•To quantify the D and L selenomethionine differences between multiple selenized yeast products using a water extraction assay

Instrumentation/ Conditions/ Materials

•Agilent Technologies 1260 infinity series LC system
•Astec CHIROBIOTIC T (25cm X 4.6mm, 5µM)
•Agilent Technologies 7700x series ICP-MS [6,7]
•Mobile phase - water:methanol:formic acid (30:70:0.02)
•Flow rate - 1 mL/min
•Column temperature - 25ºC
•Injection volume - 10µL
•Seleno-L-methionine (SeMet) standard
•Seleno-D,L-methionine (SeMet) standard
•Certified reference material - SELM-1 [9](NRC, Canada)

Procedure Optimisation;

SeMet standards of D,L SeMet and L SeMet were utilised for assay optimisation. An oxygen/argon option gas module was installed to carry out high organic solvent analysis by LC-ICP-MS. The gas percentage was varied to minimise carbon deposits on the platinum cones. The ICP-MS was a 7700x model, thus it could only tolerate a certain percentage of solvent passing into the plasma. In order to combat higher levels of solvent, such as the ~ 70 % MeOH needed for this chiral work. This involved changing the skimmer and sampling cones to platinum based skimmer and sampling cones and installing a 1mm internal diameter quartz torch. An option gas module was also installed into the ICP-MS to supply 80:20 Argon: Oxygen mix to counteract the extra carbon being deposited on the cones.

Free water soluble selenomethionine analysis:

•0.5g of Se enriched yeast samples were extracted with 5 mL deionised H_2O.
•Samples were shaken for 15 mins at 300 rpm
•Centrifuged at 4500 rpm for 10 mins.

Results

Standard concentration confirmation

D,L and L SeMet were analysed for their total Se concentration to verify their SeMet concentration by ICP-MS.

Table 1: confirmation of SeMet standards prior to analysis.

Standard	Mean [% Recovery]	S.D	R.S.D
100ppm L SeMet	99.7	1.0	1.0
100ppm DL SeMet	101.0	1.0	1.0

Selenomethionine standard confirmation by HPLC-ICP-MS

Once the oxygen/argon option gas method was optimised, the standards were analysed for purity and concentration by HPLC-ICP-MS. The L-SeMet standard was analysed first as its retention time allowed for the determination of the enantiomers in the D,L-SeMet standard. When separate standards of D,L-SeMet were measured on the HPLC-ICP-MS, the recovery of the same concentration of L-SeMet in the D,L-SeMet equalled more than 96 % of the equivalent L-SeMet standard. These calculations suggest that the standards utilised for this study were accurate and precise, when compared to the reported selenomethionine standards acquired from Sigma. Therefore, they could be utilised for D,L-SeMet quantification.

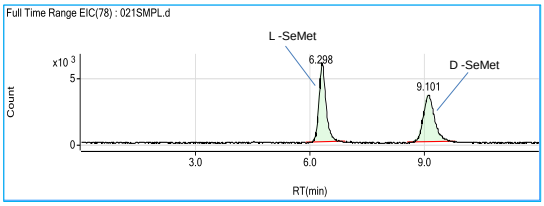
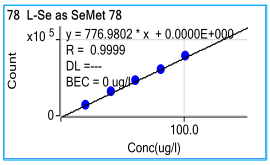


Figure 1: Resolution of D and L SeMet using Astec CHIROBIOTIC T column.



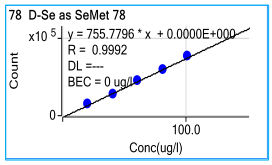


Figure 2a & b: Calibration standards of D,L SeMet. (L (52.09 %) and D (47.91%) SeMet).

Quantification of D and L SeMet in commercially available yeast

Once chromatographic optimisation for the samples was complete, analysis of three batches of four commercially available yeast products was investigated. The least concentrated enantiomer in selenised yeast, from current literature, was D-SeMet [3, 10], with ratios as high as 18/82 for D and L SeMet respectively[3]. This present study also found L-SeMet to have the highest concentration of the enantiomers. This current research follows on from our previous investigations of water soluble yeast extracts which found large variations across yeast products after a simpler water extract. Clear differences were also observed when investigating the D and L SeMet concentrations of these water extracts, Figure 3.

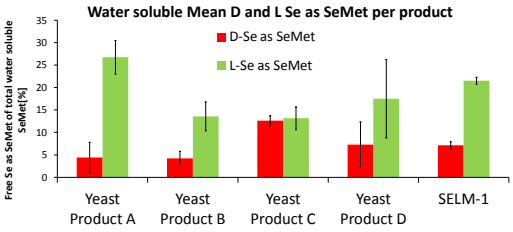


Figure 3 : Mean data of three non consecutive batches from four different yeast products. SELM-1 was also included as a certified reference material. (n=3)

3: Conclusions and future work

•ICP-MS was used to confirm the selenium concentration in SeMet standards with a mean recovery of 99.7 and 101 % for L and D SeMet respectively.
•All yeast products varied from each other, either by L or D SeMet content (or both).
•The error bars in Figure 4 highlight batch to batch variation of individual products.
•L SeMet, as expected, was the dominant enantiomer across the yeast products except for yeast product C. However, for yeast product C the difference was negligible between D and L enantiomers.
•This extraction investigated free SeMet, thus raising the question about the final stages of the yeast fermentation process (i.e. washing/spray drying steps etc). Multiple washing steps could reduce the free SeMet available for detection.
•Future work will involve identification of other selenocompounds with one or two chiral centres such as Se-methylselenocysteine (MeSeCys, one chiral centre), Selenoethionine (SeEt, one chiral centre) or Selenocystine ($SeCys_2$, two chiral centres).

Acknowledgements

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Figure A36: Poster of selenomethionine chiral speciation by HPLC.
Presented at the Irish Mass Spectrometry Society Annual Conference, 10th May 2017 – Red Cow Moran Hotel, Dublin.