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Journal of Chromatography A, 1071 (2005) 191–196
JOURNAL OF CHROMATOGRAPHY A
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Identification of the major selenium compound, Se-Methionine, in three yeast (*Saccharomyces cerevisiae)* dietary supplements by on-line narrowbore liquid chromatography–electrospray tandem mass spectrometry

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Available online 8 December 2004

Abstract

On-line monitoring of six Se-compounds was accomplished by using an XTerra MS C_{18} column coupled to electrospray tandem mass spectrometry (ES–MS–MS). In view of the nature of the compounds, the positively charged ion pairing agent tetraethylammoniumchloride (TEACl) was added to the mobile phase. The HPLC–ES–MS–MS method was optimized with six commercially available Se-compounds. Substitution of the analytical column by the narrowbore type significantly enhanced the sensitivity of the method. We were able to detect the *m*/*z* of these six molecules on-line. Furthermore, all product ions could be monitored. The method was applied to three different yeast-based supplements. They were submitted to proteolytic digestion and screened for their Se-content by HPLC–HG–AFS (hydride generation-atomic fluorescence spectrometry). By application of on-line narrowbore HPLC–electrospray tandem mass spectrometry, the main compound present in these three supplements, Se-Methionine, could be measured on its *m*/*z* and its product ions. The method can be further extended for on-line measurement of different Se-species in complex matrices

Keywords: Speciation; HPLC; ES–MS–MS; Selenium

1. Introduction

Se-supplements are being commercialized on a large scale because of their anti-oxidative properties [1]. Both organic and inorganic forms of Se are added to the growth medium of *Saccharomyces cerevisiae* (baker's yeast) to obtain these nutraceuticals. The effect of the element on human health is highly dependent on the chemical species under which it is consumed. Human intake of Se is partly dependent on the geographical region where we live. For people living in Se-depleted areas, the intake of Se-supplements may indeed be very beneficial. The question remains, however, how reliable these supplements are and whether they contain active Se-compounds. The complexity of the matrix and the low levels of Se, even in enriched samples, are the major challenges of speciation analysis. There is an urgent need for analytical techniques for separation, detection and identification of these species. The technique should allow the identification of the relevant compounds and reveal possible falsifications, i.e. when the actual chemical form present does not comply with the description. Many attempts have been made to characterize the relevant species. To date, few species present in yeast have been identified [2–9]. Signal suppression was a major problem when monitoring Se-species on-line [7]. Some results on the speciation of Se have been obtained by on-line HPLC–ES–MS(–MS). Most of them, however, were restricted to the analysis of standard solutions of Se-Cystamine (Se-Cya), Se-Ethionine, Se-Methionine (Se-Met), Se-Cystine (Se-$(Cys)_2$) and Se-MethylSelenocysteine (Se-MeSeCys). Only one product ion of each standard solution was monitored on-line with ES–MS–MS, due to the lack

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doi:10.1016/j.chroma.2004.11.046

of sensitivity [10]. The presence of Se-Met was demonstrated in a yeast extract by using retention time matching and monitoring of the molecular ion at *m*/*z* 198 by HPLC–ES–MS. As will be shown in this paper retention time and *m*/*z* determination are insufficient to characterize a molecule in such complex matrices [11]. Our earlier research, based on the combination of HPLC and HG–AFS, showed that in the proteolytic digest of a Se-supplement, one main Se-compound was present [12]. In this paper, a narrowbore column was hyphenated to ES–MS–MS to monitor Se-compounds on-line in three Se-supplements. It is known that ES–MS is often not sensitive enough to characterize a molecule. When coupling an analytical column to ES–MS, a flow split is necessary, and hence, part of the sample is then dissipated. By using a narrowbore column, whole sample can be sent to the mass spectrometer. It will be shown that this method enabled the on-line determination of all product ions of the main compound present in the different yeast matrices examined in this study.

2. Experimental

2.1. Chemicals

The formic acid used was from Vel (Leuven, Belgium). Acetonitrile was from VWR (Leuven, Belgium). The three Se-supplements investigated were: SelenoPrecise, 100 μg Se tablets from Pharma Nord (Vejle, Denmark); SeACE, 200 μg Se tablets from Wassen (Surrey, England) and Bioplex, 25 μg Se tablets from Lehning (Sainte-Barbe, France). All other chemicals used were as described in our previous work [12].

2.2. Instrumentation

For measurements with HPLC–ES–MS–MS an XTerra MS C_{18} narrowbore column with an internal diameter of 2.1 mm was used. An XTerra MS C_{18} guard column (*L* 20 mm, i.d. 2.1 mm, 5 μm particles) was applied. Both column and guard column were from Waters (Milford, MA, USA). The triple quadrupole based electrospray tandem mass spectrometer is a Quattro Micro system (Micromass, Manchester, UK) equipped with a Z-spray source. All other instrumentation was described in our previous work [12].

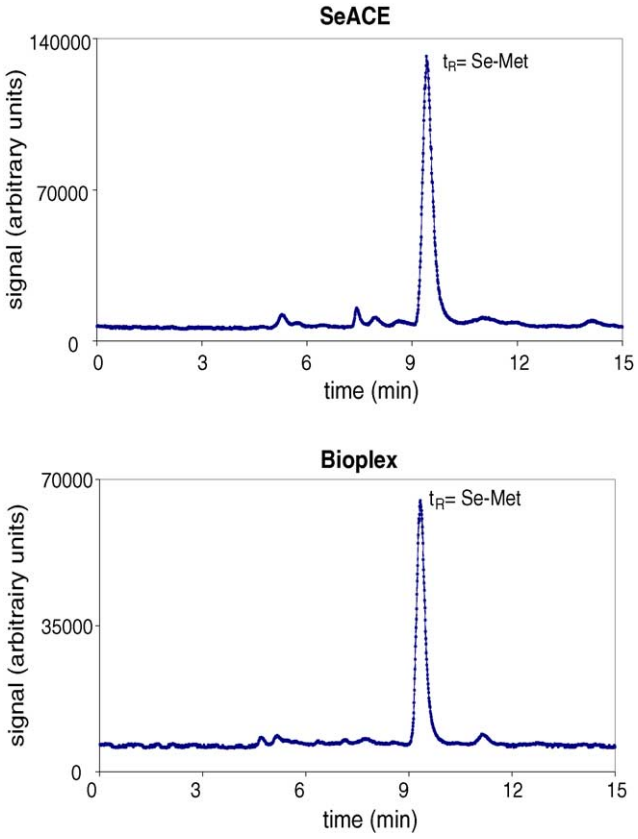

Fig. 1. HPLC–HG–AFS of enzymatic digests of SeACE and Bioplex supplements.

2.3. Standard solutions

Standard solutions for ICPMS measurements were already described [12]. Limits of detection (LODs) with pneumatic nebulization ICP–MS (PN–ICP–MS) were determined using standard solutions of 10 μg Se l^{-1} and 0.14 M HNO_3 as the blank. The LODs obtained were 0.14 μg l^{-1} for Se(IV), Se(VI), Se-Met and Se-Cya and 0.15 μg l^{-1} for Se-$(Cys)_2$ and Se-MeSeCys according to the 3 times signal-to-noise criterion.

For measurement of the product ion spectra in flow injection mode by ES–MS–MS, Se-Met, Se-Cya, Se-$(Cys)_2$ and Se-MeSeCys standard solutions were prepared with a 1 mg l^{-1} concentration of the compound. The standards were

Table 1
ES–MS–MS settings for measurement of the six Se-species

	Se-Met	Se-$(Cys)_2$	Se-MeSeCys	Se-Cya	Se(IV)	Se(VI)
Ion mode	+	+	+	+	–	–
m/*z*	198	337	184	249	129	145
Product ions	181, 152, 135, 109, 102	248	167, 149, 123, 95	204		
Capillary voltage (kV)	3.15	3.40	3.20	3.20	2.5	2.5
Cone voltage (V)	22	35	18	20	30	35
Collision energy (eV)	15	20	20	15		
Cone gas flow rate (l h^{-1})	50	50	50	50	50	50
Desolvation gas flow rate (l h^{-1})	330	350	325	325	350	350

Photomultiplier voltage: 650 V, source temperature: 120 °C, desolvation temperature: 300 °C.

diluted in a 1:1 H_2O:acetonitrile mixture. For on-line determination of the compounds and the product ions, standard solutions were made of 1 mg l^{-1} compound concentration and diluted in a 1% formic acid solution in order to enhance ion formation by protonation of the compounds.

2.4. Sample preparation

The yeast tablets were ground to powder in a stainless steel grinding-mill. The extraction procedure was described earlier [12].

2.5. Total Se determination in Se-tablets and in yeast extracts

INAA experiments showed that the total Se-concentration in the tablets was 328 μg Se g^{-1} (96 μg Se/tablet), 415 μg Se g^{-1} (190 μg Se/tablet) and 140 μg Se g^{-1} (24.9 μg Se/tablet) ±1% (±3 times signal-to-noise) for SelenoPrecise, SeACE and Bioplex supplements, respectively. Measurement of the total Se-concentration in supplements was described by Dumont et al. [12]. The total Se recoveries after proteolytic digestion were 85, 78 and 57% ±1% for SelenoPrecise, SeACE and Bioplex supplements, respectively.

2.6. HPLC–HG–AFS measurements

The conditions for separation and detection and the chromatogram with HG–AFS detection of SelenoPrecise were already presented by Dumont et al. [12]. The chromatograms of the extracts of SeACE and Bioplex are presented in Fig. 1.

2.7. (HPLC–)ES–MS(–MS) measurements

For HPLC–ES–MS–MS measurements, different scan modes were applied. The four organic species (Se-Met, Se-$(Cys)_2$, Se-Cya, Se-MeSeCys) were measured in positive ion mode, the inorganic species selenite (Se(IV)) and selenate (Se(VI)) had to be measured in negative ion mode. Product spectra were obtained for the organic species after parameter optimization (Table 1) and were in accordance to the spectra published by Lindemann et al.[10]. For Se-Met ($^+NH_3CH(COOH)CH_2CH_2SeCH_3$) *m/z* 198 was selected and 5 fragment ions could be distinguished at *m/z*: 181, 152, 135, 109 and 102. The *m/z* 181 corresponds to the loss of OH, *m/z* 152: loss of CO and H_2O, *m/z* 135: loss of CO, NH_3 and H_2O, *m/z* 109 is the ion $CH_3SeCH_2^+$ and *m/z* 102 is the ion $NH_3CH(COOH)CH_2CH_2^+$. In the product ion spectrum of Se-$(Cys)_2$ ($NH_2(COOH)CHCH_2$Se-$SeCH_2CH(COOH)NH_3^+$) (*m/z* 337) only one fragment ion was observed at *m/z* 248, corresponding to a loss of $NH_2CH(COOH)CH_3$. The product ion spectrum of Se-Cya ($NH_2CH_2CH_2$Se-$SeCH_2CH_2NH_3^+$) (*m/z* 249) also only displayed one fragment ion at *m/z* 204: this fragment ion is produced as a result of the loss of $NH_2CH_2CH_3$. The product ions of Se-MeSeCys ($^+NH_3(COOH)CHCH_2SeCH_3$) (*m/z*

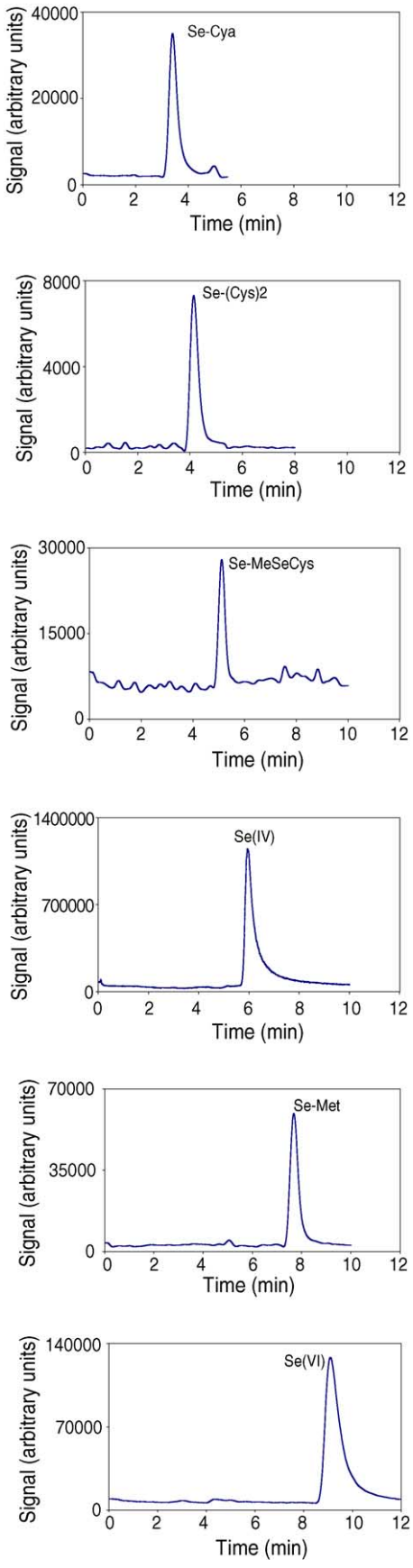

Fig. 2. HPLC–ES–MS: SIR of *m/z*: 249, 337, 184, 129, 198 and 145.

184) were found at *m/z* 167: loss of OH, *m/z* 149: loss of NH_3 and H_2O, *m/z* 123: loss of CO, NH_3 and H_2O and *m/z* 95: corresponding to the ion $SeCH_3^+$.

3. Results and discussion

3.1. Separation and detection of Se-standards

3.1.1. Optimization of the HPLC conditions for coupling with ES–MS(–MS)

For ES–MS–MS detection, the mobile phase was slightly modified: less TEACl was used, 2% methanol was added to lower the surface tension and hence to enhance desolvation. The mobile phase for use with ES–MS(–MS) was finally 0.01% TEACl + 2% methanol pH 4.5. Prior to analysis both the analytical and the narrowbore column had to be conditioned by flushing the column overnight with 0.05% TEACl solution.

3.1.2. HPLC–ES–MS of the Se-compounds

When hyphenating HPLC to ES–MS, the desolvation gas flow rate was set at 650 l h^{-1} and the desolvation temperature was raised to 300 °C. To determine the species by on-line HPLC–ES–MS a compromise was found for all organic compounds by setting the capillary voltage to 3.15 and 2.5 kV in the positive and negative ion mode respectively. For the organic species, the cone voltage and the collision energy were optimized for every single compound. The Se-compounds were first investigated by coupling the analytical column (i.d. 4.6 mm) to the electrospray probe. A flow split (40:60) was needed after the column since the electrospray source cannot handle a flow of 0.8 ml min^{-1}, such that only 40% of the sample was sent to the ES–MS. This resulted in a loss of compound and thus, loss of sensitivity. The lower signal did not give any problems for investigation of the standard-solutions. However, when measuring real biological samples, this led to major problems. A narrowbore column (i.d. 2.1 mm) was used instead. With this column a lower flow, 160 μl min^{-1} was applied which enabled sending the entire flow and thus the entire sample to the ES–MS. All species were monitored by selected ion recording (SIR) under the optimized conditions. The chromatograms obtained for the standards are given in Fig. 2. Se-Met was measured at *m/z* 198, Se-$(Cys)_2$ at *m/z* 337, Se-Cya at *m/z* 249, Se-MeSeCys at *m/z* 184, Se(IV) at *m/z* 129 and Se(VI) at *m/z* 145. Limits of detection for HPLC–ES–MS were determined as follows: the guard column with 2.1 mm i.d. was coupled to the ES–MS. Every compound monitored in positive ion mode was dissolved in a 1% formic acid solution and was brought onto the column separately. The compounds were eluted with a 1:1 H_2O:acetonitrile mixture and measured at the *m/z* of their molecular ion. The blank solution for LOD determination in positive ion mode was a 1% formic acid solution and was measured 10 times. For determination of the LOD in negative ion mode, milliQ water was measured 10 times. The LODs obtained were 14.8 μg l^{-1} for Se-Met, 3.0 μg $l^{-1}$for Se-$(Cys)_2$, 13.1 μg l^{-1} for Se-MeSeCys, 1.3 μg l^{-1} for Se-Cya, 14.8 μg l^{-1} for Se(IV) and 2.7 μg l^{-1} for Se(VI) (according to the 3 times signal-to-noise criterion) for 10 μl injections.

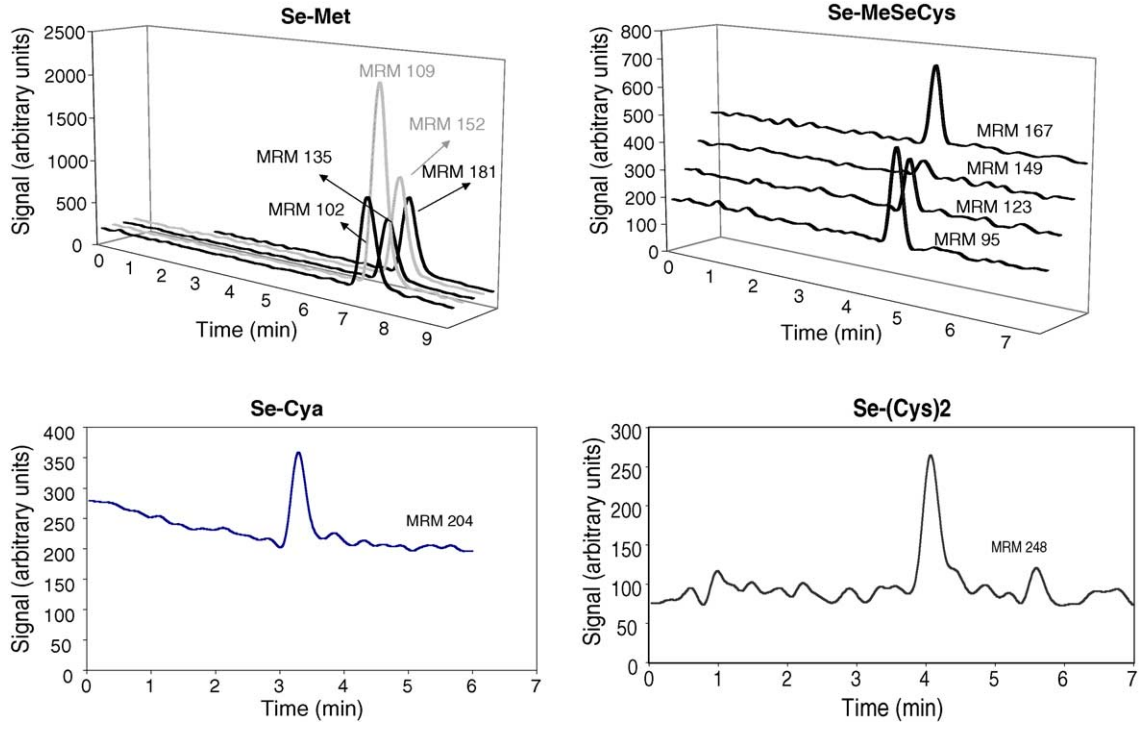

Fig. 3. HPLC–ES–MS–MS: MRM s of Se-Met, Se-MeSeCys, Se-Cya and Se-$(Cys)_2$.

3.1.3. HPLC–ES–MS–MS of the Se-compounds

A compound present in a biological sample can be identified by combination of its retention time, the mass of its molecular ion and monitoring its fragment ions. All organic species were analyzed by on-line HPLC–ES–MS–MS for their products. This was done by multiple reaction monitoring (MRM), in which the precursor ion and a product ion are selected. This mode delivers the best signal-to-noise ratios. For Se-Met, the MRM of transitions $198 \rightarrow 181$, $198 \rightarrow 152$, $198 \rightarrow 135$, $198 \rightarrow 109$ and $198 \rightarrow 102$ were measured according to their product ions. For Se-$(Cys)_2$ only one MRM was monitored: transition of m/z $337 \rightarrow 248$. Se-MeSeCys is characterized by MRM of transitions: $184 \rightarrow 167$, $184 \rightarrow 149$, $184 \rightarrow 123$, $184 \rightarrow 95$. Finally for Se-Cya, one MRM can be measured: MRM of transition $249 \rightarrow 204$. The sensitivity was significantly enhanced by application of the narrowbore column. Problems were observed especially when monitoring the MRMs of the transitions of Se-$(Cys)_2$ and Se-Cya. This might be due to the fact that the Se-compounds do not get charged so easily. This disadvantage was overcome by solving all organic compounds in a 1% formic acid solution. Sensitivity for all compounds was enhanced by a factor two. Stability studies, in which the Se-compound was dissolved in a 1% formic acid solution, showed that no species transformation occurred during at least 48 h. The results obtained for these standard solutions are shown in Fig. 3. These data demonstrate the ability to detect all precursor ion/product ion pairs for the species of interest. Another important fact is that this method allows the use of the isotopic pattern of Se in the MRM and SIR measurements. When the compound has only one product ion, the same precursor ion/product ion pair can be measured on another isotope of Se. This was done for Se-$(Cys)_2$ by measuring for instance MRM of transition $337 \rightarrow 248$ and MRM of transition $335 \rightarrow 246$ and SIR of m/z 337 and of m/z 335 Se-$(Cys)_2$ can be measured on its 2 most abundant isotopes ^{80}Se and ^{78}Se, respectively.

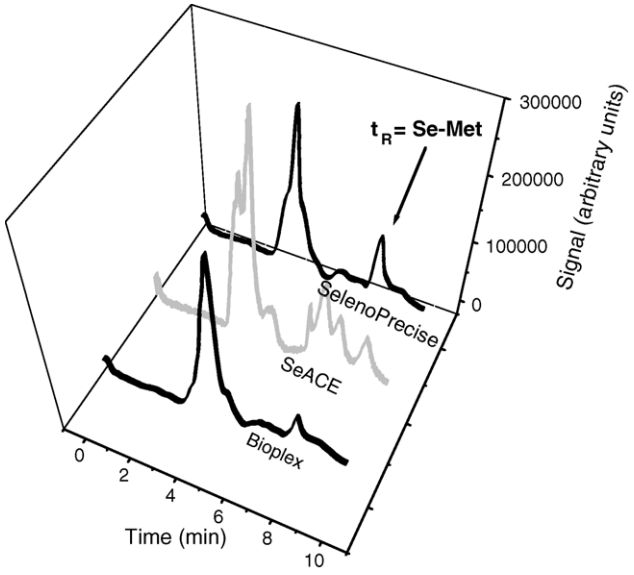

Fig. 4. SIR of m/z 198 in Bioplex, SeACE and SelenoPrecise.

3.2. Speciation of Se in Se-supplements

3.2.1. On-line monitoring of Se-Met in yeast supplements

According to the results obtained by HPLC–HG–AFS there is one major Se-compound present in the enzymatic di-

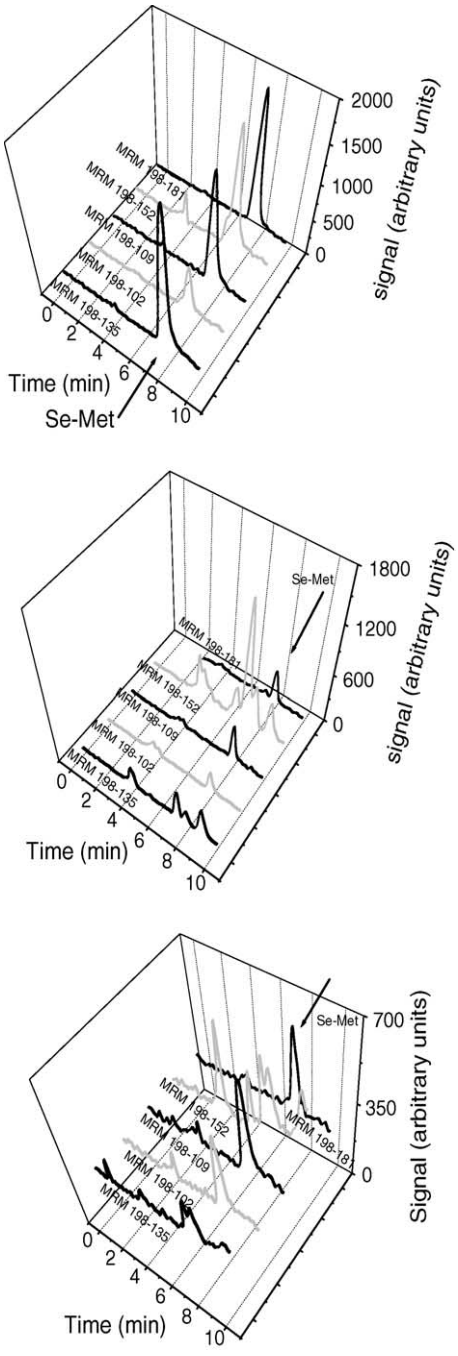

Fig. 5. HPLC–ES–MS–MS chromatograms of SelenoPrecise (top), SeACE (middle) and Bioplex (bottom).

gest of the 3 supplements. Standard addition of Se-Met to the extracts showed that the t_R of the compound matched with the t_R of Se-Met. In order to identify this compound, structural information was needed. Formic acid was added to the three extracts (final concentration 1%) to enhance ion formation. A 10 μl aliquot of the filtered extract was brought onto the column and analyzed by SIR at *m/z* 198 to verify the presence of Se-Met in the extracts (Fig. 4). This chromatogram gave different peaks. This showed that ES–MS is insufficient to identify the compound, since more compounds in the extracts have *m/z* 198. According to the results of HPLC–HG–AFS, only one peak contained Se. Only the peak corresponding to t_R = Se-Met, according to the HPLC–ES–MS results for a Se-Met standard solution, could thus be Se-Met. This proved that further molecular information is needed for the characterization of a compound in such a complex biological matrix. The method developed for on-line measurement of the product ions of Se-compounds was now applied to yeast extracts. In the three different extracts the five possible precursor ion/product ion transitions for Se-Met were measured. A 10 μl aliquot of the filtered sample in a 1% formic acid solution was injected. Similar experiments with the analytical column showed a lack of sensitivity, since only two product ions could be measured, those from the two most abundant fragments (181 and 152) in Fig. 3. Therefore experiments were done on a narrowbore XTerra MS C_{18} column (i.d. 2.1 mm) coupled to the electrospray probe so sensitivity was enhanced. HPLC and ES–MS–MS settings were as in Table 1. In Fig. 5, the HPLC–ES–MS–MS chromatograms are given for the three extracts of the different yeast samples. In these chromatograms, we can see in case of SelenoPrecise, SeACE and Bioplex that the MRMs of all transitions: 198 → 102, 198 → 109, 198 → 135, 198 → 152, 198 → 181 can be measured with sufficient sensitivity. MRM of transition 198 → 102 is characteristic for Se-Met since Se is lost during this fragmentation step. It is also obvious that some of the transitions occur at a t_R of Se-Met and additionally at another t_R. These peaks correspond to molecules having the same *m/z*, loosing a fragment of the same mass, but do not contain Se (according to the HPLC–HG–AFS chromatograms). The reason here for is that biomolecules have functional groups (e.g. NH_3, COOH, . . .) which can all be lost during fragmentation. This again demonstrates the difficulty of analysis of such complex matrices.

Acknowledgements

E. Dumont is a grant holder of the Institute for the promotion of innovation by Science and Technology in Flanders (IWT). Louis Mees is thanked for doing the INAA experiments.

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