Analysis of a complex natural product extract from ginseng – Part I: Structure elucidation of ginsenosides by rapid resolution LC–ESI TOF with accurate mass measurement

Application Note

Edgar Nägele

Abstract

Since prehistoric times extracts from herbs have been used for medical treatment of disease. Their activity and effects on humans were found by trial and error over generations. A good example of achieved efficiency is traditional Chinese medicine (TCM). In Western medicine drugs derived from natural origin are gaining importance due to their potential. However, Western pharmaceutical quality standards require a deep knowledge about the ingredients in medicine based on natural products.

This Application Note will demonstrate the use of the rapid resolution LC with rapid resolution high throughput (RRHT) columns for the separation of the ingredients found in a complex ginseng root extract and the measurement of accurate masses by an ESI orthogonal acceleration time-of-flight MS (oaTOF) for the structure elucidation. The use of a rapid resolution LC system together with an ion trap MS for structure elucidation will be discussed in part two of this work[8].

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Introduction

Crude extracts of herbal and animal origin have been used for medical treatment of disease in all ancient cultures around the globe since prehistoric time. Their activity for treatment of different diseases and other effects on humans were found by trial and error over hundreds of years and the knowledge about this medicine was inherited from generation to generation. A good example of the efficiency achieved during this process of optimization is the herbal based traditional Chinese medicine (TCM). Since these drugs are often complex mixtures containing hundreds of chemically varied substances with different effects or synergisms a quality control and quality assurance of medical potency is very difficult. A widely used and accepted method by the U.S. Food and Drug Administration (FDA) and World Health Organization (WHO) is chromatographic fingerprinting[1,2].

In addition to the importance of the chromatographic fingerprint, which is capable of identifying a particular herb and distinguishing between closely related species in a qualitative manner, the quantitative analysis of medical plant extracts is also gaining importance. For traditional medicines a quantitative analysis is crucial to their quality control and to determine the absolute content of pharmaceutical effective substances as well as potentially toxic undesirable natural substances[3]. In Western medicine drugs derived from natural origins are gaining importance due to their potential. However Western pharmaceutical quality standards require a deep knowledge about the ingredients in medicine based on natural products. Since traditional herbal medicines often contain hundreds of substances with only a few bioactive compounds it is necessary to develop new strategies to screen these plant extracts for biologically active compounds and for their pharmacological effectiveness on animal or cellular models as well as receptor and enzyme based tests[4]. A famous Asian herb, which has been used in herbal medicine for more than 5000 years is the ginseng (Panax species) root. Pharmacological effects of ginseng which have been reported are, for example, stimulatory and inhibitory effects on the central nervous system (CNS), antistress, antihyperglycemic, antineoplastic and immunomodulatory effects[5]. The main active compounds of the ginsenosides are triterpene saponins from which more than 80 have been isolated and characterized during the past years. An enormous amount of work has been done during the last 30 years to develop analytical methods for the analysis of ginseng extracts and medical formulations. The method of choice for the analysis of complex natural product extracts, like those derived from the ginseng root, is high performance liquid chromatography (HPLC)[6]. LC/MS equipment such as LC/ESI oaTOF for accurate mass measurement and LC/ion trap or LC/triple quadrupole instruments for structure elucidation by MS/MS and MS^n are currently being used to determine the complex and similar structures of ginsenosides[7]. This Application Note will demonstrate the use of the Agilent 1200 Series Rapid Resolution LC system with RRHT columns for separating the ingredients found in a complex ginseng root extract and the measurement of accurate masses by an ESI oaTOF MS. For the structure elucidation CID fragmentation was carried out and the measured accurate masses were used to calculate empirical formulas of the fragments. The use of the high resolution LC system together with an ion trap MS for structure elucidation by MS^n will be discussed in part two of this work[8].

Experimental

Equipment

- Agilent 1200 Series binary pump SL with degasser. This pump has the capability to deliver a pressure of up to 600 bar, which is necessary to perform high resolving HPLC analysis on a 1.8-µm particle size RRHT column to get the best resolution performance.
- Agilent 1200 Series high performance autosampler SL with thermostat. This autosampler is especially designed to work together with the 1200 Series binary pump SL at the lowest delay volume.
- Agilent 1200 Series thermostatted column compartment (TCC). This TCC is ready for use together with the high pressure binary pump with optional separate heat exchangers and post column cooling under optimized delay volume conditions and alternating column regeneration with an optional 2-position/10-port valve.
- Agilent 1200 Series diode-array detector SL (DAD). This DAD is capable of acquiring data at a sampling rate up to 80 Hz. This device has a built-in data storage capability.

- Agilent 6200 Series MSD TOF. Orthogonal acceleration time-of-flight mass spectrometer with dual sprayer interface for mass calibration to acquire molecular masses with highest accuracy. This time-of-flight mass spectrometer is capable of acquiring data at 40 Hz and pos/neg switching.
- Picard TOF software A02.00. Software used for data acquisition with the TOF LC/MS system.
- Analyst Software. Software for TOF and UV data analysis.
- Column. ZORBAX SB C18, 2.1 x 150 mm, 1.8 µm

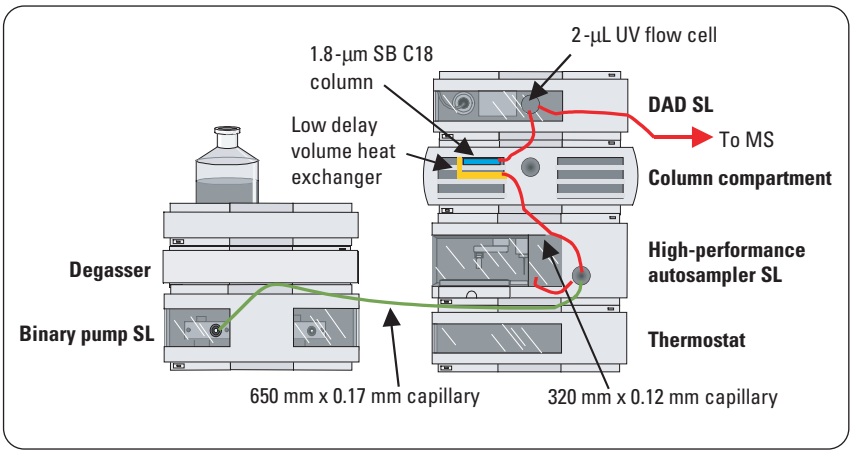

Figure 1
Agilent 1200 Series binary LC system for MS using a low delay volume configuration.

Sample

Powdered freeze-dried Asian ginseng root (1g) (Panax ginseng) was treated ultrasonically for 30 minutes in 10 mL methanol, filtered and directly used for analysis.

The set-up of the Agilent 6200 Series MSD TOF system is shown in figure 1. In this set-up the Agilent 1200 Series binary pump SL is connected to the Agilent 1200 Series high performance autosampler SL with a 0.17-mm i.d. stainless steel capillary. To reduce delay volume, the seat capillary in the Agilent 1200 Series high performance autosampler SL has an 0.12-mm i.d., which is the same kind of capillary that connects the low delay volume (1.4 µL) heat exchanger in the Agilent 1200 Series thermostatted column compartment to the column. A 2-µL cell is built into the Agilent 1200 Series diode-array detector SL for UV detection. The outgoing capillary is connected directly to the sprayer of the electrospray source at the time-of-flight mass spectrometer, which is capable of aquiring spectra at 40 Hz. This instrument set-up is optimized to achieve the highest possible resolution, which is demonstrated by the UV analysis of a complex natural product extract obtained from Asian ginseng root (Panax ginseng) (see below). The full performance of the Agilent 1200 Series binary system in the high resolution configuration is demonstrated in another publication[9]. It is also possible to use this system in a high throughput environment by making minor changes[10].

Methods:

- The Agilent 1200 Series binary pump SL was operated under the following conditions:
  Solvent A: water + 0.1 % TFA
  Solvent B: AcN + 0.1 % TFA
  Flow: 0.5 mL/min
  Gradient: 0 min 5 % B, 1 min 5 % B, 60 min 85 % B, 61 min 95 % B, 70 min 95 % B
  Stop time: 70 min
  Post time: 15 min
- The Agilent 1200 Series high performance autosampler SL was used to make injections of 10 µL sample and the samples were cooled to 10 °C. The sample loop was switched to bypass after 1 minute to reduce delay volume.
- The Agilent 1200 Series thermostated column compartment, equipped with the 1.4-µL low delay volume heat exchanger, was set at 50° C.
- The Agilent 1200 Series diode-array detector SL was operated at 80 Hz for data acquisition at a wavelength of 220 nm/4, ref. 360/100 with a 2-µL flow cell, 30-mm path length.
- Agilent 6200 Series MSD TOF was operated under the following conditions:
  Source: ESI in positive mode with dual spray for reference mass
  Dry gas: 12 L/min
  Dry Temp.: 200 °C
  Nebulizer: 35 psi
  Scan: 200-1300
  Fragmentor: 150 V or 300 V for CID
  Skimmer: 60 V
  Capillary: 3000 V

Conditions for both experiments:

Pumps

Solvent A:	H_2O + 0.1 % TFA
Solvent B:	ACN + 0.1 % TFA
Gradient:	10 % to 95 % ACN in 40 min, hold for 1 min
Flow rate:	0.4 mL/min

Autosamplers

Injection volume: 3 µL

Thermostatted column compartments

Temperature: 50° C

Detectors

DAD 2-µL cell and 20 Hz, 220 nm

Results and discussion

To compare the resolution performance of the Agilent 1200 Series Rapid Resolution LC system to an Agilent 1100 Series standard LC system, the analysis of a complex natural product extract was performed on an Agilent 1100 Series system with its maximum pressure at 400 bar equipped with a 5-µm particle size column and in comparison on an Agilent 1200 Series system with its maximum pressure at 600 bar equipped with a RRHT column with a 1.8-µm particle size. The system backpressure was about 520 bar using the 2.1 x 150 mm, 1.8-µm column. The resulting UV chromatograms acquired at 220 nm clearly demonstrate the better resolution of the peaks on the Agilent 1200 Series system (figure 2). The peak width (FWHM) of the majority of the peaks in the UV chromatogram is below 0.1 min with baseline separation. This excellent resolution, which can be achieved with the Agilent 1200 Series pump in combination with the RRHT column results in significantly more MS information useful for compound identification.

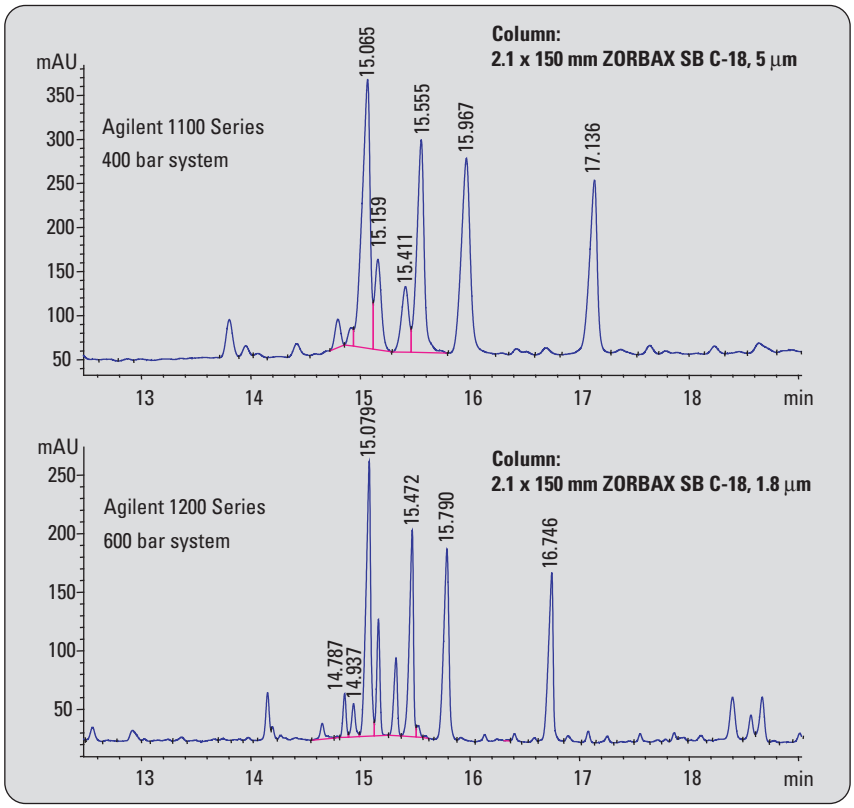

Figure 2
Ginseng extract using a 400 bar system with a 5-µm particle column and using a 600-bar system with a 1.8-µm particle column.

After separation of the individual compounds, which are components of the crude complex extract from the ginseng root, by means of rapid resolution HPLC on a 1.8-µm particle size RRHT column, they are subjected to accurate mass measurement by means of an ESI oaTOF. The main constituents elute between 20 and 33 minutes and are marked as ginsenosides Re, Rf, F_{11}, Rb_1, Rc, Rb_2 and Rd in the base peak chromatogram (figure 3). The ESI oaTOF data of the ginsenosides Rb_1, Rc and Re were analyzed in more detail for structure elucidation while the species dependent ginsenosides Rf and F_{11} are discussed in another publication[11].

The ginsenoside Rb_1 eluting at 27.7 min was identified by its protonated molecular ion at *m/z* 1109.6129 and calculation of the corresponding empirical formula with a mass accuracy of -1.90 ppm relative to 2.10 mDa. A loss of one molecule of water leads to the ion at *m/z* 1091.6012 with -0.91 ppm mass accuracy. A CID experiment was performed for structure elucidation at an elevated skimmer voltage, which presented addition-

al information in the TOF spectrum (figure 4). The loss of one of the glucose chains resulted in the ions at *m/z* 785.5047 and *m/z* 325.1136 with mass accuracies of 0.53 ppm and -0.39 ppm, respectively. A series of ions resulting from a consecutive loss of water from the ion *m/z* 785.5047 was also identified with high mass accuracy. Further loss of the second glucose chain from the molecule resulted in the ion at *m/z* 425.3784 with -0.14 ppm mass accuracy. This ion is derived from the triterpenoid core structure common to all ginsenosides. A loss of one molecule of water resulted in the ion at *m/z* 407.3679 with 0.10 mDa and -0.30 ppm mass accuracy. This set of CID fragments together with the molecular ion and the set of calculated empirical formulas confirm the structure of the ginsenoside Rb_1. The fragmentation pattern is shown in figure 4 and the mass accuracies and empirical formulas are summarized in table 1.
The ginsenoside Rc with 29.3 min retention time was identified after empirical formula calculation by

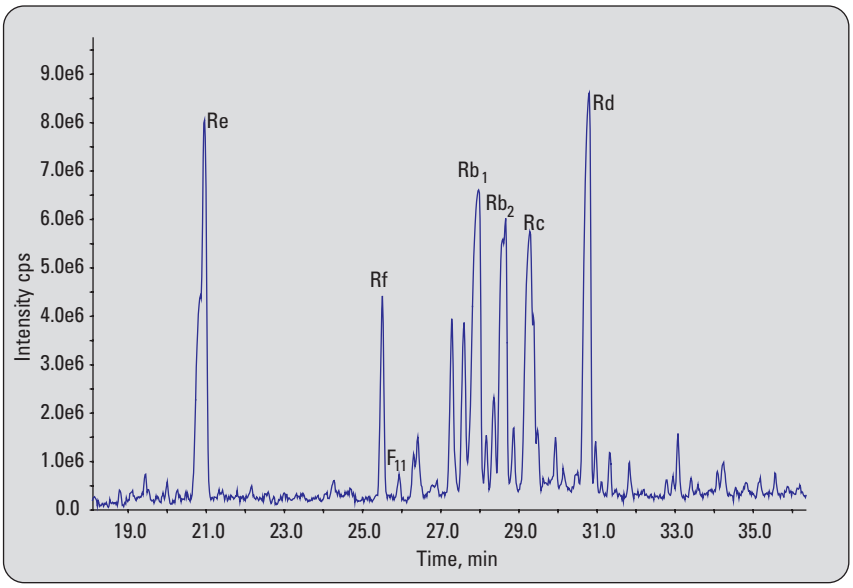

Figure 3
RR-LC-TOF basepeak chromatogram of the area containing the main components of the Asian ginseng root extract.

Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
1109.6129	1109.6108	$C_{54}H_{93}O_{23}$	2.10	-1.90
1091.6012	1091.6002	$C_{54}H_{91}O_{22}$	1.00	-0.91
785.5047	785.5051	$C_{42}H_{73}O_{13}$	-0.40	0.53
767.4950	767.4946	$C_{42}H_{71}O_{12}$	0.40	-0.58
749.4854	749.4840	$C_{42}H_{69}O_{11}$	-1.40	1.88
425.3784	425.3783	$C_{30}H_{49}O$	0.10	-0.14
407.3679	407.3678	$C_{30}H_{47}$	0.10	-0.30
343.1248	343.1240	$C_{12}H_{23}O_{11}$	0.80	2.23
325.1136	325.1135	$C_{12}H_{21}O_{10}$	0.10	-0.39

Table 1
Empirical formulas and achieved mass accuracies for the structure elucidation of ginsenoside Rb_1 by ESI oaTOF.

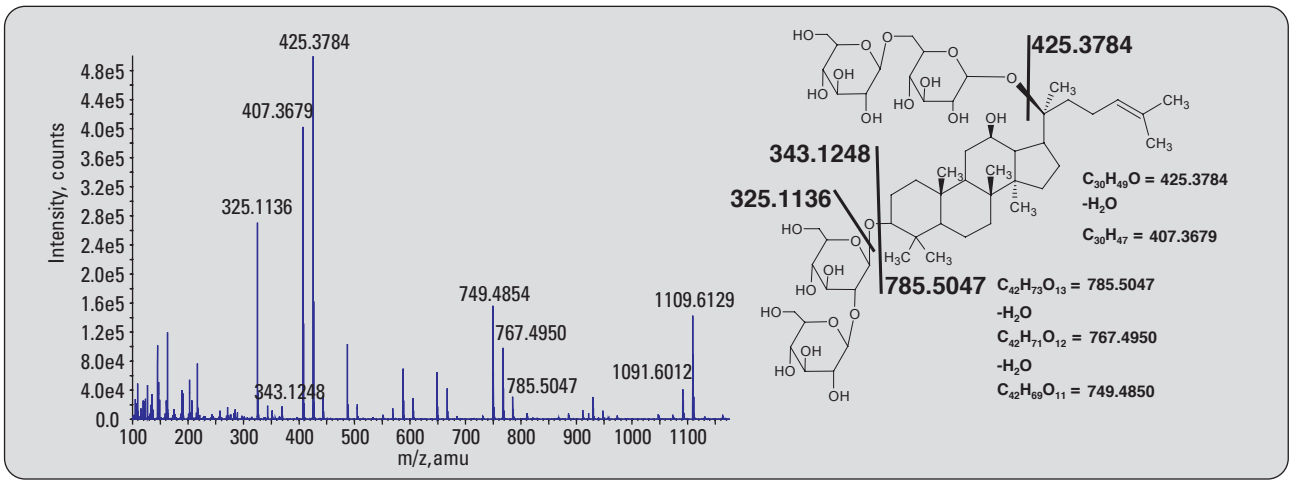

Figure 4
Accurate mass measurement of ginsenoside Rb_1 and its CID fragments by ESI oaTOF.

its molecular ion at *m/z* 1079.5991 and a product obtained by the loss of a molecule of water at *m/z* 1061.5888 with mass accuracies of 1.02 ppm and 0.78 ppm, respectively. The fragmentation obtained at a higher skimmer voltage starts with a loss of the sugar residues arabinose and glucose followed by a loss of water from the remaining molecule fragment (figure 5). The fragmentation of the sugar moieties resulted in the initial ions at *m/z* 785.5060 and at *m/z* 755.4936 with mass accuracies of -1.12 ppm and 1.26 ppm, respectively. After complete loss of all saccharide moieties the triterpenoide core structure was detected at *m/z* 425.3785 with high mass accuracy -0.37 ppm for the calculated empirical formula. The cleaved glucose chain was detected at *m/z* 325.1124 with 0.22 pmm mass accuracy. The fragmentation pattern is shown in figure 5 and the achieved mass accuracies for all fragments are summarized in table 2. The ginsenosides Rc and Rb_2, which elute at 28.7 min retention time are structure isomers with the same empirical formula and molecular mass. In the ginsenoside Rc the sugar arabinose is in the furanose form and in the ginsenoside Rb_2 the arabinose is in its pyranose form.

The last of the main ingredients, which was classified in the examined ginseng root extract is ginsenoide Re. This compound, which elutes at 20.8 min, was identified by empirical formula calculation from the mass at *m/z* 947.5585 with a high mass accuracy of 0.60 mDa or -0.59 ppm (figure 6). The CID fragmentation gave further evidence for the identity of this substance. After cleavage of the saccharide moieties there are two remaining main fragments. The fragment obtained after a loss of a molecule of glucose with *m/z* 767.4957 was measured with a mass accuracy of -1.50 ppm. A consecutive loss of water yields the ion at *m/z* 749.4855. After a further loss of the glucose disaccharide the fragment of the triterpenoide core structure at *m/z* 441.3739 with a mass accuracy of

Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
1079.5991	1079.6002	$C_{53}H_{91}O_{22}$	-1.10	-1.02
1061.5888	1061.5896	$C5_3H_{89}O_{21}$	-0.88	0.78
785.5060	785.50.51	$C_{42}H_{73}O_{13}$	0.90	-1.12
767.4939	767.4946	$C_{42}H_{71}O_{12}$	-0.70	0.85
749.4846	749.4840	$C_{42}H_{69}O_{11}$	0.60	-0.81
755.4936	755.4946	$C_{41}H_{71}O_{12}$	-1.00	1.26
737.4830	737.4840	$C_{41}H_{69}O_{11}$	-1.00	1.34
719.4723	719,4734	$C_{41}H_{67}O_{10}$	-1.10	1.56
425.3785	425.3783	$C_{30}H_{49}O$	0.20	-0.37
407.3679	407.3678	$C_{30}H_{47}$	0.10	-0.30
325.1134	325.1135	$C_{12}H_{21}O_{10}$	-0.10	0.22

Table 2
Empirical formulas and achieved mass accuracies for the structure elucidation of ginsenoside Rc by ESI oaTOF.

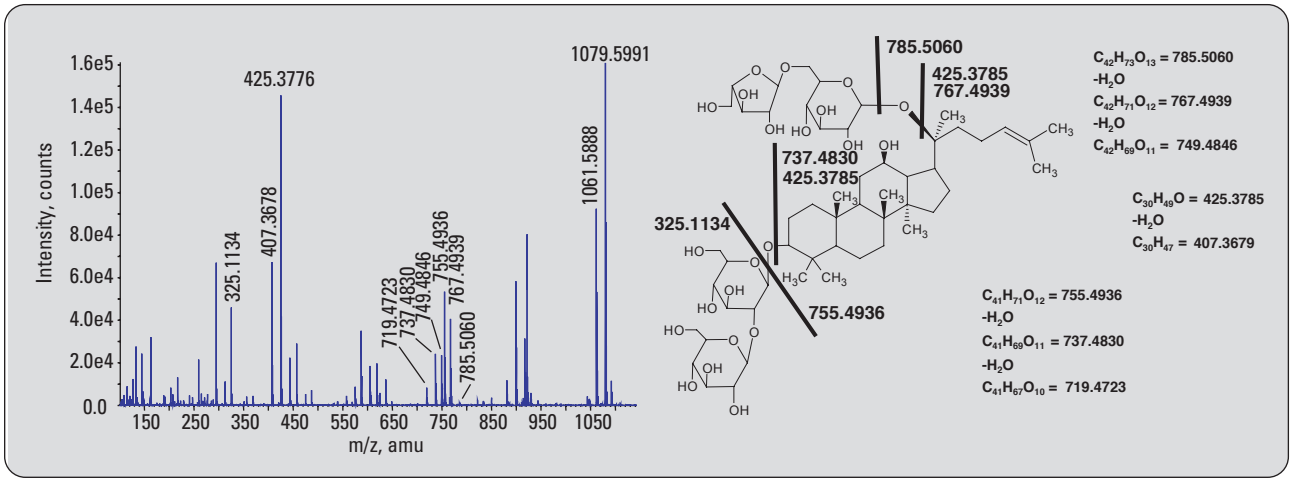

Figure 5
Accurate mass measurement of ginsenoside Rc and its CID fragments by ESI oaTOF.

0.35 ppm occurred. After a further successive loss of water, this fragment yields to the ions at *m/z* 423.3625 and *m/z* 405.3519 with 0.45 ppm and 0.55 pmm respectively. The calculated empirical formulas and mass accuracies of the measured fragments are summarized in table 3.

Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
947.5585	947.5579	$C_{48}H_{83}O_{18}$	0.60	-0.59
767.4957	767.4946	$C_{42}H_{71}O_{12}$	1.10	-1.50
749.4855	7494840	$C_{42}H_{49}O_{11}$	1.50	-2.01
441.3731	441.3733	$C_{30}H_{49}O_{2}$	-0.20	0.35
423.3625	423.3627	$C_{30}H_{47}O$	-0.20	0.45
405.3519	405.3521	$C_{30}H_{45}$	-0.20	0.55

Table 3
Empirical formulas and achieved mass accuracies for the structure elucidation of ginsenoside Re by ESI oaTOF.

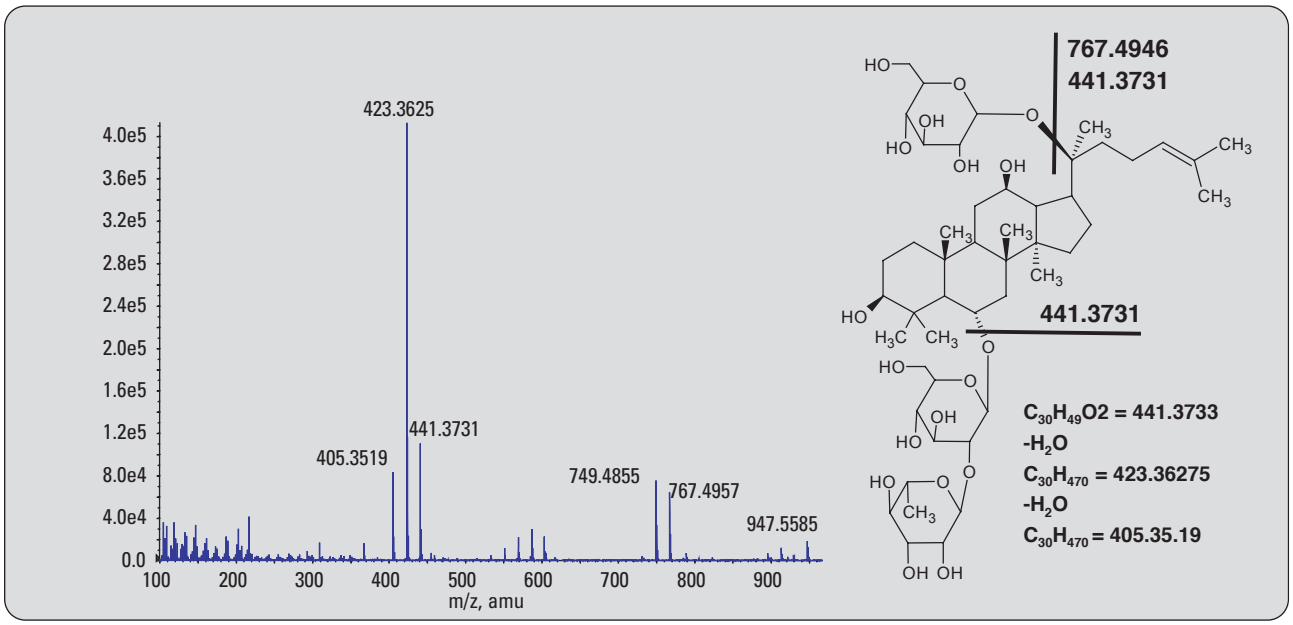

Figure 6
Accurate mass measurement of ginsenoside Re and its CID fragments by ESI oaTOF.

Conclusion

The featured application with the Agilent 1200 Series Rapid Resolution LC system together with the Agilent 6200 Series MSD TOF proves its capability for use in structure elucidation of natural products in highly complex extracts from plant origins. In this application a highly complex extract from ginseng root was analyzed with the Agilent 1200 Rapid Resolution LC/TOF system. A comparison to the predecessor Agilent 1100 Series system clearly demonstrated the higher resolution that is achieved by the new system equipped with a 1,8-µm particle size column. Complex structures of three ginsenosides, which are the main compounds of the extract, could be elucidated by the interpretation of the obtained TOF spectra. The proposed structures were confirmed by empirical formula calculation of the molecular ions as well as for the fragments of the molecules obtained by CID. All measured masses have accuracies in the lower single digit range and therefore confirm the structures with highest confidence. With this detailed knowledge about the ingredients of the natural plant extract it is possible to use the high resolution LC/TOF technology to monitor the content of an extract before usage in a pharmaceutical formulation. This is achieved in a fully automated manner and confirms the compound's identity by measuring its mass with highest accuracy and empirical formula confirmation.

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Edgar Nägele is Application Chemist at Agilent Technologies, Waldbronn, Germany.

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