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Speciation Analysis of Selenium in *Candida utilis* Yeast Cells Using HPLC-ICP-MS and UHPLC-ESI-Orbitrap MS Techniques

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Abstract: Selenium plays a key role in the proper metabolism of living organisms. The search for new selenium compounds opens up new possibilities for understanding selenometabolome in yeast cells. This study was aimed at the identification of compounds containing selenium in the feed yeasts *Candida utilis* ATCC 9950. Yeast biomass was kept in aqueous solutions enriched with inorganic selenium (20 mg·L^{-1}) for 24 h. Speciation analysis of the element was performed using the HPLC-ICP-MS and UHPLC-ESI-Orbitrap MS techniques. The obtained selenium value in the yeast was 629 μg·g^{-1}, while the selenomethionine value was 31.57 μg·g^{-1}. The UHPLC-ESI-Orbitrap MS analysis conducted allowed for the identification of six selenium compounds: dehydro-selenomethionine-oxide, selenomethionine, selenomethionine-NH_3, a Se-S conjugate of selenoglutathione-cysteine, methylthioselenoglutathione, and 2,3-DHP-selenocysteine-cysteine. In order to explain the structure of selenium compounds, the selected ions were subjected to fragmentation. The selenium compounds obtained with a low mass play a significant role in the metabolism of the compound. However, the bioavailability of such components and their properties have not been fully understood. The number of signals indicating the presence of selenium compounds obtained using the UHPLC-ESI-Orbitrap MS method was characterized by higher sensitivity than when using the HPLC-ICP-MS method. The obtained results will expand upon knowledge about the biotransformation of selenium in eukaryotic yeast cells. Future research should focus on understanding the entire selenium metabolism in cells and on the search for new transformation pathways for this element. This opens up new possibilities for obtaining functional food, rich in easily absorbable selenium sources, and constituting an alternative to dietary supplements based on this compound found primarily in inorganic form.

Keywords: selenium; UHPLC-ESI-Orbitrap-MS; *Candida*; yeast

1. Introduction

Selenium (Se) is a significant trace element for all organisms. Research on the role of this element in numerous biological systems has been rapidly developing. This element is included in 35 selenoproteins. Selenium, as a structural element of a large group of selenoproteins with antioxidative properties, helps in the prevention of cell damage caused by the effect of various metabolites which contribute to the development of numerous diseases (joint cartilage dystrophy, cardiovascular system diseases). Selenium is a cofactor of numerous enzymes, including glutathione peroxidase, and thioredoxin reductase [1]. This element is important for the prevention of the immunosuppressive effect of oxidative stress. Selenium exhibits its highest activity as a free radical scavenger [2];

furthermore, it is considered to be a preventive factor for the proliferation and development of tumor cells [3]. Monomethylated selenium forms, in particular Se-methylselenocysteine (SeMSCys) and γ-glutamyl-Se-methylselenocysteine, are precursors of methylselenol, which exhibits strong anticarcinogenic activity [4]. Selenium participates in the regulation of thyroid functions [5] and plays a significant role in the immune system [2]. Due to these properties, selenium has been the subject of many studies in this era of increased incidence of neoplastic, autoimmune, and cardiovascular diseases.

The European diet is characterized by its low selenium content due to the low occurrence of this element in the environment. Plants are the main recipients of selenium from the soil, and constitute the main source of the element in the diet of humans and animals. Based on the current state of knowledge, a rational diet ensuring coverage of the demand for all necessary food compounds is recommended. Thus, with growing interest in pharmaceutical supplementation with various key microelements, considerable emphasis has been placed on nutritional supplementation with selenium and functional food design.

Selenium content in foods is variable. The element typically occurs in organic form, such as selenomethionine and selenocysteine [6]. The bioavailability of the element is primarily determined by its chemical form and, to a lesser degree, by its total concentration or the physiological conditions of various organisms (age, health state). Organic forms of the element are characterized by higher bioavailability and lower toxicity than inorganic selenium forms [7,8]. The range of selenium concentration between necessary and toxic doses for living organisms is very narrow. Excessive selenium uptake may lead to disturbances in an organism's homeostasis. Knowledge about selenium speciation in yeast cells constitutes the key to understanding the metabolic pathways of the element, and demonstrates the favorable role of yeast in finding methods for enriching diets with this deficit element. Yeasts cultured on selenium-enriched media are used as the basis for additives to food and feeds rich in the element [9]. In summary, a diet should provide all nutrients in quantities that meet the standards established and recommended on the basis of accepted scientific data.

Yeasts have become some of the best studied eukaryotic organisms. However, despite the knowledge and experience of scientists working globally on these cells, the rate of discovery of new metabolic pathways is low. However, thus far not all possible metabolic pathways of selenium transformations in yeast cells have been identified. Thus, the possibility of conducting qualitative and quantitative analyses of selenium compounds found in numerous biological systems (e.g., yeasts) is very important. The knowledge gained from these mechanisms is key to understanding the action of bioaccumulation and the functioning of new metabolic pathways consisting in transformations of the element in cells [9,10].

Research performed on various speciation forms of selenium compounds that may occur in yeast cell biomass requires the application of numerous analytical methods and techniques. First and foremost, combined techniques are applied, in which separation methods are accompanied by various detection methods. High-performance liquid chromatography (HPLC) is used, combined with inductively coupled plasma mass spectrometry (ICP-MS). The use of high-performance mass spectrometry with an Orbitrap analyzer can provide accurate masses, upon which metabolite structures can be quickly and precisely characterized and identified. The UHPLC-ESI-Orbitrap MS method possesses a special advantage, i.e., its high resolution and sensitivity. Moreover, to determine selenium compounds, ultra-high-performance liquid chromatography (UHPLC) coupled with triple quadrupole mass spectrometry (QqQ) is used. Triple quadrupole is the most frequently used MS analyzer for food applications coupled with UHPLC, although other analyzers, such as triple quadrupole mass spectrometry, quadrupole ion trap, time of flight (TOF), quadrupole LC-MS/MS, or Orbitrap, have also been utilized [11].

The development of numerous norms and guidelines concerning nutrition has been based on the performance of total selenium concentration measurements to determine the safety and nutritional value of individual food products [12]. Elemental quantification alone does not provide sufficient information. The current state of knowledge indicates that the health-promoting and toxic effects of

this element may stem from the effect of various correlated processes. Thus, it is important to develop reliable analytical methods characterized by high sensitivity to conduct selenium speciation analyses in yeast cells and to explain selenium transformation mechanisms in cells. The present study was aimed at conducting a speciation analysis of selenium in *Candida utilis* ATCC 9950 yeast cells using HPLC-ICP-MS and UHPLC-ESI-Orbitrap-MS techniques.

2. Results and Discussion

2.1. Selenium Content in Yeast Biomass

Research conducted on the processes of element accumulation by yeast cells is of significance for the production of functional foods enriched with microelements. Among the additives used in nutrition, dried yeasts can constitute a rich source of selenium. It is possible to enrich food products and feeds with selenium, which constitutes another source of the element in the nutrition of humans and farm animals.

The cell biomass of *Candida utilis* ATCC 9950 yeasts obtained after culture on media containing industrial waste products (potato juice, 5% glycerol) constituted a natural adsorbent utilized in the process of selenium binding from aqueous solutions. The total selenium content after 24 h culture determined in *C. utilis* yeast cell biomass was 629 $\mu g \cdot g^{-1}$. Extraction efficiency was approximately 67%.

The total selenium content was determined for the later speciation identification of selenium compounds found in *C. utilis* feed yeasts (ATCC 9950). In the chromatographic conditions conducted, the study results obtained demonstrated the presence of clear peaks and a good separation efficiency throughout the process, which lasted 20 min (Figure 1).

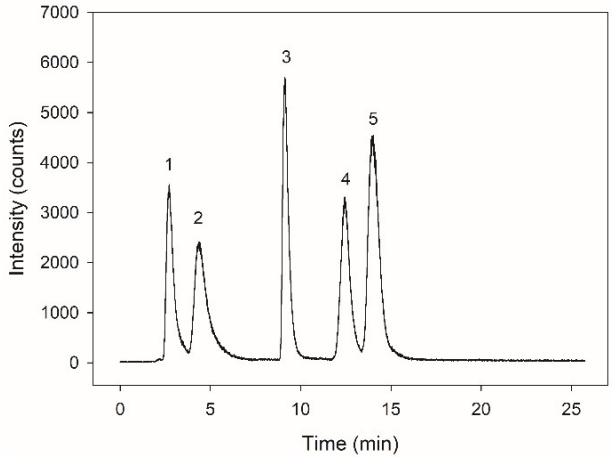

Figure 1. Chromatogram obtained for a standard mixture with 500 ppb concentration Se: 1: methyl-Se-cysteine, t_R = 2.6 min, 2: Se-methionine, t_R = 4.2 min, 3: Se(IV), t_R = 7.1 min, 4: glutamyl-Se-methylcysteine, t_R = 11.3 min, 5: Se(VI), t_R = 13.3 min.

As can be seen from literature data, yeasts are capable of binding large amounts of selenium, up to 6000 $\mu g \cdot g^{-1}$ [13]. The study of Bierla et al. [9] demonstrated that Torula yeast biomass was capable of accumulating selenium in the range of 3600 to 4000 $\mu g \cdot g^{-1}$ and the selenomethionine content obtained in these yeasts ranged from 270 to 440 $\mu g \cdot g^{-1}$. These results are comparable to those published by Kieliszek et al. [10], which indicated that *C. utilis* ATCC 9950 grown in a bioreactor on a YPD medium enriched with selenium at a dose of 20 $mg \cdot L^{-1}$ are capable of binding 427.5 $\mu g \cdot g^{-1}$ after 24 h culture. After the second day of culture, the selenium content in the biomass was 1804 $\mu g \cdot g^{-1}$. The experiments performed by Stabnikova et al. [14] demonstrated that *Saccharomyces cerevisiae* baker's yeast grown for 72 h on media supplemented with sodium(IV) hydroselenite (12 $mg \cdot L^{-1}$) only bound 203 $\mu g\ Se^{4+} \cdot g^{-1}$. The above relationships, which present the capacity of yeasts to bind a large amount of selenium, were confirmed in the study of Pankiewicz et al. [15]. Treating yeast cells with a pulsating electric field

(PEF) with an electric field intensity of 3.0 kV·cm^{-1} led to an increase in selenium (Se^{4+}) content in the cellular biomass of *Saccharomyces cerevisiae* 11 B_1 yeasts up to a value of 42.80 mg·g^{-1}.

Figure 2A,B presents the chromatograms obtained for an extract from *C. utilis* ATCC 9950 yeast prior to and after sample concentration (sample concentration was performed to analyze individual fractions with subsequent usage of the UHPLC-ESI-Orbitrap-MS system). Chromatographic signals obtained by using the HPLC ICP-MS coupled system (Figure 2A,B) for *C. utilis* ATCC 9950 biomass extract had a higher intensity for a peak with a retention time of 2.2 min corresponding to an unknown compound. This was likely a SeMet form occurring in an oxidized form (SeMetO) [16,17]. An additional peak, with a retention time of 2.6 min, corresponds to Se-methyl-SeCys; however, its presence should have been confirmed using other mass spectrometry techniques, such as ESI-QqQ-MS-MS, and/or ESI-Orbitrap-MS. Figure 2A,B presents a signal from Se-methionine with a retention time of 4.2 min and several unidentified selenium forms.

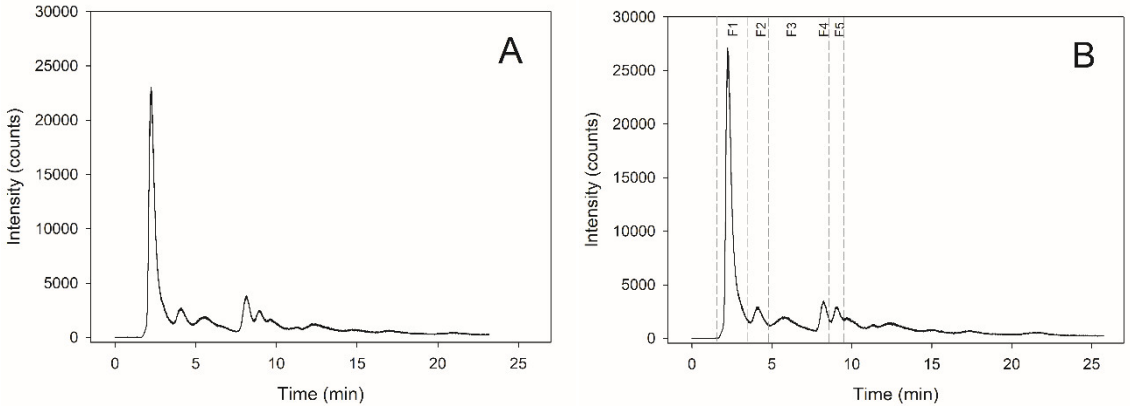

Figure 2. Chromatogram obtained for extract-*C. utilis* ATCC 9950 (**A**) and concentrated extract *C. utilis* ATCC 9950 (**B**).

Yeast cells, depending on the culture time and selenium concentration in the experimental substrate, can metabolize inorganic selenium found in SeO_3^{2-} form to its organic or elemental form [18,19]. The presence of inorganic selenium forms at levels above 2% in yeast biomass may indicate the low quality of such a yeast preparation [20]. Organic selenium complexes (e.g., selenoamino acids) are believed to be the best bioavailable form of the element [1]. Organic compounds are 85–95% assimilated by organisms as compared to inorganic forms of the element (40–50%) [21]. Such micronutrient organic forms produced by yeasts are more easily absorbable and are utilized by organisms to a greater extent [8].

Organic selenium supplementation during the production of supplements for humans and animal feeds is gaining in popularity. It is a safe and bioactive form of this precious element. Individual social groups have unreasonable diets due to cultural or culinary preferences or religious traditions. Such behavior may lead to the occurrence of dietary errors (deficiency, overconsumption), and thus to numerous diet-related diseases. The use of supplements is aimed at the enhancement of health and the maintenance of proper homeostasis in the organism. The above presented information may constitute a source for a wide range of scientific research explaining both the influence of chemical and physical forms of the addition of selenium on the metabolism and the health state of organisms. The proposed method of bioaccumulation of selenium by yeast creates wide possibilities for further use. The acquired knowledge about the forms of selenium present in the biomass of yeast and the course of metabolic processes will make it possible, in the future, to select products that would constitute an effective component of the daily diet.

2.2. Selenium Speciation in Yeast Using the UHPLC-ESI-QqQ MS and UHPLC-ESI-Orbitrap MS Methods

The UHPLC-QqQ-MS method was used for the quantitative analysis of selenium compounds present in yeast extracts. By using the UHPLC-ESI-QqQ-MS method, the presence of Se-methionine (Figure 3a) was confirmed in the sample, which was performed by registering fragmentation passages characteristic of the compound. Figure 3b presents a chromatogram for SELM-1 certified material. The sample of *C. utilis* ATCC 9950 was not found to have passages typical of Se-methyl-SeCys. This led to the conclusion that the yeast sample did not contain Se-methyl-SeCys.

In the case of selenomethionine in *C. utilis* ATCC 9950 yeasts, the content of this amino acid was at about the level of 31.57 $\mu g \cdot g^{-1}$. The selenomethionine level obtained in the reference material–SELM−1 (selenium-enriched yeast certified reference material) was 2480.37 $\mu g \cdot g^{-1}$ (77%) The recovery of SeMet from products introduced on the market depends on the physical and chemical properties of a sample, which may stem from the drying process conducted [9]. Experiments carried out by Bierla et al. [22] showed that commercial yeast (*Saccharomyces cerevisiae*) may contain approximately 1563 $\mu g \cdot g^{-1}$ of selenomethionine. This amino acid (selenomethionine) is the main selenium-containing organic compound found in yeasts. It is considered to be the best selenium form intended for humans and animals [23]. It may constitute from 60 to 80% of the total selenium content in a biomass [21]. However, in accordance with EU regulations [24], the production of dietary supplements containing selenium may only utilize dietary supplements in the form of Se-methionine and selenium-enriched yeasts. Also, yeasts should not contain more than 2500 $\mu g\ Se \cdot g^{-1}$ in their biomass. The study conducted by Bierla et al. [9] demonstrated that *Torula* (syn. *Candida*) yeasts are capable of metabolizing selenium in a different manner from *Saccharomyces cerevisiae*, leading to elevated biosynthesis of selenohomolanthionine as the primary selenium metabolite.

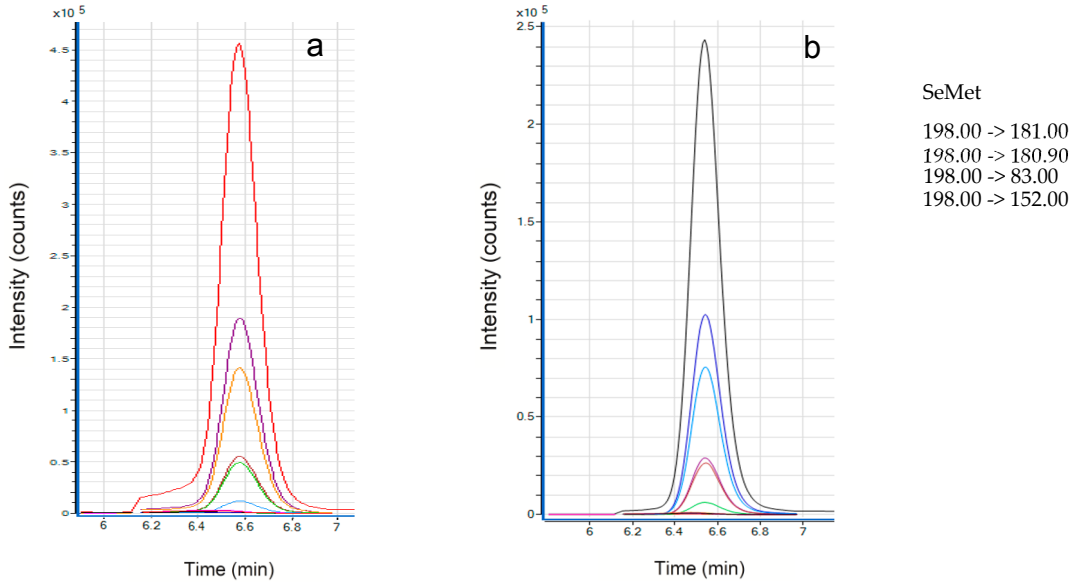

Figure 3. Chromatogram registered for extract of yeast *C. utilis* ATCC 9950 (transitions precursor ion → product ion) (**a**) and SELM−1 (selenium-enriched yeast certified reference material) (**b**) by UHPLC-ESI-QqQ-MS (the colors correspond to the intensity of the SeMet compounds, Supplementary Materials, Figure S8).

In Figure 2A,B, apart from the signals confirmed via the availability of standards, the presence of unidentified selenium forms was observed. Thus, in the subsequent part of the study, the UHPLC-ESI-Orbitrap MS method was used, primarily to identify the unknown selenium compounds as well as to confirm the presence of those forms identified with the HPLC-ICP-MS coupled technique. The absence of standards for selenium compounds meant that mass spectrometry methods with electrospray were indispensable for the identification of selenium compounds [9]. In the

method presented, ions are transferred from the liquid to the gaseous phase. The analyzed compounds in the liquid phase must be ionized to a minor degree. The efficiency of the electrospray method (ESI) improves the more polar the compound is.

For the analysis of selenium compounds using the HPLC-ESI-Orbitrap-MS method, yeast extracts were analyzed. As a result of the analysis, the presence of Se-methionine and its metabolites, previously identified via the HPLC-ICP-MS method, was confirmed. In addition, other selenium compounds were identified using the HPLC-ESI-Orbitrap-MS method. The identified compounds found in the collected fractions are presented in Table 1.

Table 1. Selenium compounds identified in yeast extract using the UHPLC-(ESI)-Orbitrap-MS technique.

Fraction	Exp. *m/z*	Theo. *m/z*	Δppm	Formula	Name	RT/min
F1	195.9876	195.9870	3.06	$C_5H_{10}NO_2Se^+$	Dehydro-Selenomethionine-oxide	-
F2	198.0032	198.0028	2.02	$C_5H_{12}NO_2Se^+$	Selenomethionine	6.59
F2	180.9767	180.9875	−59.67	$C_5H_{10}NO_2Se^+$	Selenomethionine—NH_3	6.59
F3	475.0405	475.0396	1.89	$C_{13}H_{23}N_4O_8SSe^+$	Se-S conjugate of cysteino-selenoglutathione	10.14
F3	402.0236	402.0232	0.99	$C_{11}H_{20}N_3O_6SSe^+$	Methylthioselenoglutathione	13.88
F4, F5	376.9922	376.9916	1.59	$C_9H_{17}N_2O_7SSe^+$	2,3-DHP-selenocysteine-cysteine	12.08

The search for and identification of unknown selenium compounds is presented on the basis of the example of selenometabolite precursor identification: Se-S conjugate of selenoglutathione -cysteine, selenomethionine, methylthioselenoglutathione, and 2,3-DHP-selenocysteine-cysteine. The HPLC-ESI-Orbitrap-MS chromatograms obtained were searched for the presence of a suitable isotope envelope characteristic of chemical compounds containing selenium in their composition. The element has 26 isotope forms, of which only six are durable [25]. The most significant ones have a mass from 74 to 82.

The HPLC-ICP-MS method utilized allowed for the identification of five selenium compounds using commercial standards in extracts obtained from *C. utilis* ATCC 9950 yeast biomass (Se-methyl-SeCys, SeMet, SeO_3^{2-}, γ-glutamyl-Se-methyl-SeCys, and SeO_4^{2-}). The results are in line with the previously published results of Bierla et al. [20] and Kieliszek et al. [10]. In chromatograms obtained from yeast extracts, the presence of unidentified, overlapping signals could be discerned. In such a situation, the need to identify them arises, which requires additional analytical methods.

Yeasts are microorganisms, which are capable of binding selenium ions from the extracellular environment and which accumulate them in their structures. They are undoubtedly among those microorganisms the biochemical activity of which is most frequently used for numerous biotechnological processes [26]. Yeast enriched in selenium contains over 60 unique compounds with this element [27]. The identification of individual metabolic pathways in which inorganic selenium transformation to organic forms occurs would contribute to broadening the understanding of selenometabolome in yeasts.

The signal present on the chromatogram obtained from *C. utilis* yeast extract using the HPLC-ICP-MS method (Figure 2B, fraction F1) and subsequently identified using the HPLC-ESI-Orbitrap-MS method consisted of dehydro-selenomethionine-oxide (*m/z* 195.9876). In fraction 2, the presence of SeMet–NH_3 (*m/z* 180.9767) and SeMet (*m/z* 198.0032) was determined. In the case of the F3 fraction, the presence of two different compounds was determined: an Se-S conjugate of selenoglutathione-cysteine (*m/z* 475.0405), and methylthioselenoglutathione (*m/z* 402.0236). In the F4 and F5 fractions, the presence of only one compound was found: 2,3- dihydroxy-propionyl(DHP)-selenocysteine-cysteine with *m/z* 376.9922. Probably due to their low concentrations, other selenium components present could not be identified.

Identification analyses of *C. utilis* yeast extract confirmed the presence of Se-methionine, which was identified using the HPLC-ICP-MS method. As an example, in an image of the fragmentation spectrum for an ion with *m/z* 198.0032 (Figure 4) corresponding to selenomethionine (Supplementary Materials, Figure S8), the presence of selected selenium components was found: $C_2H_5Se^+$ with a similar theoretical value *m/z* 108.9698 (ethylselenurane), $C_4H_7Se^+$ with *m/z* 134.9934 (selanylbut−1-ene), and $C_5H_9O_2Se^+$ with *m/z* 181.0291 ((4-methoxy-4-oxobutan-2-yl)selanyl) (Figure 4

and Supplementary Materials, Figure S8). The first compound is formed by intracellular regroupings of dimethylselenide or dimethylselenosulfenate, creating the C_2H_5Se cluster. A compound with a mass close to 181.0291 was identified in black mustard–*Brassica nigra* [28].

In the case of methylthioselenoglutathione (*m/z* 402.0236), fragmentation of this selenium component (Figure 5) demonstrated the presence of the following ions: $C_5H_8NO_3^+$, $C_3H_8NSSe^+$, $C_6H_{10}NO_3SSe^+$, and $C_6H_{13}NO_3SSe^+$. The presence of the first has been confirmed in the biomass of *S. cerevisiae* CNCM I-3060 yeast [29]. It was the detected γ-Glu fragment included in the glutathione structure. Glutathione (GSH) is one of the most important molecules for cellular defense against chemically reactive toxic compounds or oxidative stress, which can be produced by high selenium concentrations occurring in culture media [30]. This protective function partially stems from its participation in conjugation reactions [31]. The three last compounds have not yet been presented.

The conjugated 2,3-DHP-selenocysteine and glutathione form is one of the most common forms containing selenium, and occurs in almost all yeasts enriched with the element [32]. The transformation of the above compound via metabolic processes leads to the formation of 2,3-DHP-selenocysteine-cysteine in yeast biomass. Based on the analyses of mass spectrum fragmentation conducted (Figure 6), it was possible to identify individual compound ions characterized by molecular weights $[M + H]^+$ = from 83.9780 to 255.8982. The first compound corresponded to the γ-Glu fragment with a loss of formic acid [29,33]. The last molecular weight presented corresponded to the occurrence of 2,3-dihydroxypropionyl-selenocysteine. The compound was identified in an extract from sunflower sprouts enriched with selenium [34].

In the cellular biomass of *C. utilis* ATCC 9950 yeast, an Se-S conjugate of selenoglutathione-cysteine with *m/z* 475.0405 mass was determined. Fragmentation of the spectrum of this compound led to the formation of seven ions (Figure 7). The ion presented with the chemical formula $C_8H_{16}N_3O_5SSe^+$ ($[M + H]^+$ = 345.9487) was responsible for the occurrence of two residues of γ-Glu [29]. Selenium compounds with *m/z* 199.0382 and 230.9933 have not been previously reported.

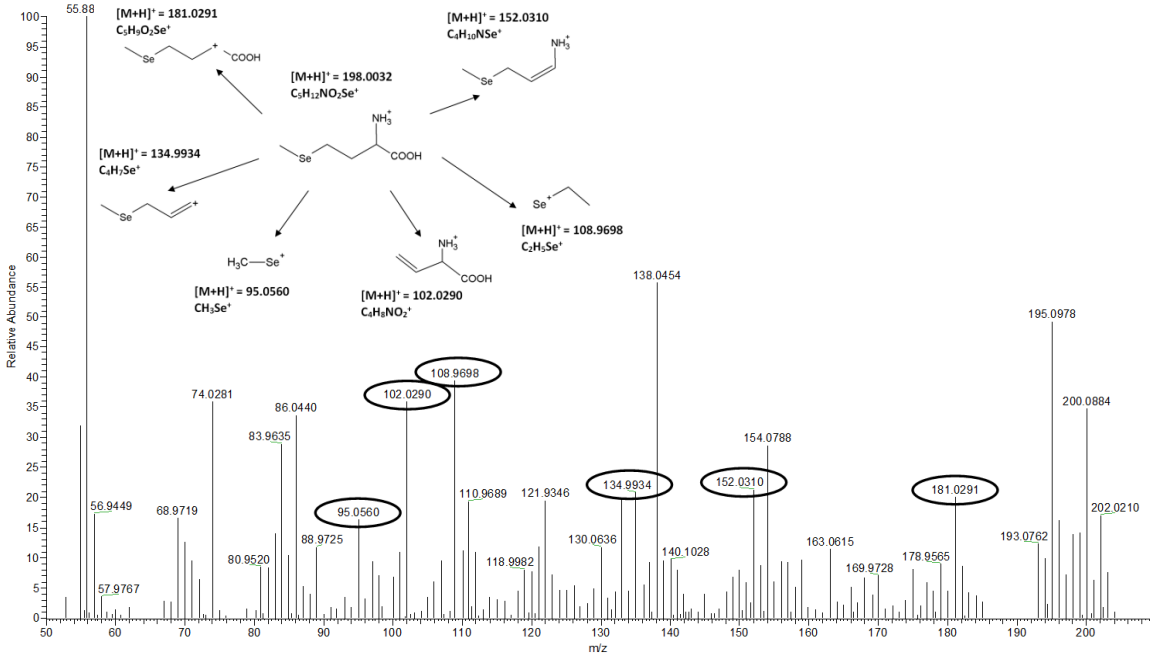

Figure 4. Fragmentation spectrum for ion with m/z = 198.0032 and fragmentation pathway.

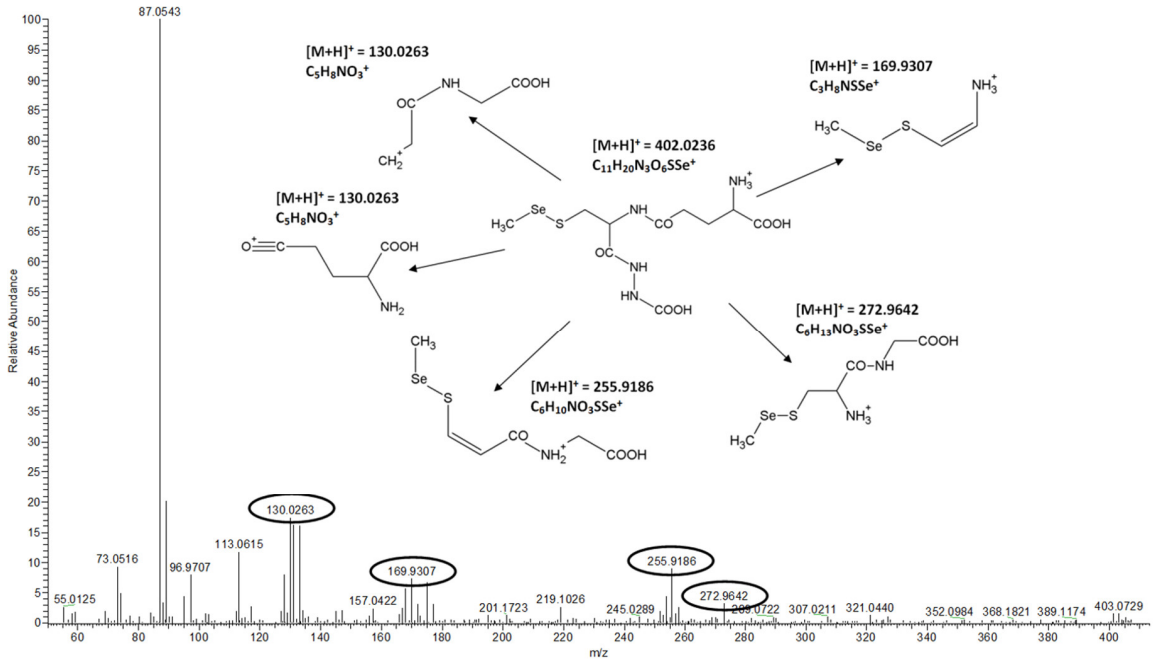

Figure 5. Fragmentation spectrum for ion with m/z = 402.0236 and fragmentation pathway.

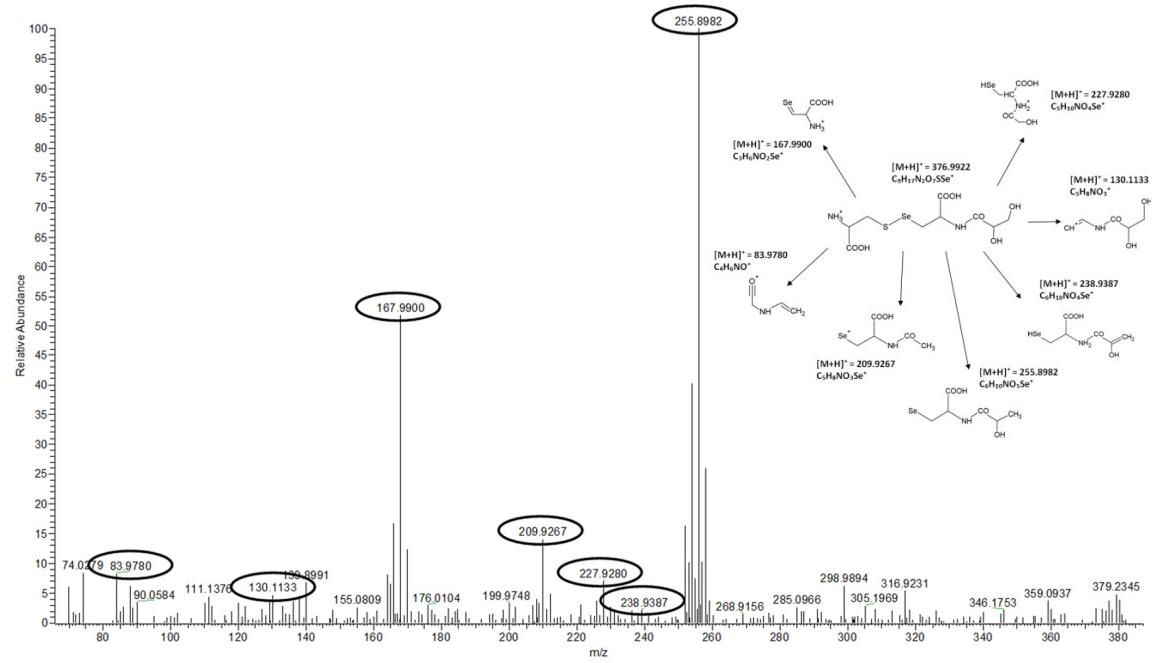

Figure 6. Fragmentation spectrum for ion with m/z = 376.9922 and fragmentation pathway.

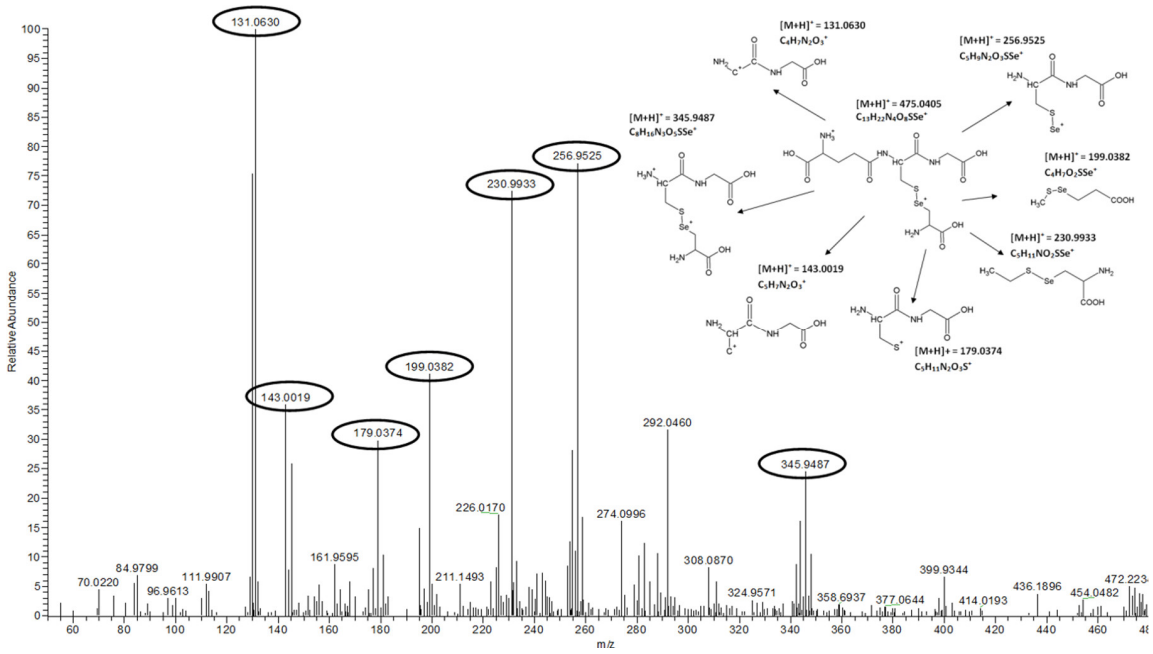

Figure 7. Fragmentation spectrum for ion with *m/z* = 475.0405 and fragmentation pathway.

In summary, the results obtained encourage further analyses on the identification of new selenium compounds in the biomass of various yeast species. Appropriate use of analytical methods and yeast culture conditions will allow for and enhance the understanding of the differences in metabolic processes of selenium transformation to its organic forms. Such an approach is associated with the pursuit of the discovery or creation of new products and will make it possible to obtain a dietary supplement enriched in a suitable form of the element. Future research should focus on an analysis of compounds, which will likely occur in commercially sold dietary supplements, and water-soluble, low-molecular selenium compounds. Biotechnological research has great potential for the discovery of new phenomena or a better understanding of those already studied and for further improvement of research. Thus, new, exciting possibilities in selenium metabolism may be identified.

3. Materials and Methods

3.1. Biological Material

The study utilized a *Candida utilis* ATCC 9950 yeast strain originating from the collection of pure cultures of the Division of Food Biotechnology and Microbiology of Warsaw University of Life Sciences-SGGW. Biological material was stored on YPD slants at a temperature of 6–8 °C.

Candida utilis yeast biomass was obtained after a 24-h shaken culture (SM-30 B, Edmund Bühler GmbH, Bodelshausen, Germany), at a vibration amplitude of 200 cycles·min^{-1} in media consisting of waste potato juice with 5% glycerol. The biomass obtained was rinsed with sterile distilled water twice and centrifuged (3000× *g*, 10 min, +4 °C, Centrifuge 5804R Eppendorf, Hamburg, Germany). The biomass obtained with 10 g weight was used to inoculate aqueous solutions enriched with selenium at the amount of 20 mg·L^{-1}. Culture was conducted for 24 h at 28 °C on a shaker with a vibration amplitude of 200 cycles·min^{-1}. After the culture process, the yeast biomass was filtered and then centrifuged (3000× *g*, 10 min, +4 °C). The biomass obtained was lyophilized and stored for further analyses.

3.2. Extraction of Selenium Compounds from Yeast Biomass

Yeast samples with a weight of 0.2 g were extracted in 6 mL H_2O with the addition of 20 mg protease (protease from *Streptomyces griseus* type XIV, Sigma-Aldrich, Warsaw, Poland) and 20 mg

lipase (lipase from *Candida rugosa*, Sigma-Aldrich, Warsaw, Poland). Extractions were conducted in a thermostat for 16 h at 37 °C on a magnetic stirrer. Following extraction, the samples were centrifuged for 10 min (EBA-20 from Hettich Zentrifugen, Kirchlengern, Germany), and then the supernatant was filtered through 0.45 μm membrane filters. Prior to chromatographic separation, the extracts were stored at −20 °C.

To calculate the efficiency of the extraction process, the extract samples obtained were mineralized in a closed microwave system. For mineralization, 1 mL extract and 5 mL 65% of nitric(V) acid were used. Prior to determination, the samples were additionally diluted. All the solutions and standards were prepared using Milli-Q-Gradient high purity deionized water (Millipore, Burlington, MA, USA). The efficiency of extraction for *C. utilis* was approximately 67%.

3.3. Determination of Selenium Content in Yeasts Using ICP-MS

To determine the total selenium content in yeast, an inductively coupled plasma mass spectrometer ICP-MS (NexION 300D Perkin Elmer Sciex, Thornhill, ON, Canada) was used. The yeast samples were wet mineralized in a microwave mineralizer (UltraWAVE, Milestone). When performing determinations with the ICP-MS technique, the calibration curve method for external standards was applied, which was prepared using 1% nitric(V) acid and a selenium standard (Sigma-Aldrich, Warsaw, Poland). The Se content in each sample was calculated as the mean of the results obtained for five repetitions. In the measurements, a selenium-82 isotope was monitored. The limit of detection (LOD) and limit of quantification (LOQ) of the procedure for selenium obtained with the ICP-MS technique ($mg \cdot kg^{-1}$) were 0.085 $mg \cdot kg^{-1}$ and 0.141 $mg \cdot kg^{-1}$, respectively.

3.4. Speciation Analysis of Selenium Using HPLC-ICP-MS

To separate and identify selenium compounds present in yeast extracts, a coupled HPLC-ICP-MS technique was used (HPLC-Agilent Technologies, Santa Clara, CA, USA; ICP MS-Elan 6100 DRC ICP MS, Perkin Elmer SCIEX, Waltham, MA, USA). To separate selenium compounds, an anion type PRP-X100 exchange column measuring 250 mm × 4.1 mm × 10 μm (Hamilton, Reno, NV, USA) was used. The mobile phase flow rate was 1 $mL \cdot min^{-1}$ at 22 °C. A total of 100 μL of sample was injected onto the chromatographic column. The mobile phase applied during the separation of the selenium compounds was ammonium acetate 5 $mmol \cdot L^{-1}$ and 150 $mmol \cdot L^{-1}$ with pH 4.7 dissolved in deionized water. During separation, a gradient elution was applied for 21 min [11].

To identify selenium compounds in yeast extracts using the coupled HPLC-ICP-MS technique, five selenium standards were used: Se-methyl-SeCys, SeMet, SeO_3^{2-}, γ-glutamyl-Se-methyl-SeCys, and SeO_4^{2-} (Sigma-Aldrich, Munich, Germany). The chromatogram obtained for the selenium standards using the coupled HPLC-ICP-MS system is presented in Figure 1. To confirm the correctness of the selenium sample preparation, selenium-enriched yeast (SELM-1), a certified reference material from the National Research Council Canada, was used for the analysis of its forms.

3.5. Selenium Compound Quantitative Analysis Using UHPLC-QqQ-MS

A UHPLC-QqQ-MS/MS coupled system was used for the quantitative analysis of the selenium compounds present in yeast extracts and in the SELM−1 certified reference material. Measurements were performed in MRM mode (Multiple Reaction Monitoring) using a UHPLC Agilent 1290 Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 150 mm × 4.6 mm HyperCarb column with a particle size of 5 μm (Thermo Scientific, Waltham, MA, USA) coupled to a 6460 Triple Quad LC QqQ mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

3.6. Speciation Analysis of Selenium Using UHPLC-ESI-Orbitrap-MS

Measurements were conducted using an Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA, USA) coupled to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). In order to perform the selenium compound identification using the

UHPLC-ESI-Orbitrap-MS system, eluates were collected to vials at times corresponding to the retention times of selenium signals previously recorded using HPLC-ICP-MS. The volumes of the fractions collected differed depending on the recorded selenium peak width.

The fractions obtained were concentrated in a vacuum centrifuge to dryness and re-suspended in 2% aqueous formic acid at a final volume of 25 µL. Chromatographic separation of the standard mixture and samples was performed using gradient elution. The mobile phase consisted of 0.1% formic acid solution in water (A) and 0.1% formic acid solution in acetonitrile (B). The samples were loaded directly onto a HyperCarb analytical column (150 mm × 4.6 mm, 5 µm, Thermo Scientific, Waltham, MA, USA) at a solvent flow rate of 0.5 mL min^{-1} 3% (B). The injection volume was 20 µL. Separation was performed with an increasing linear gradient of the B solvent after 24 min in the following conditions: 0 min, 3%; 16 min, 50%; 17 min, 50%, 19 min, 90%; 19.01 min, 3% B; and 23 min, 3%. The eluted compounds were ionized in positive ion mode at 3.9 kV voltage in a heated HESI ion source. The parameters of the ion source were optimized based on the total values of the ionic current. The TIC values were as follows: shield gas flow 25 L·min^{-1}, auxiliary gas flow 15 L·min^{-1}, transfer capillary temperature 350 °C, and sprayer temperature 300 °C. MS analyses were performed in an Orbitrap Fusion™ Tribrid™ single-stage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The device was operated using a heated electrospray interface (ESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive (ESI+) and negative ion modes (ESI−). Mass spectra from tests were recorded using an Orbitrap mass analyzer at 60.000 resolution in the range *m/z* 100–1200. Data analyses were performed using XCalibur 3.0 (Thermo Scientific, San Jose, CA, USA) software for the presence of isotope standards characteristic of compounds containing selenium. MS/MS spectra were interpreted to confirm the presence of known compounds and to explain the structure of unknown selenium compounds present in yeast samples. In order to obtain structural standards, the ChemSpider database was further applied.

4. Conclusions

The use of the HPLC-ICP-MS and UHPLC-ESI-Orbitrap-MS/MS methods allowed for the speciation of selenium metabolites in extracts obtained from the cellular biomass of *C. utilis* ATCC 9950 yeast. The presence of four selenium compounds, for which standards were commercially available, was confirmed using the HPLC-ICP-MS method, by matching the retention times of the recorded selenium signals to the standards. Due to the possibility of signal overlap and the inaccuracy of retention times during HPLC-ICP-MS, each time a formal identification using a more accurate method in ionization conditions through electrospray should be considered. The Orbitrap MS system is a stand-alone mass spectrometry, designed for high-accuracy, high-resolution full scans. The high resolution of the Orbitrap MS combined with good mass accuracy provides good sensitivity and selectivity in analyses (yeast biomass). Using the UHPLC provides a superior chromatographic resolution, leading to improved sensitivity. The identification of selenium metabolites and their fragmentation behavior provides valuable and new information for a better understanding of the action of selenium metabolism in yeast cells. Moreover, structures for compounds with $C_3H_8NSSe^+$, $C_6H_{10}NO_3SSe^+$, and $C_6H_{13}NO_3SSe^+$ formulas have been proposed and found in *C. utilis*, which have not yet been reported. In conclusion, selenium present in yeast in organic form is characterized by better bioavailability than inorganic forms. The chemical form of selenium consumed in a diet is more important for dietetics than the content of this element in the diet.

Supplementary Materials: Available online at http://www.mdpi.com/2076-3417/8/11/2050/s1. Figure S1: Chromatograms registered for a mixture of selenium standards: (a) XIC for Se-methyl-selenocysteine, [M + H]+ *m/z* 166.9604; (b) XIC for Selenomethionine, [M + H]+ *m/z* 198.0026; (c) XIC for Selenocystine, [M + H]+ *m/z* 336.9194; (d) XIC for γ-glutamyl-Se-methyl-selenocysteine, [M + H]+ *m/z* 313.0293, Figure S2: MS spectrum recorded for Se-methyl-selenocysteine (SeMetCys) ion, *m/z* = 183.9871 (t_R = 5.19) along with the isotopic envelope characteristic of selenium compounds, Figure S3: MS spectrum recorded for Selenocysteine (SeCys) ion with *m/z* = 336.9194 (t_R = 5.63) along with the isotopic envelope characteristic of selenium compounds, Figure S4: MS spectrum recorded for Selenomethionine (SeMet) ion with *m/z* = 198,0026

(t_R = 6.58) along with the isotopic envelope characteristic of selenium compounds, Figure S5: MS spectrum recorded for γ-glutamyl-Se-methyl-selenocysteine ion *m/z* = 313.0293 (t_R = 10.58) along with the isotopic envelope characteristic of selenium compounds, Figure S6: Fragmentation spectra of reference substances (Se-methyl-selenocysteine), Figure S7: Fragmentation spectra of reference substances (Selenocysteine), Figure S8: Fragmentation spectra of reference substances (Selenomethionine), Figure S9: Fragmentation spectra of reference substances (γ-glutamyl-Se-methyl-selenocysteine), Table S1: LOD (detection limit) and LOQ (quantification limit) for Se-methionine (SeMet) and selenomethyl-selenocysteine (SeMetCys) obtained through UHPLC–QqQ-MS/MS [mg·kg^{-1}].

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Sample Availability: Samples of the compounds are available from the authors.