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DETERMINATION OF SELECTED SELENOAMINO ACIDS IN BEER BY CAPILLARY ISOTACHOPHORESIS

OZNACZANIE WYBRANYCH SELENOAMINOKWASÓW W PIWIE TECHNIKĄ IZOTACHOFOREZY

Abstract: A rapid, simple and reliable capillary isotachophoresis (ITP) with conductivity detection was used to determine the trace quantities of organic compounds of selenium - selenomethionine (Se-Met) and selenocystine (Se-Cys) - in various types of beer. The content of Se-Met in beer is between $59 \cdot 10^{-3}$ g dm^3 (Czarne beer) and $510 \cdot 10^{-3}$ g dm^3 (Żywiec Porter beer), while the content of Se-Cys ranges from $84 \cdot 10^{-3}$ g dm^3 (Czarne) to $247 \cdot 10^{-3}$ g dm^3 (Żywiec Porter).

Keywords: isotachophoresis, beer, selenomethionine, selenocystine

Selenium is found in organisms as a trace element, though it is indispensable to their correct functioning. It is supplied in organic form, mainly as selenomethionine and selenocysteine, as well as inorganic form, as selenate(VI), M_2SeO_4 and selenate(IV), $MSeO_3$. However, various selenium derivatives of sulphuric amino acids have been detected in plants and animals [1]. Selenium facilitates assimilation of vitamin E and regulates its physiological functions. This element is indispensable in the work of the heart muscle and blood vessels, stimulates the immune system and retards tissue aging processes. Besides taking part in enzymatic reactions protecting cells from the effects of free radicals, selenium has immunodulatory, anti-inflammatory and antiviral effects. It protects the organism from poisoning by heavy metals, eg Fe, Cd or Pb, by forming metal selenides (M_2Se) with them. It has also been found to have anticarcinogenic (antitumor) properties, so it plays an important role in the prevention of neoplasmic diseases [2].

In organisms selenium plays mainly a biochemical role, as a component of enzymic proteins. There is no more comprehensive evidence for potential protection against tumours by different diet components than that which concerns selenium [3]. In over 90% of scientific research on the anticarcinogenic effects of selenium, selenate(IV) or

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selenomethionine (Se-Met) are used. It has been proved that Se-Met is much less toxic than inorganic selenium compounds [4].

In 1996 Clark et al [5] discovered that supplementing the diet of a group of people with selenized brewer's yeast caused a decrease of nearly 50% in overall cancer morbidity and mortality.

Organisms take selenium primarily with their food. Popular products considered rich in selenium are yeast and garlic [6]. Moreover, in natural food products selenium most often occurs in chemical combination with proteins, so food products with high protein content, such as meat, fish, fish products and especially offal, are the richest sources of selenium. Selenium-rich food products most often mentioned in other studies include tomatoes, cucumbers, broccoli, cabbage, celery, onion, egg yolk, bran, wheat, barley and shellfish [3].

Due to their anticarcinogenic effects, selenoamino acids constitute a highly significant group of compounds, so their determination in foods is very important. Nearly all separation techniques are used to analyse selenoamino acids. Good results are achieved using such techniques as gas chromatography (GC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) [7]. Many papers describe the coupling techniques used to separate selenoamino acids, mainly selenomethionine and selenocystine, in such products as garlic, onion or yeast. These are GC coupled to mass spectrometry (MS) [8] and HPLC coupled to mass spectrometer and inductively coupled plasma (ICP MS) [9-14]. Techniques used for selenium speciation are mainly ion chromatography with a variety of detectors: atomic absorption spectrometry (AAS) [15], inductively coupled plasma emission spectrometry [4, 16], hydride generation atomic absorption spectrometry (HG-AAS) [17], ultraviolet treatment-hydride generation atomic fluorescence spectrometry (UV-HG-AFS) [18-20] or hydride generation inductively coupled plasma mass spectrometry [21] and capillary electrophoresis coupled mainly with ICP MS [1, 22-25] and electrospray ionisation mass spectrometry (ESI MS) [22, 26].

Isotachophoresis is an electroseparation technique based on differences of migration velocities of analytes in an electrical field. In the ITP mode, only cations or anions can be separated. After analytes are introduced between two different boundaries, a leading electrolyte (LE) and a terminating electrolyte (TE), the sharp focsusing of individual zones is observed. If a steady state is attained, and zones are separated, all zones will be moving at a constant migration velocity [27].

This technique is used to a much lesser extent, despite its advantages of low cost, minimal or no sample preparation, especially in the case of the samples with complex matrix, and easy miniaturization.

TPI is a powerful technique for analyzing not only simple inorganic or organic samples but also biochemical samples. For example this technique has been applied to the analysis of amino acids. In most cases analysis has been performed on acidic amino acids as anions. A wide range of amino acids were investigated with a variety of aqueous electrolyte systems with alkaline pH levels [7, 28, 29].

In the literature on using ITP in determining amino acids there is only one paper [28], concering selenoamino acids. Using a ITP - capillary zone electrophoresis (CZE) coupling technique miniaturized system they determined Se-Met, selenoethionine (Se-Et), and Se-Cys, at a level of several micromoles per litre. The isotachophoresis was used to distribute and preseparate the sample components.

In the literature on selenium content in food there are no publications concerning selenoamino acids concentration in beer, despite the fact that beer is undoubtedly a rich source of this compound. For this reason the present study aimed to work out an analytical method using isotachophoresis, a relatively fast and low-cost technique, to determine Se-Met and Se-Cys in selected types of beer.

Materials and methods

Apparatus, reagents and samples

Isotachophoretic separations were performed using the Electrophoretic Analyser EA 102 (Villa Labeco, Spisska Nova Ves, Slovakia) equipped with column-coupling system consisting of two capillaries with conductivity detectors. The analytical capillary (160 x 0.3 mm ID) was connected with a preseparation capillary (90 x 0.8 mm ID). Capillaries of fluorinated ethylene-propylene copolymer were used. The analyser was equipped with a sample valve of 30 mm^3 fixed volume. Separations were performed at a room temperature.

5-bromo-2,4-dihydroxybenzoic acid and seleno-L-methionine (98%) (Se-Met) from Sigma-Aldrich (Steinheim, Germany), ethanolamine, 1,3-bis[tris(hydroxymethyl)methylamino]propane (BTP), Mowiol (40-88), β-alanine and seleno-L-cystine (98%) (Se-Cys), sodium hydroxide (30%) all from Fluka (Buchs, Switzerland), methanol from J.T. Baker (Holland) and barium hydroxide from Chem-Lab (Belgia). All chemicals were of analytical grade. Deionized bidistilled water was used in the preparation of the electrolyte systems, stock solutions of all standards and beer samples.

Beers in bottles (Table 1) were purchased from local retail outlets.

Table 1

Beers

Name	Kind	Producer (importer)	Brewing place and racking	Extract contents [%]
MILLER	light	Brewing Company S.A.	Italy	11.2
PILSNER URQUELL	light	Brewing Company S.A.	Czech Republic	11.8
PERONI NASTRO AZZURRO	light	Brewing Company S.A.	Italy	lack of data
LECH PREMIUM	light	Brewing Company S.A.	Poland	11.1
DOG IN THE FOG	light	Brewing Company S.A.	Poland	lack of data
TYSKIE	light	Brewing Company S.A.	Poland	11.7
REDD'S	light	Brewing Company S.A.	Poland	lack of data
ŻYWIEC PORTER	dark	Brewing Group Żywiec S.A.	Poland	22
CZARNE	dark	Brewing „FORTUNA" Sp.zo.o.	Poland	12.7

Isotachophoretic conditions

Composition of the electrolyte system used for performing isotachophoretic separation is shown in Table 2.

Table 2

Composition of the electrolyte system used for performing isotachophoretic selenoamino acids separations

	Leading ion	4 mM 5-bromo 2,4-dihydroxybenzoic acid
Leading electrolyte	Counter ion	ethanolamine
	Co-counter ion	2 mM BTP
	Additive*	0.05% Mowiol (40-88)
	pH	9.4
Terminating electrolyte	Terminating anion	10 mM β-alanine
	Counter ion	Ba^{2+}[added as $Ba(OH)_2$]
	pH	10.5

*To suppress the electroosmotic flow

Prior to use, all buffer solutions were degassed with an ultrasonic bath Model Polsonic 3 (Polsonic, Poland). The separations performed were achieved using control program shown in Table 3.

Table 3

Separation program used for performing ITP

Step	Time [s]	Current [μA]
1	300	250
2	300	250
3	70	250
4	150	50
5	700	50

Sample preparation

Standard solutions of selenomethionine and selenocystine were prepared by dissolving $2 \cdot 10^{-3}$ g of each preparation in 10 cm^3 of water. Working standards solutions (from $1 \cdot 10^{-3}$ to $15 \cdot 10^{-3}$ g·dm^{-3}) were freshly prepared daily by diluting appropriate aliquots of the stock solutions in water.

For ITP analysis beer samples were diluted 1:20 for bright beers and 1:40 for dark beers respectively, degassed in an ultrasonic bath for 70 min to remove CO_2 and adjusted to pH 9.5 by addition of 30% NaOH. Then the water was added to give a final volume of 10 cm^3.

Results and discussion

Choosing electrolytes

For isotachophoretic determination of selenoamino acids the electrolyte system developed by Evearst et al [7] was used for amino acids separation and later used for preliminary separation of synthetic mixtures of these compounds by Grass et al [28] using ITP technique. Since pH of these solutions is high there is a possibility of carbon dioxide absorption from the air leading to carbonate formation during isotachophoretic separation. This problem (to minimize interference due to this absorption) was solved adding 5-bromo-2,4-dihydroxybenzoic acid (leading ion of a mobility similar to carbonates) to the leading buffer while the terminating buffer was prepared using barium ions from barium hydroxide being the counter ion.

During preliminary experiments it was found that time elapsed since the preparation of electrolytes influenced the shape of a blank test curve on the recorded isotachophoregrams. The longer the time the smaller the difference of height between leading and terminating electrolyte showing additional steps most probably being products of electrolyte decomposition. This problem was solved by freezing fresh electrolytes and taking each time only the amount necessary for current experiments.

Isotachophoretic analysis

For the chosen electrolyte system (Table 2) isotachophoretic separation conditions for selenoamino acids mixture were optimized. Figure 1 shows recorded isotachophoregram with steps corresponding to selenocystine and selenomethionine, respectively. Other steps correspond to impurities of buffer solutions. The relative step heights (RSHs), counted as the ratio of the step height of the analyte to the step height of the terminator, values being the base for qualitative analysis were determined for both selenoamino acids. For Se-Met RSH is 0.60 while for Se-Cys RSH is 0.17.
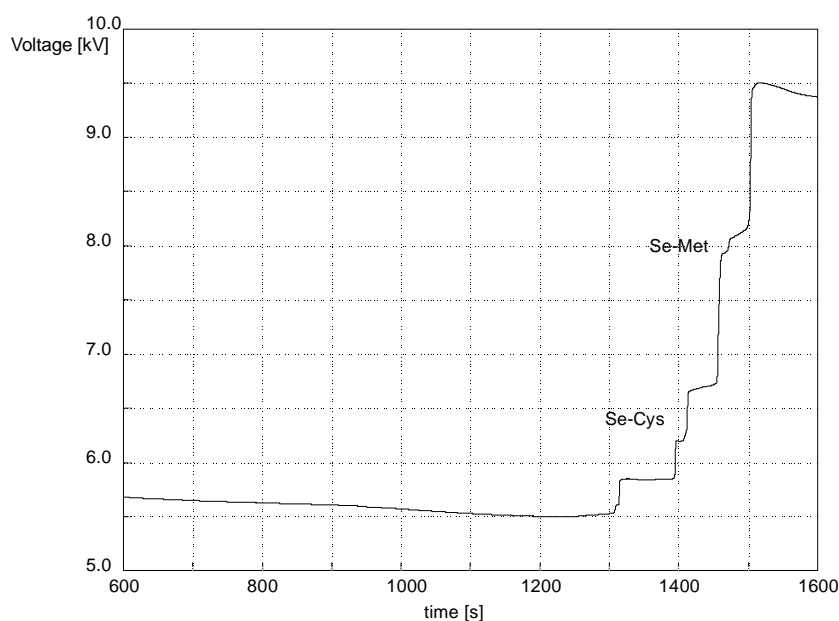

Fig. 1. Isotachopherogram of sample containing $1 \cdot 10^{-4}$ $g \cdot dm^{-3}$ Se-Met and Se-Cys. The experimental conditions employed are detailed in Tables 2 and 3

Quantitative analysis was performed using standard addition method that minimizes the matrix influence on results of determination. Usually selenoamino acid content was first estimated using calibration curve.

Calibration using standard addition method was performed for beers preparing beer solutions with 25, 50 and 100% addition of Se-Met and Se-Cys.

Following validation parameters were determined using calibration curves (standard solutions concentrations 1, 2.5, 5, 7.5, 10, $15 \cdot 10^{-3}$ $g \cdot dm^{-3}$): linearity, detection limit and

quantification limit. Though calibration curves for Se-Cys and Se-Met (Table 4) show high correlation coefficients suggesting linearity of detector response in the selenoamino acids concentration range examined, linearity of detector response towards these analytes was determined. For this purpose for each signal recorded during preparation of calibration curves concentrations of selenoamino acids were calculated using calibration curve equations. Calculated concentrations were compared with expected values (known concentrations of the solutions used in preparation of calibration curve). Calculated values show that standard solutions having concentration $1 \cdot 10^{-3}$ and $2.5 \cdot 10^{-3}$ $g \cdot dm^{-3}$ are outside linearity range of the detector being calibrated.

Table 4

Regression parameters calculated from Se-Cys and Se-Met calibration curve

	Se-Cys	Se-Met
Parameter	Value	Value
Slope [$s/10^{-3}g \cdot dm^{-3}$]	0.89	0.91
y-intercept [s]	1.14	1.08
Correlation coefficient	0.97	0.99
Standard deviation for slope coefficient [$10^{-3}g \cdot dm^{-3}$]	0.03	0.02
Standard deviation for y-intercept [s]	0.26	0.16
Residual standard deviation [$10^{-3}g \cdot dm^{-3}$]	0.81	0.51

Detection limits for Se-Cys and Se-Met were calculated using calibration curves. Regression parameters in Table 4 were calculated using appropriate functions of MS EXCEL program.

Using calibration curve parameters and the following equation: LOD = (3.3s)/b (where b is the slope coefficient of the calibration curve, s is the residual standard deviation for calibration curve (s_{xy}) or the residual standard deviation for y-intercept of calibration curve (s_a)), the following values of LOD were calculated taking into account residual standard deviation for y-intercept - s_a as well as residual standard deviation for slope coefficient - s_{xy}: s_a - LOD = $0.96 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Cys, s_a - LOD $0.59 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Met, s_{xy} - LOD = $2.99 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Cys, s_{xy} - LOD = $1.84 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Met.

Apparent difference between the calculated values is correct. Taking into account residual standard deviation gives higher LOD value because in this case not only y-intercept variation but also variation of slope coefficient is taken into consideration. Therefore, the mean value of measurements can be used as the determined LOD value: LOD $\approx 2 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Cys, LOD $\approx 1.2 \cdot 10^{-3}$ $g \cdot dm^{-3}$ Se-Met.

Using these values the limit of quantification (LOQ), ie the smallest amount or concentration of analyte that can be quantified using this method was calculated according to the equation: LOQ = 3 LOD. The calculated limit of quantification is $6 \cdot 10^{-3}$ $g \cdot dm^{-3}$ for Se-Cys and $3.6 \cdot 10^{-3}$ $g \cdot dm^{-3}$ for Se-Met. These values explain the source of so high deviation from linearity for calibration curve points in the low concentration range.

Selenoamino acids determination in various beers

The aim was to determine selenoamino acids concentrations in various beers. Before isotachophoretic analysis beer was prepared according to the procedure described in section

Sample preparation. Examples of isotachophoregrams for Miller and Żywiec Porter beer are shown in Figures 2 and 3. Se-Cys and Se-Met concentration was determined using on the basis of standard additions. Results obtained for Se-Cys and Se-Met are given in Table 5.
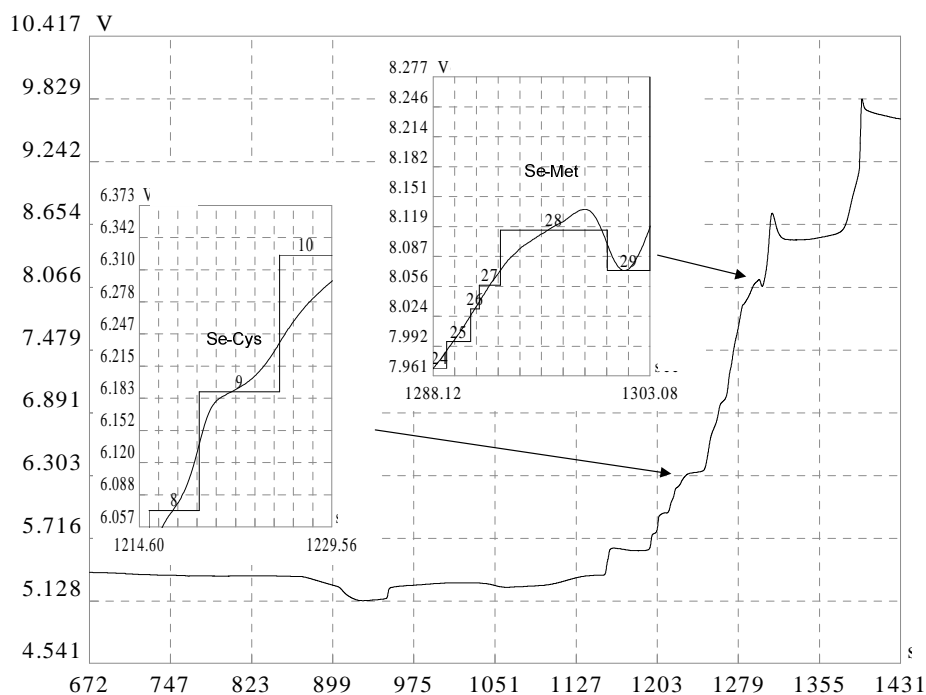

Fig. 2. Isotachopherogram of Miller beer, records of analytical capillary. The experimental conditions employed are detailed in Tables 2 and 3

Analyzing concentrations of Se-Cys obtained it was found that Redd's is the beer containing lowest concentration of this amino acid ($43.5 \cdot 10^{-3}$ g·dm^{-3}). Traditional light beers brewed in Poland, namely Lech Premium and Tyskie Se-Cys concentration was $100 \cdot 10^{-3}$ g·dm^{-3}. No such correlation (similar Se-Cys concentration in the same kind of beer from the same country) was found for Italian beers Miller ($120 \cdot 10^{-3}$ g·dm^{-3}) and Peroni Nastro Azzuro ($85 \cdot 10^{-3}$ μg·dm^{-3}). The picture is the same for sweet beers brewed in the same brewery: Dog In The Fog ($220 \cdot 10^{-3}$ g·dm^{-3}) and Redd's ($44 \cdot 10^{-3}$ g·dm^{-3}). The highest content of Se-Cys was found in dark beer Żywiec Porter ($247 \cdot 10^{-3}$ g·dm^{-3}).

A correlation between Se-Cys concentration and malt content can be observed (Table 1). Beers having malt content of 11% have Se-Cys concentration about $110 \cdot 10^{-3}$ g·dm^{-3} while beers having 22% of malt have about $240 \cdot 10^{-3}$ μg·cm^{-3} of Se-Cys.

The best repeatability was found for Miller beer having standard deviation of $\pm$ 2%, while the worst repeatability was found for Redd's beer with residual standard deviation $\pm$ 19%.
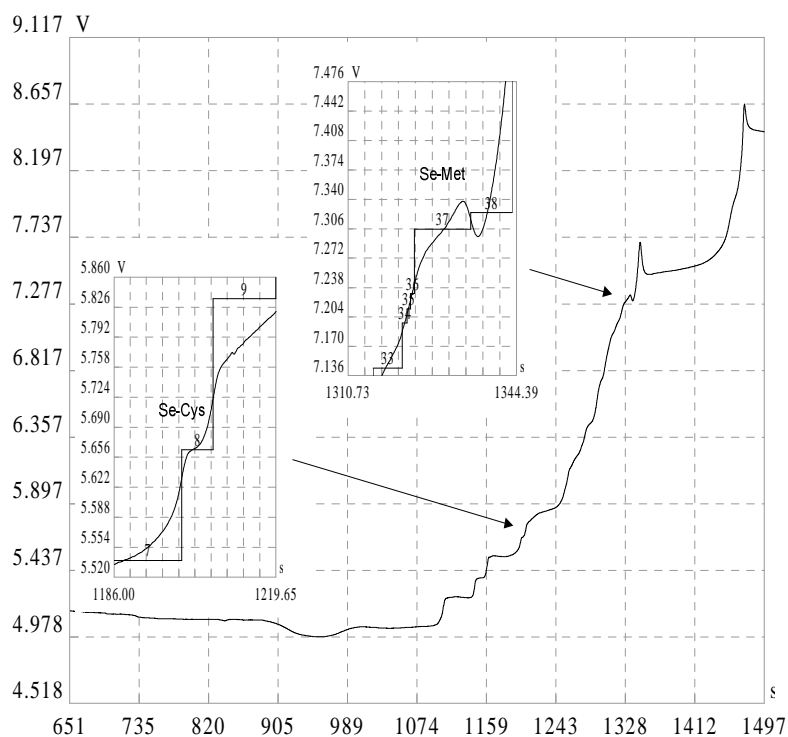
Fig. 3. Isotachopherogram of Żywiec Porter beer, records of analytical capillary. The experimental conditions employed are detailed in Tables 2 and 3

Table 5

Comparison of the content of Se-Met and Se-Cys in different types of beer

Beer	Se-Met			Se-Cys		
	Concentration $[10^{-3} \text{ g} \cdot \text{dm}^{-3}]$	SD	RSD [%]	Concentration $[10^{-3} \text{ g} \cdot \text{dm}^{-3}]$	SD	RSD [%]
MILLER	139.5	6.95	5.0	119.8	1.91	1.6
PILSNER URQUELL	165.6	8.67	5.2	132.3	7.66	5.8
PERONI NASTRO AZZURRO	131.5	19.41	14.8	84.6	9.08	12.1
LECH PREMIUM	145.1	7.40	5.1	99.4	5.4	5.4
DOG IN THE FOG	234.2	4.16	1.8	219.5	10.13	4.6
TYSKIE	83.5	10.86	13.0	92.5	4.69	5.1
REDD'S	88.8	10.05	11.3	43.5	8.29	19.1
ŻYWIEC PORTER	510.4	21.9	4.3	246.5	11.0	4.5
CZARNE	58.7	10.39	17.7	84.0	7.30	8.7

In the case of Se-Met (Table 5) Czarne is the beer having the lowest content of that substance ($59 \cdot 10^{-3}$ g·dm^{-3}) while dark beer Żywiec Porter which had the highest content of

Se-Cys has the highest content of that Se-Met too ($510 \cdot 10^{-3}$ g·dm^{-3}). Much similar content of Se-Met was found in beers brewed in Italy: Miller ($140 \cdot 10^{-3}$ g·dm^{-3}) and Peroni Nastro Azzurro ($132 \cdot 10^{-3}$ g·dm^{-3}), Czech beer Pilsner Urquell ($166 \cdot 10^{-3}$ g·dm^{-3}) and Polish beer Lech Premium (145 g·dm^{-3}). High variation in content of the examined amino acid was found for tow sweet beers from the same brewery: Dog In The Fog ($234 \cdot 10^{-3}$ g·cm^{-3}) i Redd's ($89 \cdot 10^{-3}$ g·dm^{-3}). The same as in the case of Se-Cys it was found that the higher the malt content the higher Se-Met concentration. The only beer that does not conform to that 'rule' is Czarne beer. Despite the high extract content in that beer (12%) only $59 \cdot 10^{-3}$ g·dm^{-3} of Se-Met was found.

In the case of quantification of that selenoamino acid repeatability of results is much worse than that for Se-Cys. Relative standard deviation is between $\pm 2\%$ up to as much as $\pm 18\%$. The best repeatability was achieved for Miller beer and the worse was for Redd's beer. In the case of Pilsner Urquell and Peroni Nastro Azzurro it was impossible to select 7 most similar times, that had negative influence on repeatability of results, especially for the beer brewed in Italy.

It was also found that almost all the beers examined contain more Se-Met than Se-Cys. In seems to be justified by literature reporting high Se-Met content in beer production substrates [30].

Conclusions

An analytical procedure for simultaneous determination of Se-Cys and Se-Met in beer as anions using ITP technique having short time of analysis and low operating cost (chemicals) was developed.

The determined concentrations of Se-Cys are between $43 \cdot 10^{-3}$ g·dm^{-3} for Redd's beer and $247 \cdot 10^{-3}$ g·dm^{-3} for Żywiec Porter beer while Se-Met content flash in the range of $59 \cdot 10^{-3}$ g·dm^{-3} for Czarne beer to $510 \cdot 10^{-3}$ g·dm^{-3} for Żywiec Porter beer. In almost all beers Se-Met content is higher than Se-Cys content.

It was found that the concentrations of selenoamino acids under examination are highly dependent on malt content.

Determination and quantification limits found were: Se-Cys: LOD = $2 \cdot 10^{-3}$ g dm^{-3}, LOQ = $6 \cdot 10^{-3}$ g·dm^{-3}, Se-Met: LOD = $1.2 \cdot 10^{-3}$ g·dm^{-3}, LOQ = $3.6 \cdot 10^{-3}$ g·dm^{-3}.

Acknowledgements

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OZNACZANIE WYBRANYCH SELENOAMINOKWASÓW
W PIWIE TECHNIKĄ IZOTACHOFOREZY

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Abstrakt: W pracy przedstawiono sposób oznaczania organicznych związków selenu - selenocystyny i selenometioniny - w różnych gatunkach piwa za pomocą techniki izotachoforezy (ITP). Na podstawie otrzymanych wyników stwierdzono, że technika izotachoforezy pozwala w stosunkowo krótkim czasie i przy małych kosztach analizy oznaczyć śladowe ilości selenocystyny (Se-Cys) i selenometioniny (Se-Met) w próbkach piwa. Wyznaczona zawartość Se-Met w piwie waha się w granicach od ok. $59 \cdot 10^{-3}$ g·dm^{-3} (piwo Czarne) do ok. $510 \cdot 10^{-3}$ g·dm^{-3} (piwo Żywiec Porter), a zawartość Se-Cys od ok. $84 \cdot 10^{-3}$ g·dm^{-3} (dla piwa Czarne) do ok. $247 \cdot 10^{-3}$ g·dm^{-3} (piwo Żywiec Porter).

Słowa kluczowe: izotachoforeza, piwo, selenometionina, selenocystyna