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# Selenite biotransformation during brewing. Evaluation by HPLC–ICP-MS

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ABSTRACT

Yeast (*Saccharomyces cerevisiae*) and lactic bacteria have shown their ability to accumulate and transform inorganic selenium into organo Se compounds. The objective of this work was to evaluate selenium biotransformation during brewing by using *S. cerevisiae* and *Saccharomyces uvarum* for Ale and Lager fermentation, respectively. Se-enriched beer was produced by the addition of sodium selenite (0, 0.2, 1.0, 2.0, 10.0, 20.0 μg Se $mL^{-1}$, respectively) to the fermentation media composed of yeast, malt extract and water. The alcoholic fermentation process was not affected by the presence of selenium regardless of the type of *Saccharomyces* being used. The percentage of selenium incorporated into beer, added between 1.0 and 10 μg $mL^{-1}$ was 55–60% of the selenium initially present. Se-compounds in post-fermentation (beer and yeast) products were investigated by using an analytical methodology based on HPLC–ICP-MS. For this purpose, several sample treatments, including ultrasonic-assisted enzymatic hydrolysis, in conjunction with different separation mechanisms like dialysis and anion exchange HPLC chromatography were applied for unambiguously identifying Se-species that produce during brewing. Selenomethionine was the main selenium compound identified in beer and yeast, being this species in the only case of the former not associated to peptides or proteins.



## 1. Introduction

The importance of selenium (Se) as essential trace element to the human health [1] is recognized and its deficiency related to the occurrence of several diseases [2–6]. Selenium supplementation has been widely applied for increasing selenium status of Se-deficient population, being selenium-enriched yeast supplements which have focused the greatest interest [7]. Yeast (*Saccharomyces cerevisiae*) and lactic bacteria have shown their ability to accumulate and to transform inorganic selenium to organo-Se compounds (safer and highly bioactive) [8–10]. Fermentation has been used for several authors as a process to prepare Se-enriched food. Alzate et al. [9,10] compared the different selenium species that are produced when lactic fermentation in presence of two different types of microorganisms, LAB (*Lactobacillus*) and yeast (*Saccharomyces*), take place to produce yogurt and kefir, respectively. Selenium species formed depend on the type of microorganism involved in the fermentation process, being selenocystine ($SeCys_2$) and semethylselenocysteine (MeSeCys) the main species generated by *Lactobacillus* and Selenomethionine (SeMet) by *Saccharomyces*. Recently [11], SeMet was the main selenium-containing amino acid identified in selenized white wine produced by *S. cerevisiae and Saccharomyces bayanus*.

Brewing also involves fermentation in presence of yeast. The basic ingredients of beer are water; a fermentable (convertible into alcohol) starch source, such as malted barley; a brewer's yeast to produce the fermentation; and a flavouring, such as hops. A secondary starch source (an adjunct) may be used, such as maize (corn), rice or sugar. Beers are commonly categorized into two main types, the globally popular pale lager (brewed with bottom-fermenting yeast as *Saccharomyces uvarum*) and the regionally distinct Ale (brewed with top-fermenting yeast, most commonly *S. cerevisiae*).

Research in brewing over the past three decades, has been focused on the application of different fermentation processes mainly to facilitate the beer production and consequently to reduce the production costs [12]. However, to the author's knowledge the direct synthesis of Se-compounds during brewing has never been reported before. Therefore, the aim of the present study is to evaluate whether biotransformation of selenium, added as selenite, occurs during brewing. For this purpose, an analytical methodology based on the use of HPLC–ICP-MS will be optimized. Several sample treatments in conjunction with different separation mechanisms like dialysis and anion exchange HPLC chromatography will be applied for unambiguously identifying Se-species that produce during brewing.

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**Table 1**
ICP-MS instrumental settings and HPLC separation conditions.

| | |
|---|---|
| ICP-MS instrumental parameters | |
| Forward power | 1250 W |
| Plasma gas flow rate | 15.0 L min$^{-1}$ |
| Auxiliary gas flow rate | 1.26 L min$^{-1}$ |
| Carrier gas flow rate | 1.1 L min$^{-1}$ |
| Nebulizer type | Meinhard |
| Spray chamber type Dwell time per point | Scott-double pass 200 ms |
| Data acquisition mode | Time resolved analysis |
| Isotope monitored Internal standard | $^{77}$Se, $^{82}$Se $^{73}$Ge |
| HPLC conditions | |
| Analytical column | Hamilton PRPX-100 (250 × 4.1 mm) |
| Mobile phase | 10 mM ammonium citrate pH 5 |
| Flow rate | 1 mL min$^{-1}$ |
| Injection Volume | 100 μL |

## 2. Experimental

### 2.1. Instrumentation

Ultrasonic-assisted enzymatic hydrolysis of samples was carried out in a Sonoplus ultrasonic homogenizer (Bandelin, Germany) equipped with a titanium 3 mm diameter microtip and fitted with a HF generator of 2200 W at a frequency of 20 kHz. Extracts were centrifuged in an Eppendorf centrifuge 5804 F34-6-38 (Germany). Microwave acid digestion was performed in a 1000 W MSP microwave oven (CEM MSP 1000, Matheus, NC).

HPLC–ICP-MS measurements were carried out by using a PU-2080 Plus high pressure pump (JASCO, Japan) for chromatographic separation and a Thermo-X series X7 ICP-MS for elemental specific detection. Anionic exchange separation of selenium compounds was performed on a Hamilton PRP X-100 (Reno, NE). The HPLC column was directly connected to the Meinhard nebulizer of the ICP-MS via PTFE tubing (0.5 mm i.d). The samples were injected through a six-port Rheodyne 7725i valve fitted with a 100 μL loop. The optimum chromatographic and instrumental parameters for on-line measurements with ICP-MS are summarized in Table 1.

Cell counting was carried out by using a counting chamber Neubauer improved with a, bright-lined Hirschmann Laborgeräte EM, Techcolor (dimensions 0.100 mm depth, 0.0025 mm$^2$) and a microscope (Nikon 71622, 1.25x, Japan).

Fermentation was carried out under sterilized conditions by autoclaving material and solutions at 110 °C for 20 min. Filters of 0.22 μm were used to sterilize standard solutions. Manipulation and transfers were performed in a laminar flow cabinet (Telstar Biostar 16809, air speed 0.38 ms$^{-1}$, working pressure 132 Pa mm CA).

### 2.2. Reagents and materials

Selenomethionine (SeMet), selenomethylselenocysteine (SeMetSeCys), and selenocystine ($SeCys_2$) were purchased from Sigma and dissolved with Milli-Q water with 3% hydrochloric acid for improving dissolution. Solutions of inorganic selenium were prepared by dissolving sodium selenite ($Na_2SeO_3$) and selenate ($Na_2SeO_4$), purchased from Merck, in Milli-Q water. These stock solutions were kept in dark at 4 °C. Selenomethionine–Se–oxide (SeMetO) was obtained by oxidation of selenomethionine by using hydrogen peroxide, $H_2O_2$ (35%) (Merck), and by following the procedure described by Pedrero et al. [13]. The enzymatic hydrolysis was achieved by using a non-specific enzyme, Protease XIV, purchased from Sigma–Aldrich.

Separation of selenium species for anion-exchange chromatography was performed by using 10 mM of citric acid (Sigma) in 2% (v/v) methanol of HPLC grade. All reagents used were of the highest purity. De-ionized water (18 MΩ cm) was obtained from a Milli-Q water purification system unit.

*S. cerevisae*, *S. uvarum* and malt extracts were obtained from "Special Lager" and "West Riding Wheat" home brewing kits, purchased from Micromalta S.L. The commercial beer-making yeasts were microbiologically identified by the Deparment of Microbiology of Complutense University as a pure culture of *S. cerevisiae* and *S. uvarum*.

### 2.3. Beer's fermentation

About 0.125 g of rehydrated yeast (equivalent to $9.0 \times 10^6$ *S. cerevisiae* cells mL$^{-1}$ or to $24 \times 10^6$ *S. uvarum* cells mL$^{-1}$ for Ale and Lager fermentation, respectively and 16.3 g of malt extract were placed in 50 mL erlenmeyer flasks and exposed to sodium selenite at a final concentration of 0.2–20 μg mL$^{-1}$. The resulting solutions were left to ferment for 12 days at 18 °C. After fermentation, selenized beer was separated from yeast by centrifugation at 2057 g for 20 min.

### 2.4. Total selenium determination by ICP-MS

The post-fermentation products, i.e. residual yeast and selenized beer were digested with 2.5 mL of concentrated nitric acid in an analytical microwave oven. The resulting solutions were then diluted to 25.0 mL with deionized water prior to their analysis for total selenium by ICP-MS. In addition, selenium concentration was determined in commercial beers of different brands to establish baseline Se levels. Total selenium was quantified by both external and standard addition calibrations of the signal obtained by ICP-MS following the experimental conditions summarized in Table 1. Analytical peaks were evaluated as peak area.

### 2.5. Dialysis

About 5 mL of Se-enriched beer were dialysed. The dialysis process was performed for 20 h at 4 °C against Milli-Q water using dialysis membranes with 3.5 kDa molecular weight cut-off. During this period the Milli-Q water was changed twice.

### 2.6. Selenium species determination by HPLC–ICP-MS

The extraction of Se compounds from post-fermentation products was performed by ultrasonic-assisted enzymatic hydrolysis by using our procedure as described elsewhere [11]. In brief, 5 mL of beer or 0.200 mg of yeast were placed in an Eppendorf tube and 3 mL of deionized water and 20 mg of Protease XIV were then added. Sample hydrolysis was performed at 37 °C for 2 min sonication. The extracts were centrifuged at 7500 × *g* for 20 min by using a 10 kDa cut-off filter. Finally, the supernatants were appropriately diluted prior to their analysis for selenium speciation. Identification of selenium species were done by comparing the retention time and spiking experiments. Blanks analysis were performed to evaluate the occurrence of selenium species (Se(VI) and SeMet) from impurities in the protease used for sample extraction [14]. Selenium species quantification was performed by standard addition calibration of the signal obtained by HPLC–ICP-MS following the experimental conditions summarized in Table 1. Analytical peaks were evaluated as peak area.

### 2.7. Validation of the method

In the present study a certified reference material is employed in order to validate the methodologies used. Method validation was performed by using the certified reference material SELM-1 (Se-yeast, certified for total selenium (2059 ± 64 mg kg$^{-1}$)

**Table 2**
Results for the determination of total selenium concentration in beers of different trademarks using ICP-MS ($n = 3$).

| Sample (trademark) | Se ($\mu g\,L^{-1}$) |
|---|---|
| Mahou | 12.8 ± 1.2 |
| Amstel | 17.2 ± 1.3 |
| Heineken | 12.5 ± 1.1 |
| Bucker (0% alcohol) | 9.2 ± 1.2 |
| Paulaner | 14.2 ± 2.0 |

and selenomethionine ($3389 \pm 173\,mg\,kg^{-1}$) from NRCC, Ottawa, Canada).

## 3. Results and discussion

### 3.1. Selenium concentration in commercial beers

Total selenium concentration was determined in different trademarks beers by ICP-MS following the procedure given above. Detection and quantification limits were calculated as the concentration equivalent to three and ten times the standard deviation of blank measurements ($n = 10$), respectively. Detection and quantification limits were 0.001 $\mu g\,L^{-1}$ and 0.004 $\mu g\,L^{-1}$, respectively.

As shown in Table 2, the concentration of selenium found was quite low (ppb level) and similar between all branches tested.

### 3.2. Beer production in presence of increasing selenite concentration

We have recently demonstrated that the addition of selenium (as Se(IV)) before fermentation, up to a final concentration of 20 $\mu g\,mL^{-1}$ to produce white wine [11] does not affect the efficiency of the alcoholic fermentation process. Based on these previous results, brewing was carried out in the presence of Se(IV) (added as $Na_2SeO_3$) within the range 0.2–20 $\mu g\,Se\,mL^{-1}$. After 12 days of brewing, selenium concentration was determined in the Se-beer by ICP-MS. For this purpose, two different sample treatment procedures were applied: microwave acid digestion and enzymatic probe sonication. Besides, beer was analysed without applying any pre-treatment.

The total selenium found in selenized beer after fermentation in presence of increasing selenite concentration is compiled in Table 3. The percentage of selenium incorporated into beer was about 55–60% of the level of selenium initially added (from 1.0 to 10 $\mu g\,mL^{-1}$ of Se(IV)). In contrast, increasing the Se concentration to around 20 $\mu g\,mL^{-1}$ resulted in a decrease in this percentage (to around 30%). The results obtained were independent on the type of yeast used to make beer: the top-fermenting *S. cerevisiae* and bottom-fermenting *S. Uvarum*. Table 4 summarizes comparative results obtained for Se content in Se-beer by the application of different sample treatments suggesting that selenium is not associated with peptides or proteins.

**Table 3**
Total selenium in selenized beer after 12 days Ale-fermentation ($n = 3$).

| Se (IV) added ($\mu g\,Se\,mL^{-1}$) | Se in selenized beer ($\mu g\,Se\,mL^{-1}$) | % Se |
|---|---|---|
| 0.2 | 0.086 ± 0.003 | 43 ± 5 |
| 1.0 | 0.61 ± 0.08 | 61 ± 3 |
| 2.0 | 1.1 ± 0.4 | 55 ± 4 |
| 10.0 | 6.0 ± 0.7 | 60 ± 7 |
| 20.0 | 6.0 ± 0.5 | 30 ± 5 |

**Table 4**
Results for the determination of total selenium concentration in selenized beer using ICP-MS after application of different sample treatments ($n = 3$).

| Selenium added ($\mu g\,L^{-1}$) | Acid digestion by MW ($\mu g\,Se\,mL^{-1}$) | Enzymatic hydrolysis by USP ($\mu g\,Se\,mL^{-1}$) | Without treatment ($\mu g\,Se\,mL^{-1}$) |
|---|---|---|---|
| 0.2 | 0.086 ± 0.004 | 0.084 ± 0.003 | 0.083 ± 0.002 |
| 1.0 | 0.61 ± 0.02 | 0.64 ± 0.03 | 0.60 ± 0.07 |
| 10 | 6.0 ± 0.7 | 5.8 ± 0.6 | 6.3 ± 0.7 |

**Table 5**
Percentage of biotransformation in selenized beer after dialysis (MW > 3.5 kDa) ($n = 3$).

| Selenium added ($\mu g\,mL^{-1}$) | [Se]/$\mu g\,mL^{-1}$ in selenized beer | [Se]/$\mu g\,mL^{-1}$ in selenized beer after dialyisis | % Se |
|---|---|---|---|
| 0.2 | 0.086 ± 0.004 | 0.008 ± 0.004 | 10 ± 7 |
| 1.0 | 0.61 ± 0.02 | 0.060 ± 0.004 | 10 ± 1 |
| 10 | 6.0 ± 0.7 | 0.70 ± 0.01 | 12 ± 1 |

### 3.3. Selenite biotransformation during fermentation

As a first step to a fast evaluation of whether inorganic selenium has been biotransformed during fermentation process, Se-enriched beer was dialysed through a 3.5 kDa molecular weight cut-off membrane. The results obtained shown that 88% of the Se content transversed the membrane and emerged in the dialysate outside the bag. This evidence that only a minor amount (around 10%) of the Se(IV) incorporated into beer was converted to compounds with a molecular weight higher than 3.5 kDa (Table 5). Although dialysis is a valuable analytical tool for screening biotransformation processes, however, dialysis also involves hydrophobic interactions, which could keep compounds less than the pore size from crossing.

For a better understanding of the process, anion exchange HPLC–ICP-MS previous ultrasonic-assisted enzymatic hydrolysis was used for selenium speciation in post-fermentation products, using our procedure as described above. Besides, Se-beer was analysed by LC–ICP-MS without applying any pre-treatment.

A HPLC–ICP-MS chromatogram of an enzymatic extract of Se-beer obtained by ultrasonic probe sonication under conditions given in Table 1 is shown in Fig. 2. The presence of two major peaks that matched with the retention time of SeMet oxidation product and SeMet standard (Fig. 1) were detected. Comparative Se profiles (Fig. 2) were obtained with and without application of enzymatic hydrolysis. These results are in agreement with those obtained in the above dialysis study and strongly support that selenium in Se-beer is mainly present as free selenomethionine. As expected, a main peak corresponding to selenomethionine (4–5 min) was detected in the enzymatic extract of residual yeast (Fig. 3). Unlike beer, Se-yeast provides different Se profiles, dependent on the type

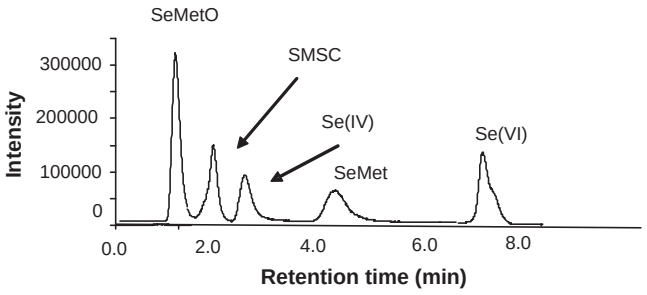


**Fig. 1.** Separation of a mixture of Se standards containing 150 $\mu g\,L^{-1}$ o each Se species by anion exchange-HPLC–ICP-MS. Chromatograms were monitored at Se $m/z$ 82.

**Table 6**
Quantification of SeMet by anion exchange HPLC–ICP-MS in post Ale-fermentation fractions ($n = 3$).

| Se-beer | | | | Se-yeast | | | Se-(beer + yeast) | | |
|---|---|---|---|---|---|---|---|---|---|
| Se added ($\mu g\,Se\,mL^{-1}$) | Se found ($\mu g\,Se\,mL^{-1}$) | SeMet ($\mu g\,Se\,mL^{-1}$) | %R | Se found ($\mu g\,Se\,mL^{-1}$) | SeMet ($\mu g\,Se\,mL^{-1}$) | %R | Se found ($\mu g\,Se\,mL^{-1}$) | SeMet ($\mu g\,Se\,mL^{-1}$ | %R |
| 0.2 | 0.086 ± 0.004 | ND | – | 0.12 ± 0.02 | 0.11 ± 0.01 | 92 ± 5 | 0.20 ± 0.05 | 0.19 ± 0.02 | 95 ± 5 |
| 1.0 | 0.61 ± 0.02 | 0.006 ± 0.003 | 1.0 ± 0.2 | 0.48 ± 0.05 | 0.46 ± 0.03 | 96 ± 8 | 1.10 ± 0.02 | 1.05 ± 0.01 | 95 ± 5 |
| 10 | 6.0 ± 0.7 | 0.12 ± 0.01 | 2.0 ± 0.3 | 4.0 ± 0.1 | 3.6 ± 0.1 | 90 ± 4 | 9.8 ± 0.2 | 10.3 ± 0.2 | 105 ± 3 |

*R* calculated as sum of species related to total selenium found.

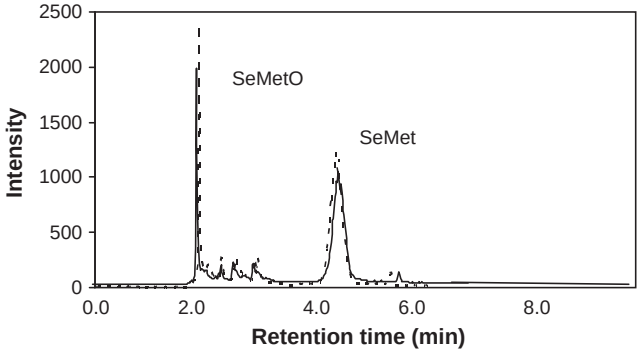


**Fig. 2.** Separation of Se-enriched ($10\,\mu g\,Se\,mL^{-1}$) beer by anion exchange HPLC–ICP-MS after 12 days Ale fermentation (A) with (dashed line) and (B) without enzymatic (straight line) hydrolysis. Chromatograms were monitored at Se *m*/*z* 82.

of sample treatment used. These results are consistent with those appearing in the literature indicating that selenium in Se-yeast is mainly present in the form of SeMet, but in this case associated with peptides or proteins. It is also interesting to note that Se-compounds produced during brewing were not dependent on the type of the commercial beer-making yeast, and therefore the type of fermentation, i.e. Lager (brewed with bottom-fermenting yeast as *S. uvarum*) and Ale (brewed with top-fermenting yeast as *S. cerevisiae)* fermentation.

### 3.4. Selenomethionine quantification

Selenomethionine in the final products after Ale fermentation in presence of 0.2, 1.0 and $10\,\mu g\,Se\,mL^{-1}$ was quantified by anion exchange HPLC–ICP-MS by using the standard addition method. Detection and quantification limits for SeMet were $0.7\,\mu g\,L^{-1}$ and $2.2\,\mu g\,L^{-1}$, respectively. The total Se content of such products was also determined for mass balance purposes. The quality assurance procedures for these analyses included the measurement of a Se-yeast reference material CRM SELM-1 for total selenium and SeMet concentration. Since, at the 95% confidence level, no significant differences were detected between the certified values and the experimental ones, the method used was considered accurate for total selenium and SeMet determination.

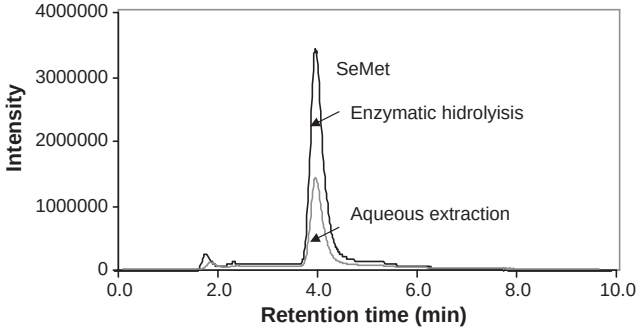


**Fig. 3.** Separation of residual selenized yeast by anion exchange HPLC–ICP-MS with and without previous enzymatic hydrolysis. Fermentation period: 12 days. Chromatograms were monitored at Se *m*/*z* 82.

As yeast is usually present in Ale beers, Se-Ale beer was analysed with and without separation of the yeast phase. As it can be observed in Table 6, SeMet concentration in the enzymatic extracts of both Se-Ale beer (without prior removal of yeast) and residual Se-yeast samples is about 95–105% of the total selenium concentration in the samples. However, the results were not as expected when SeMet was quantified in the enzymatic extract of Se-Ale beer previous separation of the yeast-phase. In this case, a proper mass balance was not achieved since SeMet accounted only 1–2% of the total selenium found. As it was previously mentioned, the HPLC–ICP-MS chromatogram obtained from an enzymatic extract of Se-beer (after yeast removal) showed the presence of a major peak that matched with the retention time of SeMet oxidation product. SeMet-oxide could be randomly produced during brewing and/or extraction step and sample storage, makes it difficult to achieve a proper mass balance. In any case, detection of both SeMet and SeMetO involves transformation of selenite into seleno amino acids during fermentation. Despite this problem, suitable results were obtained for Se-yeast and Se-Ale beer samples by using the proposed methodology, demonstrating its suitability for selenium speciation in beer samples.

## 4. Conclusions

In this study, biotransformation of selenium, added as selenite, during brewing has been shown. For this purpose, an analytical methodology based on the use of several sample treatments in conjunction with different separation mechanisms as dialysis and anion exchange-HPLC was optimized for evaluating Se-biotransformation during brewing. The proposed methodology allow us to identify and quantify total selenium and selenomethione in the products obtained after brewing (yeast and beer) at the low $\mu g\,L^{-1}$ levels. Method validation was achieved by measurement of Se-yeast reference material SELM-1.

The results presented here have evidenced selenite biotransformation during brewing by using either Lager or Ale fermentation. The administration of sodium selenite in the dosage of $1.0–10\,\mu g\,mL^{-1}$ is suitable for brewing without altering the fermentation capabilities of both *S. cerevisiae* and *S. uvarum*. Biotransformation of inorganic selenium during fermentation leads in the resulting selenized beer to SeMet which seems not to be incorporated into proteins. The results from Se-beer also demonstrated that SeMet is easily oxidized to SeMetO. SeMet randomly transformed to SeMetO by factors inherent to the medium makes it difficult to achieve a proper mass balance. Brewing is an additional example how yeast is able to transform inorganic selenium to organo-Se compounds (SeMet), safe and highly bioactive from the nutritional point of view.

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