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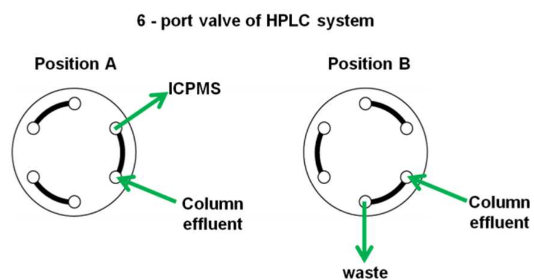

An HPLC/ICPMS method incorporating a methanol gradient and robust instrumental set-up to preclude high amounts of methanol entering the plasma

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HPLC/ICPMS with Effluent Diversion for Robust and Time-Efficient Determination of Selenium Metabolites in Human Urine

Sabine Kokarnig, Nina Kroepfl, Doris Kuehnelt*, Kevin A. Francesconi
University of Graz, Institute of Chemistry – Analytical Chemistry, Universitätsplatz 1, Graz, Austria
*Corresponding author: doris.kuehnelt@uni-graz.at

Abstract

Reversed-phase high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (RP-HPLC/ICPMS) is widely applied for the determination of the major urinary selenium metabolites such as selenosugars. Urine often also contains small amounts of non-polar Se species like dimethyl selenide (DMSe) and dimethyl diselenide (DMDSe). Although these compounds are not significant quantitatively, they present considerable analytical problems because of their enhanced signal, and long retention on reversed-phase columns. The use of solvent gradients to reduce the retention times of DMSe and DMDSe is usually hampered by the intolerance of the ICPMS towards high loads of organic solvent. We report a simple instrumental set-up that allows application of a methanol gradient program without compromising ICPMS detection. High loads of organic solvents were prevented from reaching the ICPMS by using a 6-port valve between the HPLC column and the ICPMS. The combination of this experimental set-up and the developed methanol gradient provided separation of the main urinary selenium metabolites, three selenosugars and TMSe, within 7 min, and elution of non-polar selenium species DMSe and DMDSe and re-equilibration was completed within 15 minutes. The introduction of 1 % CO_2 in argon to the nebuliser gas flow significantly improved the stability of the system. Stability over 12 hours of measurement time, tested by repeat injection (40 times) of a urine sample spiked with selenosugars and TMSe gave variations in retention times of ≤ 0.5 % and in ICPMS signal response of ≤ 2% for all four species. The method can be used to determine the main urinary selenium metabolites in the presence of non-polar selenium species within an overall analysis time, including re-equilibration, of 15 minutes instead of two hours for isocratic elution, offering great advantages for the analysis of large numbers of samples.

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34 ## Introduction

35

36 Selenium is an essential trace element with many established and purported beneficial health
37 effects.[1] Investigations into human selenium metabolism are important to understand the health
38 benefits ascribed to this element. Because urine is the major excretionary route for selenium, the
39 metabolites of selenium in this body fluid have long been a research focus.[2]

40

41 Modern methods for determining selenium species in urine are mainly based on HPLC/mass
42 spectrometry. Although molecular mass spectrometry is used to investigate novel metabolites,
43 most quantitative studies use HPLC together with elemental mass spectrometry, namely
44 inductively coupled plasma mass spectrometry (ICPMS).[3] Most urinary selenium metabolites of
45 interest are usually determined by using reversed-phase HPLC either directly[4,5,6] or in
46 combination with ion-pairing agents.[7]

47

48 Besides the major urine metabolites, selenosugars and TMSe, non-polar selenium species,
49 DMSe, DMDSe, as well as mixed selenium/sulfur compounds, have also been identified as
50 natural urinary selenium metabolites at low concentrations.[8,9,10] These compounds are also
51 formed upon degradation of selenosugars.[8] The presence in urine of DMSe and DMDSe, even
52 at trace levels, can cause substantial problems for the determination of selenosugars by
53 reversed-phase HPLC/ICPMS due to their volatility and low polarity. The volatile nature of DMSe
54 and DMDSe results in their giving a much higher response in the ICPMS - depending on the
55 type of nebuliser, up to 60-fold higher - than the non-volatile selenium species.[11] Therefore, even
56 minimal degradation of selenosugars, not even noticeable as a decrease in selenosugar
57 concentration, can result in large signals for the degradation products DMSe and DMDSe.
58 Furthermore, because of their non-polar nature, DMSe and DMDSe show strong interactions
59 with reversed-phase columns[8] necessitating chromatographic run-times of up to 2 hours under
60 isocratic conditions optimised for selenosugars. Hence DMSe and DMDSe might easily be
61 initially overlooked but could give large (interfering) signals in consecutive chromatograms of the
62 measurement sequence.

63

64 There has been to date no easy solution to these analytical problems. Separation on shorter
65 columns or even pre-columns to elute DMSe and DMDSe within a reasonable time[11,12] cannot
66 be applied due to a loss of resolution of the species of interest, selenosugars and TMSe.

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67 Addition of high amounts of organic solvents to the mobile phase to reduce the retention of
68 DMSe and DMDSe[12] is not compatible with quantitative ICPMS measurements[13] and requires
69 countermeasures (e.g. reduced HPLC flow rate, additional cooling of the ICPMS spray chamber,
70 addition of oxygen to the nebuliser gas) to avoid carbon deposits and maintain plasma
71 stability[12,14] resulting in a less robust and more complex instrumental set-up.
72
73 We present a novel reversed-phase HPLC/ICPMS method incorporating a methanol gradient
74 combined with a robust and simple instrumental set-up that avoids the introduction of high
75 amounts of methanol into the ICPMS plasma. The method allows separation of selenosugars
76 and TMSe in the presence of volatile, non-polar selenium species in 15 minutes, and hence is
77 well suited for the determination of urinary selenium metabolites in large numbers of samples.
78

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79 **Experimental**

80 *Chemicals and Reagents*

81 All solutions were prepared with Milli-Q water (18.2 MΩ cm). Ammonium formate (≥95%) and
82 formic acid (>98%, p.a.) were purchased from Roth (Karlsruhe, Germany), and methanol (HPLC
83 Gradient Grade) was obtained from J.T. Baker (Deventer, Netherlands). Selenium species
84 standards used for HPLC/ICPMS were: in-house synthesized TMSe, methyl-2-acetamido-2-
85 deoxy-1-seleno-β-D-galactopyranoside (selenosugar **1**),[15] methyl-2-acetamido-2-deoxy-1-
86 seleno-β-D-glucosopyranoside (selenosugar **2**)[15] and methyl-2-amino-2-deoxy-1-seleno-β-D-
87 galactopyranoside (selenosugar **3**).[16] Identity and purity of the synthesized compounds was
88 assured by molecular MS, NMR,[15,16] and elemental analysis,[15] and selenium purity was
89 demonstrated by HPLC/ICPMS. Dimethyl selenide (DMSe, >99%) was purchased from Fluka
90 (Buchs, Switzerland) and dimethyl diselenide (DMDSe) from Acros Organics (Geel, Belgium).
91 Stock solutions of selenosugars and TMSe were prepared in water and further diluted with water
92 prior to use. Stock solutions of DMSe and DMDSe were prepared in ethanol and further diluted
93 with water as previously reported.[11] Normal background urine (no Se-supplementation) was
94 provided by a single volunteer, who gave informed consent; it was filtered through 0.20 µm nylon
95 filters (Markus Bruckner Analysentechnik, Linz, Austria) and spiked with selenium compounds as
96 detailed below prior to analysis.

97

98 *Instrumentation*

99 The separations were performed with an Agilent 1100 HPLC system (Agilent Technologies,
100 Waldbronn, Germany) comprising a solvent degasser (G 1379A), a binary pump (G 1312A), a
101 thermostated autosampler (G 1329A), and a thermostated column compartment (G 1329A)
102 equipped with a 6-port valve controlled by the HPLC software. A reversed-phase column
103 (Atlantis dC18, 150 x 4.6 mm, Waters, Wexford, Ireland) was used with a mobile phase
104 containing 20 mM ammonium formate, pH 3.0, 3 % methanol (solvent A) and methanol (solvent
105 B). The flow rate was 1 mL min $^{-1}$ and the column temperature was at 30 °C throughout. The
106 HPLC system was connected to an Agilent 7500ce ICPMS equipped with an Ari Mist HP
107 nebulizer (Burgener Research International, Berkshire, UK). The ICPMS was operated in the
108 reaction mode (3.5 mL H_2/min) monitoring *m/z* 77, 78, 80, and 82, whereby *m/z* 78 was used for
109 data evaluation. For some applications (see below), a mixture of 1% CO_2 in argon was
110 introduced to the nebuliser gas via a T-piece immediately before the ICPMS torch as a carbon
111 source to enhance signal stability.[17]

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112 *Optimising the gradient*
113 The gradient was optimised with the assistance of an active flow splitter (Agilent, G1968D)
114 installed between the HPLC and the ICPMS, which transferred 1 % of the column effluent to the
115 ICPMS in an auxiliary flow of solvent A (Figure 1a). The methanol gradient was optimised using
116 a standard solution of DMDSe (ca 1 mg Se L^{-1}) as it is the longest retained compound (ca 120
117 min). Starting conditions were: 0 – 7 min 0 % solvent B, 7 – 8 min 0 – 100 % solvent B, 8 – 14
118 min 100 % solvent B, 14 – 15 min 100 – 0 % solvent B, 15 – 30 min 0 % solvent B. The starting
119 conditions were modified by the following steps: first, the composition of the mobile phase was
120 optimised by varying the methanol content of solvent B (60 %, 70 %, 80 %, 90 % & 100 %);
121 second, the length of time that the methanol concentration was held at its maximum value was
122 reduced from 6.0 to 0.5 min in half minute steps; and third, optimum re-equilibration time was
123 determined by testing times from 15 to 2 min in half minute steps. After each optimisation step
124 with the standard solution of DMDSe, a urine sample spiked with selenosugar 1, 2, 3, and
125 TMSe, DMSe and DMDSe at ca 1 mg L^{-1} (because only 1 % of the column effluent was directed
126 to the ICPMS) was injected (10 µL) to test the effect of the parameter on the separation in a
127 urine matrix. All samples and standards were measured in triplicate.
128
129 *Performance of the method under long-term operation*
130 Applicability and stability of the developed method (40 injections over a 12 hour period) was
131 tested with the instrumental set-up shown in Figure 1b by repeated analysis of a urine sample
132 (specific gravity: 1.008) spiked with selenosugars **1**, **2**, **3**, and TMSe, each at concentrations of
133 10 µg Se L^{-1} (2 mL of urine spiked with 20 µL standard solutions containing 1 mg Se L^{-1} of each
134 compound). Additionally, this experiment was performed under conditions where 1 % CO_2 in
135 argon was used as optional gas (40 injections over a 12 hour period). In both experiments a
136 TMSe drift control standard solution (20 µg L^{-1}) was injected every five samples; injection volume
137 was always 20 µL (urine and TMSe standard).
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139 **Results and Discussion**
140 With isocratic reversed-phase HPLC[16] the four main urinary selenium metabolites selenosugars
141 1, 2, 3 and TMSe are separated in less than 7 min, but DMDSe has a retention time close to 2 h
142 (Figure 2). We aimed to convert this isocratic method into a gradient elution method by raising
143 the methanol content after elution of selenosugar 2 in order to shorten the retention times of
144 DMSe and DMDSe without altering the separation of the selenosugars and TMSe.
145
146 *Optimising of the gradient*
147 For the optimisation of the methanol gradient, monitoring of DMSe and DMDSe was essential.
148 Since only limited amounts of methanol can be introduced into the plasma under normal
149 operating conditions, a flow splitter was placed between the HPLC system and the ICPMS
150 transferring only 1 % of the column effluent to the plasma in an auxiliary flow of solvent A (Figure
151 1a). With this set up it was possible to monitor the retention behavior of DMSe and DMDSe
152 during the optimisation of the methanol gradient without needing special precautions with
153 respect to ICPMS detection. First, the percentage of methanol (60 - 100 % in 10 % steps)
154 necessary to elute DMDSe, the compound retained longest on the column, within a reasonable
155 time was investigated. With a gradient of up to 60 % methanol, DMDSe had a retention time of
156 16 min, and this decreased to 11.1 min with the gradient up to 100 % methanol. The retention
157 time for DMDSe with a gradient up to 80 % methanol was only marginally longer (12.2 min
158 instead of 11.1 min); since the pressure fluctuations were lower at 80 % methanol, we
159 considered these conditions to be the most suitable and applied them to subsequent
160 optimisation steps.
161
162 Next, the time that the gradient was held at 80 % of methanol was tested. A minimum of 1.5 min
163 at 80 % of methanol was necessary to elute DMDSe from the column; this was also the case for
164 DMDSe in the urine matrix. However, as the retention time of DMDSe may vary with other urine
165 matrices, a small safety margin was added so that a holding time of 2 min at 80 % methanol was
166 adopted.
167
168 Testing different re-equilibration times revealed that a re-equilibration time as low as 2 min gave
169 stable retention times for the selenosugars and TMSe. However, because the pressure in the
170 HPLC system required 4.5 min to return to its initial value, we employed a re-equilibration time of
171 4.5 min. The optimised conditions (Table 1) resulted in a total analysis time of 15 minutes per

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172 sample, which is an 8-fold decrease compared to isocratic elution (Figure 2) without affecting the
173 separation of the selenium metabolites of interest. Although the method was not tested for other
174 non-polar selenium compounds, such as mixed selenium/sulfur species, our previous work has
175 shown that these compounds elute between DMSe and DMDSe under isocratic reversed-phase
176 conditions,[8] and hence they would also be efficiently removed from the column with the
177 developed method.
178
179 *Performance of the method under long-term operation*
180 Having optimised the methanol gradient with the assistance of high concentrations of selenium
181 species and the flow splitter (which directed just 1 % of the HPLC effluent to the ICPMS), we
182 were now in a position to forego the flow splitter and apply the method directly to urine samples.
183 Thus, the experimental setup was changed so that 100 % of the column effluent was directed to
184 the ICPMS during elution of the analytes of interest, namely the three selenosugars and TMSe.
185 The LOQs (0.25 µg Se L^{-1} for each species) using this optimised method were suitable for its
186 application to baseline urine samples. This was accomplished by installing the 6-port valve of the
187 HPLC system between the column and the ICPMS, and sending the column effluent to the
188 ICPMS for the first 7.3 min of the HPLC runtime (position A, Fig. 1b). Then, the column effluent
189 was directed to the waste for 6.2 minutes, during which time the methanol content increased to
190 80 %, which prevented DMSe, DMDSe and high levels of methanol from reaching the ICPMS
191 (position B, Fig. 1b). After a chromatographic run-time of 13.5 min, the valve was diverted back
192 to position A for the remaining 1.5 minutes to allow the system to stabilise.
193
194 The robustness, stability and precision of the method were tested by repeated measurement (40
195 injections over 12 h) of a urine sample spiked with all three selenosugars and TMSe at a
196 concentration of 10 µg Se L^{-1} each. We note that this species distribution does not necessarily
197 reflect the natural situation in urine, where selenosugars 2 and 3 are usually minor in
198 comparison to selenosugar 12 and the significance of TMSe is highly variable[17] and, therefore,
199 impossible to mimic. However, the relative amounts of these main selenium metabolites have no
200 effect on the focus and outcome of the current study; we chose to use the same concentration
201 for all 4 species to ensure comparability of the results. Precision (RSD) was <0.5 % for retention
202 times and ca 5 % for signal intensity. This stability test was repeated, but with the addition of 1 %
203 of CO_2 in argon, which yielded comparable retention time precision and improved signal
204 precision of ca 2 % (Table 2, Figure 3).

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205

206 In summary, we report a reversed-phase HPLC/ICPMS method for urine analysis that
207 accomplishes complete removal of the troublesome selenium species DMSe and DMDSe
208 without distorting separation of selenosugars and TMSe. The method is robust, time-efficient
209 and well suited for the analysis of large batches of urine samples. Since DMSe and DMDSe are
210 also formed in the course of in vitro experiments with cell cultures[18,19,20,21] our method is likely to
211 be useful for applications other than urine analysis. It could also prove valuable for speciation
212 analysis of other elements such as arsenic where some species can show unacceptably long
213 retention times under reversed-phase conditions.[22]

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Table 1: Optimised methanol gradient for eluting non-polar species DMSe and DMDSe while maintaining separation of selenosugars and TMSe.

<table>
<tr><th>Time [min]</th><th>% Solvent B</th><th>Time [min]</th><th>Effluent diversion mode</th></tr>
<tr><td>0 – 6.5</td><td>0</td><td>0 – 7.3</td><td>Position A</td></tr>
<tr><td>6.5 – 7.5</td><td>0 – 80</td><td rowspan="3">7.3 – 13.5</td><td rowspan="3">Position B</td></tr>
<tr><td>7.5 – 9.5</td><td>80</td></tr>
<tr><td>9.5 – 10.5</td><td>80 – 0</td></tr>
<tr><td>10.5 – 15</td><td>0</td><td>13.5 – 15.0</td><td>Position A</td></tr>
</table>

* due to the dead volume it takes about 1.5 min until the mobile phase reaches the plasma.

Table 2: Stability of optimised methanol gradient for retention times and signals of all species (10 µg Se L^{-1} each) without (Experiment 1) and with (Experiment 2, performed on a different day) the addition of 1 % CO_2 in argon as optional gas (n=40 over a 12 h period in each case).

Species	Experiment 1			Experiment 2		
	Retention Time [min]	Retention Time RSD [%]	Peak area RSD [%]	Retention Time [min]	Retention Time RSD [%]	Peak area RSD [%]
selenosugar 3	2.31	0.2	4.6	2.35	0.2	1.7
TMSe	2.92	0.5	4.5	2.99	0.4	1.7
selenosugar 1	4.94	0.2	5.4	5.08	0.2	2.0
selenosugar 2	6.00	0.2	4.5	6.22	0.3	1.8

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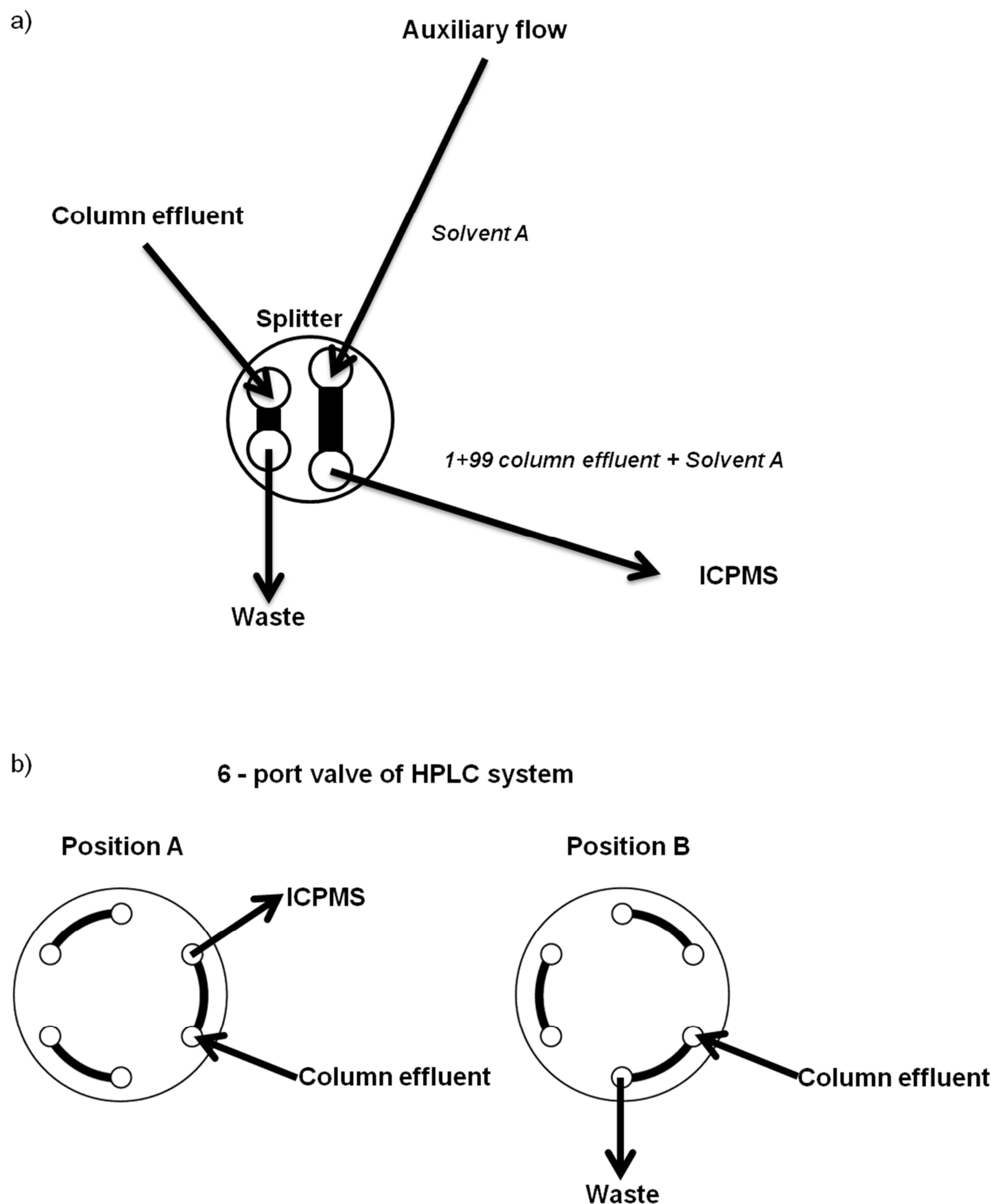

Figure 1. (a) Instrumental setup used for the first part of the method development – optimising the methanol gradient. The column effluent of the HPLC system was introduced into an active flow splitter and 1 % of the effluent was transferred to the ICPMS in an auxiliary flow of solvent A.

(b) Instrumental setup for the effluent diversion mode and application to urine samples. In position A (0 – 7.3 min and 13.5 to 15 min) the column effluent is transferred to the ICPMS and in position B (7.3 – 13.5 min) it is transferred to the waste.

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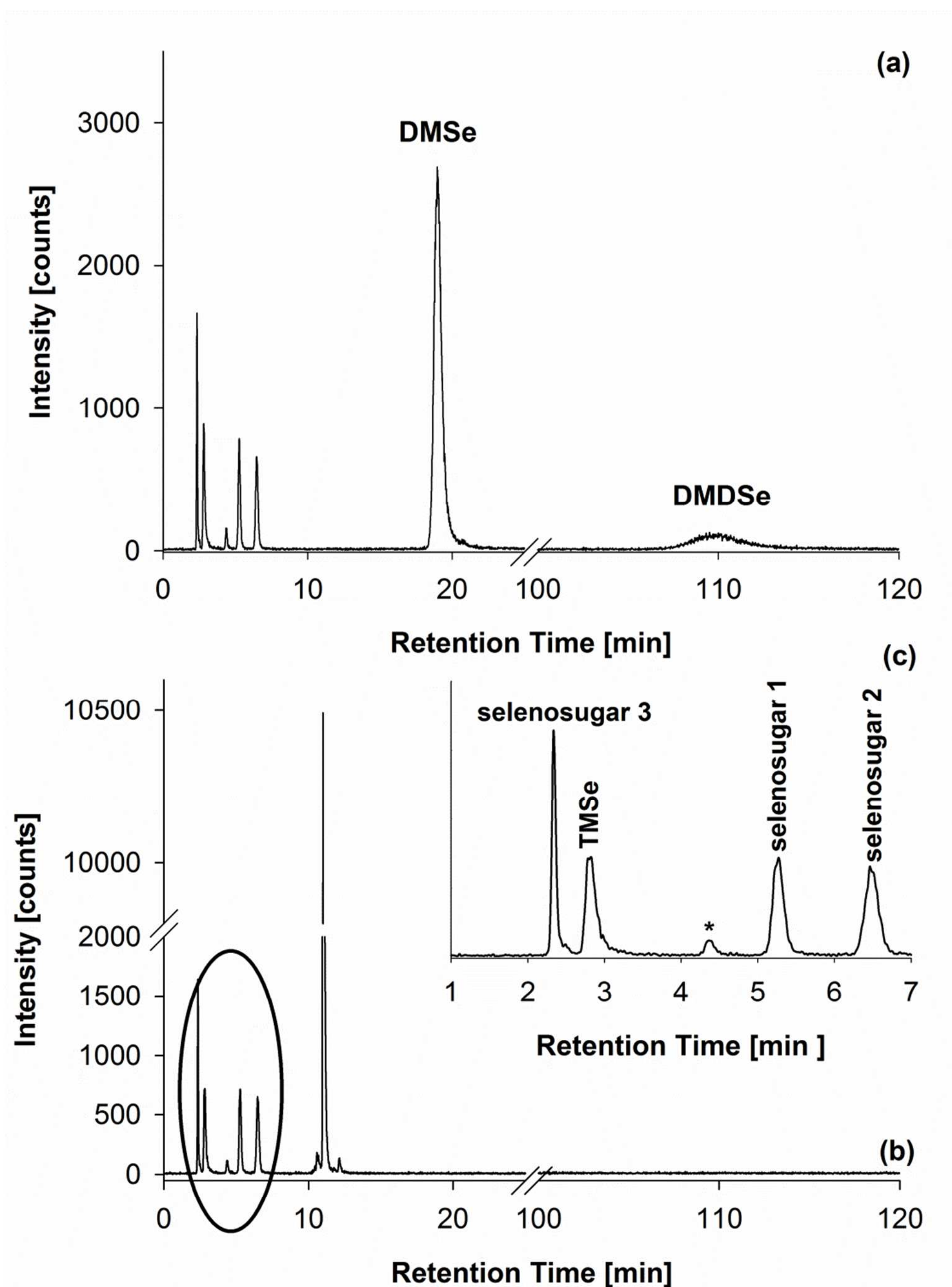

Figure 2. RP-HPLC/ICPMS chromatograms of a urine sample spiked with selenosugars **1**, **2**, and **3**, TMSe, DMSe and DMDSe (ca 1 mg Se L^{-1} each, only 1% of column effluent directed to the ICPMS) recorded with the instrumental setup shown in Figure 1a. (a) isocratic elution with 20 mM ammonium formate, pH 3.0, 3 % of methanol (b) gradient elution as detailed in Table 1, (c) zoom of the time period in which the main urinary metabolites elute (circled in (b)). * impurity in the standard solution of selenosugar 2, which had formed during long time storage of the aqueous stock solution.

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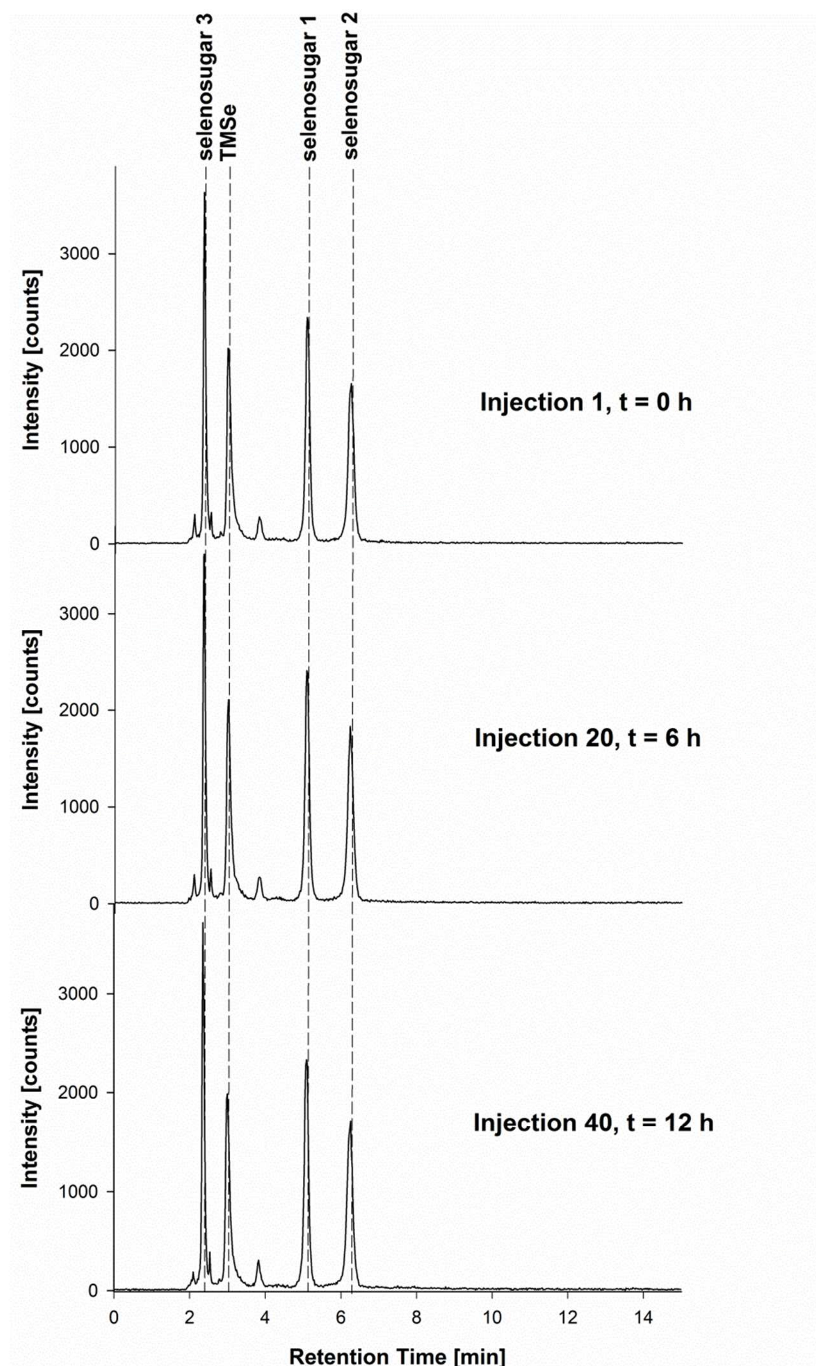

Figure 3. RP-HPLC/ICPMS chromatograms showing stability of the methanol gradient. A urine sample spiked with selenosugars **1**, **2**, and **3**, and TMSe (10 µg Se L^{-1} each) was injected (10 µL) 40 times over a 12 h period. Examples are shown for injection at t=0 h, t= 6 h (injection N° 20) and t= 12 h (injection N° 40).

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