Comparative Study of Extraction Techniques with Ammonia or Methanol/Water for the Isolation of Ginsenosides Using HPLC/MS

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Z. Naturforsch. **60b,** 205 – 210 (2005); received June 16, 2004

Roots of ginseng plants have been extracted with liquid ammonia or methanol/water (60:40 v/v). The extracts were characterized by means of a gradient HPLC/MS method and the results were compared with each other. The ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1 and Ro including the malonyl ginsenosides m-Rb_1, m-Rb_2, m-Rc and m-Rd could be identified. Contrary to previous reports suggesting that during the extraction with liquid ammonia malonyl ginsenosides are totally converted to neutral ginsenosides, it could now be demonstrated by use of mass spectrometry that even after an extraction time of 4 h malonyl ginsenosides can be detected. However, the amount of malonyl ginsenosides was reduced by about one order of magnitude. The neutral ginsenosides were not changed by liquid ammonia.

The mass spectrometric detection has been performed by use of the quasi-molecule ion $[M+Na]^+$ and some specific fragment ions.

Key words: Ginsenosides, Extraction, Liquid Ammonia Extraction, Methanol/Water, HPLC/MS

Introduction

In China, mixtures of ginseng roots have already been applied in traditional medicine as remedies and tonics for thousands of years. The positive effects of these extracts, for example the activation of the natural resistance of the organism, are mainly attributed to the ginsenoside constituents.

For the isolation of the ginsenosides different methods are known. Since the ginsenosides – as polar compounds – are quite soluble in polar solvents they are often extracted with water or water/alcohol mixtures. According to reference [1], the examination of the roots regarding the identity of the ingredients and the determination of their concentration are performed by methanol/water extraction. With some procedures, however, sensitive ingredients may thereby be destroyed, hence, recently more gentle extraction procedures such as with supercritical solvents (SFE) like carbon dioxide or, but less frequently, with ammonia have been applied [2 – 5]. For the extraction of ginseng roots with liquid ammonia specific information is not yet available. Therefore, this procedure must be evaluated regarding not only the yields of ginsenosides [5] but also their possible chemical conversions under the given pressure and temperature conditions (334 K, 20 bar).

Through consideration of the retention time and by MS/MS experiments, the applied HPLC/ESI-MS technique (high pressure liquid chromatography/electrospray-mass spectrometry) guarantees a safe identification of the analytes, even if no reference substances are available. The MS application is even essential if it is necessary to determine analytes in trace concentrations or to monitor reactions for completeness, *e.g.* regarding claims [4, 5] that malonyl ginsenosides during the extraction with liquid ammonia are changed to the corresponding neutral ginsenosides. The characterization of the ginsenoside composition of the extracts allows the determination of the origin of the roots whether they are Oriental or American ginseng [6 – 8].

The aim of this study is to show whether there is a change in the respective characteristic fingerprint of the ginsenoside composition caused by sample treatment and extraction conditions.

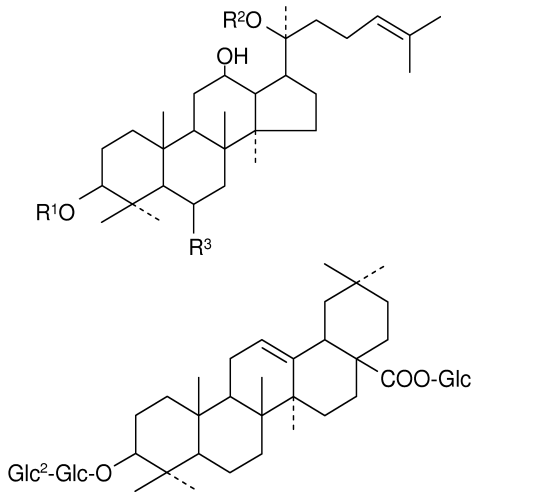
Results and Discussion

The ammonia extracts of the dried root-powder of the species Panax ginseng C. A. Meyer from South Ko-

Table 1. Structures of ginsenosides under study.

Ginsenoside	R^1	R^2	R^3
Rb_1	Glc^2-Glc	Glc^6-Glc	H
Rb_2	Glc^2-Glc	Glc^2-Arap	H
Rc	Glc^2-Glc	Glc^2-Araf	H
Rd	Glc^2-Glc	Glc	H
m-Rb_1	Glc^2-Glc^6-Ac	Glc^6-Glc	H
m-Rb_2	Glc^2-Glc^6-Ac	Glc^2-Arap	H
m-Rc	Glc^2-Glc^6-Ac	Glc^2-Araf	H
m-Rd	Glc^2-Glc^6-Ac	Glc	H
Re	H	Glc	O-Glc^2-Rha
Rf	H	H	O-Glc^2-Glc
Rg_1	H	Glc	O-Glc

Glc = β-D-glucose
Rha = α-L-rhamnose
Arap = α-L-arabinose (pyranose)
Araf = α-L-arabinose (furanose)



Ro

rea were cleaned on a solid-phase column and characterized by gradient HPLC/ESI-MS. The results were compared with those of a methanol/water extraction (60 vol-% methanol) of a former study [5].

For the mass-spectrometric identification, the observation of typical masses, such as the quasi-molecule ions, and/or of some specific fragment ions is desirable. Former studies proved that the fragmentation of the ionized ginsenosides leads to glycosides, water or both components [6–10]. In order to be able to select the corresponding ions for the mass-spectrometric detection of the ginsenosides, mass spectra of some standards of the ginsenosides, as far as existing, were measured and archived in a separated library. Table 1 shows the structural formulas of the ginsenosides.

Characterization of the individual ginsenosides

In contrast to the use of an isocratic HPLC method with UV detection [5] applied before the HPLC/MS studies including a gradient HPLC method led to changed retention times. However, the order of elution of the ginsenosides is retained unchanged under both HPLC conditions with exception of the ginsenosides Rg_1 and Re at the beginning of the chromatogram. The HPLC/MS chromatograms of the aqueous mixture of the ginsenoside standards Rb_1, Rb_2, Rc, Rd, Re, Rf and Rg_1 are given in Fig. 1. The chromatograms show the relative intensity of the ion current of a specific mass

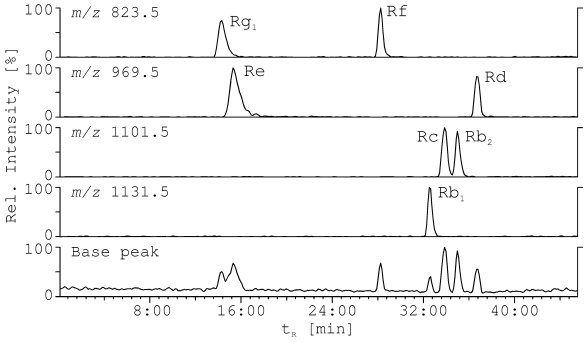


Fig. 1. HPLC/MS chromatograms of an aqueous mixture of the ginsenoside standards Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1 (see also Table 2).

versus retention time. Additionally in Fig. 1 the base peak chromatogram is shown below the mass traces. In this chromatogram the mass peaks with the particular highest intensity were extracted from all mass spectra and combined as mass trace. In contrast, the averaged intensities of all mass peaks occurring in the scanned mass area are recorded as the normally used total ion current (TIC) mass trace. Because of the high background in the mass spectra the TIC is not suitable as representation form in this case.

After evaluation of the spectra a number of ion masses could be assigned to certain $[M+Na]^+$- and fragment ions (Table 2).

The quasi-molecule ions $[M+Na]^+$ are used predominantly for the mass spectrometric identification of

Table 2. Relative intensities of important ions in %. The retention times refer to the HPLC-run of the methanol extract.

	Rg_1	**Re**	**Ro**	**Rf**	**Rb_1**	**Rc**	**Rb_2**	**Rd**	**m-Rb_1**	**m-Rc**	**m-Rb_2**	**m-Rd**
t_R [min]	*14.0*	*15.0*	*23.6*	*27.8*	*32.6*	*33.5*	*34.8*	*36.0*	*22.3*	*23.2*	*24.0*	*25.4*
$[M+Na]^+$	**823.5**	**969.5**	**979.5**	**823.5**	**1131.5**	**1101.5**	**1101.5**	**969.5**	**1217.5**	**1187.5**	**1187.5**	**1055.5**
	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
***m/z* 203**	3.0	12.8	1.5	22.7	3.5	6.0	2.7	10.5	3.6	2.1	2.3	3.3
217	3.0	11.2	2.3	3.5	6.8	8.5	4.9	19.5	7.8	6.2	5.9	4.6
223					4.9	7.5	3.2	12.6				
231									8.1	8.3	7.4	7.5
249									2.9	3.0	2.1	2.2
309									5.9			6.0
325		1.0			6.5	7.5	3.2	12.6				
330						0.6	2.7					
360					4.9							
393			4.7						2.0	3.4	2.3	1.4
405	3.2	14.4		5.7								
407		2.0			16.5	27.5	13.4	63.2	40.7	33.8	24.8	39.5
423	5.5	44.0		11.9								
425					16.5	30.0	15.4	52.6	30.0	31.0	23.0	33.5
435									0.6	2.0	1.3	1.2
439			83.3									
441	1.9	13.2	9.2	3.5								
443					1.1	2.8	1.6	4.1	1.3	0.8	1.0	1.4
453									2.5	1.9	2.4	3.7
471									5.9	2.0	5.9	2.8
487					3.2	2.8	1.6	5.8				
505						2.8						
533		5.2			1.4	2.6	1.2	5.1				
569		8.8			3.5	3.3	2.0	10.5				
587		6.4			4.3	7.5	4.9	7.4				
605					6.5	10.5	6.1	11.6	4.1	5.5	27.8	4.9
621	4.3	3.4										
643	3.6											
645								4.9				
649					3.0	2.6	2.4	5.3				
684	9.1											
749		6.4			3.0	7.5	3.2	14.7				
767		3.6			1.4	1.8	3.2	2.5				
789		48.0			9.2	20.5	12.7	57.9				
805	2.7	15.6		23.5				8.4				
807								5.8				
817			15.0						3.3			
831		2.7						11.6				
835									4.8	6.2	5.6	6.0
849		3.4						3.8				
853									2.6	2.0	2.9	2.7
870									9.6	5.9	10.0	4.3
875									44.4	55.2	44.4	70.0
891									10.0	8.6	6.3	1.6
929					1.4	2.1	2.4	10.0				
935		25.6										
951		10.0						12.1				
952					1.0	1.5	0.7	4.5				

the single ginsenosides. The ion with the mass $m/z =$ 1131.5 could only be assigned to the ginsenoside Rb_1 unambiguously. The other $[M+Na]^+$-ions are representative for two ginsenosides in each case – $m/z = 823.5$ for Rg_1 and Rf, $m/z = 969.5$ for Re and Rd, *m/z* 1101.5 for Rc and Rb_2 – however, they have to be identified unequivocally under consideration of their retention times.

With electrospray-ionization, in presence of sodium ions the formation of $[M+Na]^+$ ions are preferred contrary to $[M+H]^+$ also for ginsenosides. The kind of fragments produced from these ions depends on

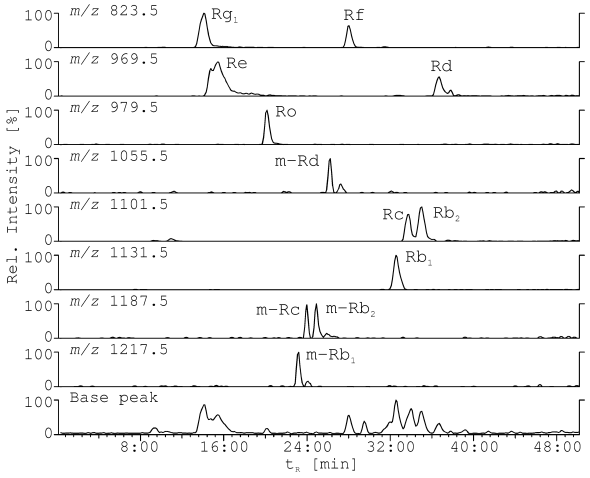


Fig. 2a. Chromatograms of the individual $[M+Na]^+$ ions of the ammonia extract after 4 h extraction time.

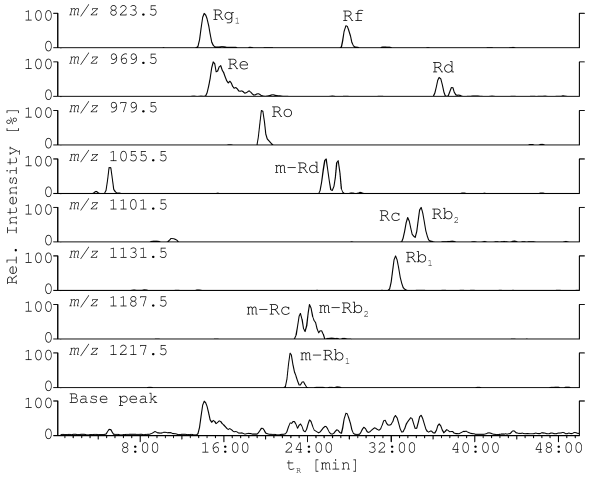


Fig. 2b. Chromatograms of the individual $[M+Na]^+$ ions of the methanol extract after 4 h extraction time.

the type of the quasi-molecule ion. The formation of sodium Na^+-adducts are also possible with the fragments. Applying HPLC/MS, fragment ions are not observed very frequently or only in low intensities, and because of the relatively high background they are usually not suitable for detection.

However, when fragment ions exist and when they are typically for a substance class – the ions appear with all representatives of this group – these can be used as an additional assistance for identification. Having fragment ions of the same mass such as *m/z* 789.5 and no confirmation is given by the retention time, then different structures can be confirmed by the use of the MS/MS-technique.

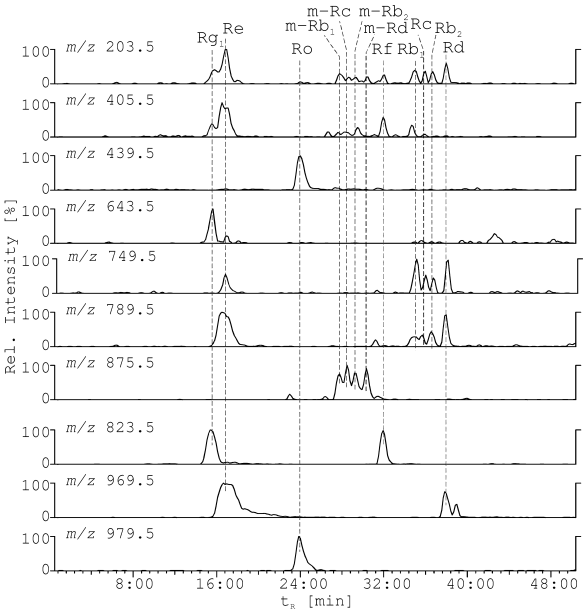


Fig. 2c. HPLC/MS chromatogram of the methanol extract with the mass traces of significant fragment ions of the ginsenosides and the quasi-molecule ion of the ginsenoside Ro.

Characterization of the extracts

The extracts of the ammonia- and the methanol extraction (in each case 4 h extraction time) were characterized with HPLC/MS in the same way. The mass traces of the quasi-molecule ions $[M+Na]^+$ are presented for the ammonia extract in Fig. 2a and for the methanol extract in Fig. 2b. All seven ginsenosides are detectable in the extracts (see Fig. 1).

The HPLC/MS analysis of both extracts show that the malonyl ginsenosides must be considered to be the main components. In Table 2 the mass spectrometric data and the retention times of the ginsenosides as well as the malonyl ginsenosides and the ginsenoside Ro, a triglycoside of oleanolic acids are presented. The relative distribution of the neutral ginsenosides, malonyl species and Ro can be taken from Table 3. The intensities of the quasi-molecule ions of Rg_1 to Rd as well as Ro are comparable for both extracts and mount up to 10^5 for Rg_1 to Rb_2. The intensities for Rd and Ro are lower in each case. The four malonyl ginsenosides show an intensity ratio of approximately 1:1:1:1 for both extracts, however, the NH_3-extract shows about an order of magnitude lower values (about 10^4).

The fragment ion *m/z* 203 (Table 2; Fig. 2c), a glucose/sodium-adduct $[Glc+Na]^+$ [6], can be formed

Table 3. Intensities of the quasi-molecule ions of the ginsenosides in comparison between ammonia and methanol extract. The retention times refer to the HPLC run of the methanol extract.

Ginsenoside	$[M+Na]^+$	t_R	Intensities of $[M+Na]^+$	
	m/z	[min]	NH_3 extract	MeOH extract
Rg_1	823.5	14.0	$1.40 \cdot 10^5$	$2.17 \cdot 10^5$
Re	969.5	15.0	$9.20 \cdot 10^4$	$9.81 \cdot 10^4$
Rf	823.5	27.8	$8.97 \cdot 10^4$	$1.02 \cdot 10^5$
Rb_1	1131.5	32.6	$1.61 \cdot 10^5$	$1.47 \cdot 10^5$
Rc	1101.5	33.5	$8.49 \cdot 10^4$	$9.81 \cdot 10^4$
Rb_2	1101.5	34.8	$1.08 \cdot 10^5$	$1.37 \cdot 10^5$
Rd	969.5	36.0	$5.17 \cdot 10^4$	$4.66 \cdot 10^4$
m-Rb_1	1217.5	22.3	$1.09 \cdot 10^4$	$1.22 \cdot 10^5$
m-Rc	1187.5	23.2	$1.12 \cdot 10^4$	$1.00 \cdot 10^5$
m-Rb_2	1187.5	24.0	$1.16 \cdot 10^4$	$1.02 \cdot 10^5$
m-Rd	1055.5	25.4	$9.35 \cdot 10^3$	$6.65 \cdot 10^4$
Ro	979.5	23.6	$2.85 \cdot 10^4$	$3.91 \cdot 10^4$

from all ginsenosides. As well, the fragment ion *m/z* 217 appears in all ginsenosides.

In each case the mass spectra of the 20(*S*)-protopanaxadiols Rb_1, Rc, Rb_2 and Rd contain the specific fragment ions $m/z = 223$, 487, 649, 929 and 952 as well as $m/z = 325$, the latter appears with about 1% relative intensity with Re, too (Table 2). For the ginsenosides of the triol type Rg_1, Re and Rf, the fragment ions $m/z = 405$, 423 and 441 are characteristic [6, 7]. Furthermore, they are apparently also indicated by the mass $m/z = 805$, although an ion of this nominal mass is also registered for Rd. In the case of Rg_1 and Rf the fragment ion originates by separation of water from the quasi-molecule ion [9]. This ion appears in the mass spectra of the ammonia- and the methanol extract with high intensity. The malonyl ginsenosides m-Rb_1, m-Rc, m-Rb_2 and m-Rd are characterized by the fragments of $m/z = 231$, 249, 435, 453, 471, 835, 853, 870, 875 and 891. Remarkable high intensities are reached for the ion of $m/z = 875$.

For malonyl ginsenosides and 20(*S*)-protopanaxadiols ions with $m/z = 407$, 425, 443 and 605 are likewise characteristic, whereas the ions $m/z = 407$ and 425 appear most frequently. Other fragment ions of the malonyl ginsenosides are missing for the 20(*S*)-protopanaxatriols.

The detection sensitivity of the HPLC/MS technique is particularly suitable for the identification of the oleanolic acid derivate Ro that, in contrast to the HPLC/UV detection [5], could only be identified by mass spectrometry. Ro is both in the ammonia- and the methanol extract and could be detected by a noticeable $[M+Na]^+$-ion $m/z = 979$ and the fragment ions $m/z = 393$, 439 [7], 441 and 817 (Fig. 2c, Table 2, 3).

For identification of m-Rd the ion $[C_{51}H_{84}O_{21} + Na]^+$ with $m/z = 1055.5$ was observed. Since, as already mentioned before, the order of elution corresponds with only two exceptions to that of both different HPLC systems, the first peak of the double-signal is assigned to the malonyl ginsenoside m-Rd with $m/z = 1055.5$. With the mass trace $m/z = 1187.5$ m-Rc and m-Rb_2 are detected unequivocally and with $m/z = 1217.5$ the compound m-Rb_1. The ion m/z 1187.5 is assigned to the empirical formula $[C_{56}H_{92}O_{25} + Na]^+$, the ion $m/z = 1217.5$ to $[C_{57}H_{94}O_{26} + Na]^+$.

In comparison to the results of a study with an extraction in pure water, the concentrations of individual analytes in both described extracts are higher by up to a factor of 10.

Specification of the regional provenance

In contrast to American ginseng the Oriental ginseng as well as the Asian (Korean) ginseng contains the ginsenoside Rf [7, 8]. In spite of the same molecular mass as that of Rg_1 ($m/z = 823$), Rf can be identified on the basis of retention time because it retards with approximately 28 min, nearly twice as long as Rg_1 (see also Table 2, Fig. 2a, 2b). Additionally, the masses $m/z = 405$, 423 and 441 can be detected as characteristic fragment ions for the 20(*S*)-protopanaxatriol Rg1, Re and Rf [7]. The order of retention is given in Fig. 2c.

F_{11}, a 24(*R*)-pseudo-ginsenoside, is a typical indication for American Ginseng. In a previous study [5] it was only detected by UV spectroscopy so that F_{11} could not be analyzed with the lack of a necessary chromophore. As expected, a verification with mass spectrometry confirms that result. This is not a surprise because F_{11} occurs in Asian Ginseng as an ingredient only in traces $< 0,0001\%$ [8]. Though F_{11} and Rf have the same molecular mass and also very similar retention times, nevertheless, the identification can be achieved by their significant fragment ions [7, 8]. One of these ions, $m/z = 439$ could be assigned to Ro on the basis of the retention time and other specific ions. However, it is to be noticed that from F_{11} this ion can only be formed from the $[M+H]^+$ ion and not from the Na^+ adduct [8].

Conclusion

The comparison of the extraction methods show that the extraction with methanol/water (60:40, v/v) is a

suitable method preferably for the isolation of the original ginsenoside composition. If the conversion of the malonyl ginsenosides to the neutral ginsenosides Rb_1, Rc, Rb_2 and Rd is preferred, the ammonia procedure is the method of choice. Both procedures permit an unambiguous assignment of the origin of the ginsengs.

With this work it could also be shown that HPLC/MS is a suitable method for the analysis of the ginsenosides, on the one hand because the natural materials are not changed by additional sample handling, on the other hand because of the outstanding determination limits. Thereby, the ginsenoside Ro could be identified in both extracts in absence of a reference standard. This is a decisive advantage of mass spectrometry [5]. An effective chromatographic separation of the analytes is advantageous, therefore, the gradient technique is to be preferred over the isocratic HPLC method.

As with the determination of Ro the detection power of the HPLC/UV method was also not sufficient in order to determine that 10% of the malonyl ginsenosides still existed despite the 4 h extraction procedure in ammonia.

Experimental Section

Sample treatment

The ginseng roots of five-year old plants were received from South Korea. The treatment of the roots and the extraction procedures (in each case 4 h extraction time) are described in [5]. The solvents of the extracts were evaporated by a rotary evaporator to dryness. For the clean-up procedure the residual products were solved in 100 ml of bi-distilled water and cleaned on a solid phase as described in [5]. The cleaned products were stored in methanol (5 ml).

HPLC/ESI-MS system

The HPLC used for HPLC/MS was a high pressure gradient system with two Knauer Maxi-Star K-1000 and a dynamic mixer (Knauer, Berlin, Germany). The flow rate was set to 110 μl/min and then reduced to 1.5 μl/min by an Accurate Splitter (IC 400 VAR, LC Packings, Amsterdam, Netherlands), resulting in a back pressure of about 150 bar at initial conditions. The separation was performed on a 150×0.1 mm i.d. column filled with C18 packing material GromSil 120 ODS 3 CP, 5 μm particle size (Grom, Rottenburg-Hailfingen, Germany). The eluent composition was H_2O/acetonitrile (80:20 v/v) for A and H_2O/acetonitrile (20:80 v/v) for B. The following HPLC-gradient was used: 0 min 100% A, 20 min 80% A, 50 min 0% A. Acetonitrile was HPLC grade (Roth, Germany). The eluents were degassed using helium.

The HPLC/MS experiments were carried out on a Finnigan MAT 90 double-focussing mass spectrometer (Finnigan, Bremen, Germany), using the electrospray ionization source ESI-II (Finnigan, Bremen, Germany). The temperature of the transfer capillary was set to 250 °C. 2-propanol/water (4:1 v/v) with 3 μl/min was used as sheath liquid. The electrospray voltage was set to approximately 3.8 kV. The sample was introduced by means of a syringe pump through a fused silica capillary (50 μm i.d.). The CID fragmentation was achieved by increasing the tube-lens voltage from 100 V (chromatograms in Fig. 2a/b) to 120 V (Fig. 2c).

Acknowledgements

The authors thank the DFG (main-focus program, Overcritical fluids as solvent – and reaction agents), the 'Bundesministerium für Bildung und Forschung' as well as the 'Ministerium für Wissenschaft und Forschung des Landes NRW' for financial support.

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