Analysis of a complex natural product extract from ginseng – Part III: Species differentiation of ginseng plants and authentication of ginseng products by LC/MS

Application Note

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Abstract

Since prehistoric times herbal extracts have been used for the treatment and prevention of disease. The same plant often shows different patterns of active ingredients depending on the region, climate, growing conditions or sub-species. Some plants used in traditional Chinese medicine (TCM) are a good example of this behavior. A typical plant is the ginseng root, which is widespread in various sub-species on the entire Asian and American continents. This Application Note will demonstrate the use of the Agilent 1200 Series Rapid Resolution LC system with Rapid Resolution High Throughput (RRHT) columns for the separation of the ingredients found in a complex ginseng root extract. Detection and identification of the different compounds by LC/ESI orthogonal acceleration time-of-flight (oaTOF) and LC/ion trap mass spectrometry was implemented for the differentiation of the regional or biological origin. The possibility to provide evidence for the authenticity of a ginseng product is also demonstrated by determining the ingredient profile based on known structural information of the ingredients.

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Introduction

Traditional natural medicine from different and regionally separated cultures often uses the same plant species for the preparation of herbal-based extracts and tinctures for the treatment of disease. The plants used, which are often cultivated as sub-species or grown under different conditions, show a distinctive pattern of active ingredients depending on these influences. A typical botanical example for this behavior is the ginseng root (*Panax spec.*), which has been used in traditional Chinese medicine (TCM) for thousands of years. This herb is widespread in various sub-species on entire Asian and American continents[1]. The method of choice for the analysis of complex natural product extracts like the ginseng root is high performance liquid chromatography (HPLC)[2]. The main active compounds are triterpene saponins called ginsenosides of which more than 80 have been isolated and characterized during the past several years. 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 used for the determination of complex and similar structures of ginsenosides[3]. It is possible to differentiate between the origin and the ginseng sub-species and to confirm the authenticity of pharmaceutical ginseng products using fragmentation patterns obtained by means of tandem mass spectrometry of the pharmaceutical active ingredients[4]. This Application Note will demonstrate the use of the Agilent 1200 Series Rapid Resolution LC system with Rapid Resolution High Throughput (RRHT) columns for the separation of the ingredients found in a complex ginseng root extract from Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*). Detection and identification of various compounds by LC/ESIoaTOF accurate mass measurement and LC/ion trap MS^n mass spectrometry was implemented for the differentiation of the regional or biological origin. The possibility to provide evidence for the authenticity of a ginseng product is also demonstrated by determining the ingredient profile based on known structural information of the ingredients. The detailed structure elucidation of ginsenoides by means of ESI oaTOF for accurate mass measurement including CID fragmentation and ion trap MS^n is described in detail in parts I and II of this study[5,6.]

Experimental

Equipment

- Agilent 1200 Series binary pump SL with degasser. This pump has the capability to perform high resolving HPLC analysis on a 1.8-µm particle size column to get the best resolution performance.
- Agilent 1200 Series high performance autosampler SL (h-ALS SL) with thermostat. This autosampler is especially designed to work together with the binary pump SL with lowest delay volumes.
- Agilent 1200 Series thermostatted column compartment (TCC). The TCC is ready for use with the binary pump SL. Optional extras include separate heat exchangers for pre-column heating and post-column cooling under optimized delay volume conditions and a 2-position/10-port valve for alternating column regeneration.
- Agilent 1200 Series diode array detector SL (DAD). The DAD is capable of acquiring data with a sampling rate of up to 80 Hz.
- Agilent 6210 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 with pos/neg switching.
- Agilent 6330 Ion Trap. Ion trap mass spectrometer for MS^n tandem mass spectrometric experiments with scan rate up to 26,000 m/z per second and automated data-dependent MS^n capabilities.
- The software used for LC/ion trap instrument control was ChemStation B01.03, ion trap software 5.3, and for data analysis the ion trap data analysis software 3.3.
- The software used for LC/TOF instrument control was the Mass Hunter Workstation A02.00 for data acquisition and AnalystQS for data analysis.
- Column: ZORBAX SB C18, 2.1 X 150 mm, 1.8-µm

Sample

1. Powdered freeze-dried Asian ginseng root (1 g) (*Panax ginseng*) and powdered freeze-dried American ginseng root (1 g) *(Panax quinquefolius*) were treated in an ultra sonic bath for 30 minutes in 10 mL methanol, filtered and directly used for analysis.

2. Syrup-like Korean ginseng extract pharmaceutical product (ILHWA Co., LTD, Korea) was dissolved (1 g) in 100 mL water/MeOH (1/1, v/v) and used directly after filtration.

System set-up

The set-up of the LC/MS system is shown in figure 1. The Agilent 1200 Series binary pump SL is connected to the Agilent 1200 Series h-ALS SL with a 0.17-mm i.d. stainless steel capillary. To reduce delay volume, the seat capillary in the h-ALS SL has 0.12 mm i.d. The same kind of capillary connects to the low delay volume (1.6 µL) heat exchanger in the TCC, which is connected to the column. For UV detection a 2-µL flow cell is built into the DAD SL. The outgoing capillary is directly connected to the sprayer of the electrospray source at the ESI oaTOF or to the ion trap mass spectrometer. This instrument set-up is optimized to achieve the highest possible resolution, which is demonstrated by the comparative UV analysis of a complex natural product extract obtained from Asian ginseng root (*Panax ginseng*)[5]. Using the 2.1 x 150 mm, 1.8-µm column, the system back pressure was typically about 560 bar. The peak width (FWHM) of the majority of the peaks in the UV chromatogram was below 0.1 min with baseline separation. The full performance of the Agilent 1200RR LC system in the high resolution configuration is outlined in a separate performance note[7]. It is also possible to use this system with minor changes in a high throughput environment[8].

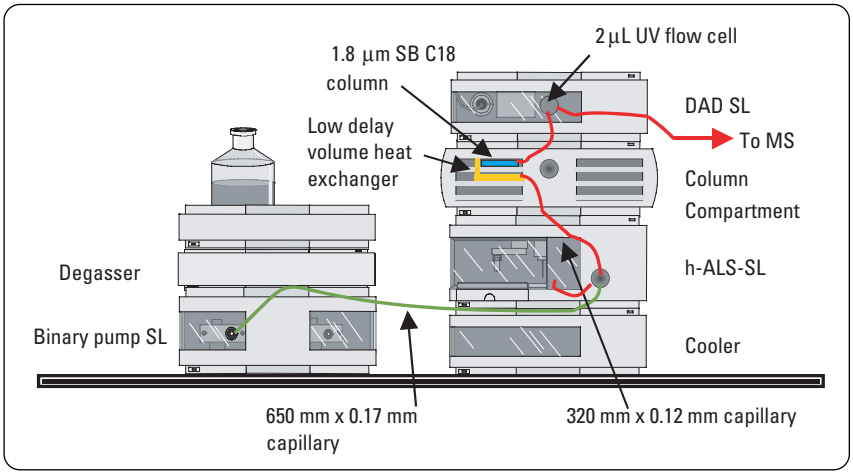


Figure 1
Agilent 1200 binary LC system for MS in low delay volume configuration.

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 sample injections of 10 µL and the samples were cooled to 10 °C. The sample loop was switched to bypass after one minute to reduce delay volume.
- The Agilent 1200 Series thermostatted column compartment was adjusted to 50 °C, equipped with the 1.6-µL low delay volume heat exchanger.
- 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 the 2-µL flow cell, 3-mm path length.
- The TOF mass spectrometer 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

- The ion trap mass spectrometer was operated under the following conditions:

Source:	ESI in positive mode.
Dry gas:	5.0 L/min.
Dry temp.:	300 °C
Nebulizer:	15 psi
Target:	150,000
Max. accum. time:	100 ms
Scan:	200-1300
Averages:	2. Automated MS/MS and MS^3

Results and discussion

The separation of the individual compounds in the samples of Asian ginseng (*Panax ginseng*) and American ginseng *(Panax quinquefolius*) using high resolution HPLC on a 1.8-µm particle size column unravels the differences in the individual composition of the crude plant extracts from the different ginseng sub species. Obviously, the differences lie not only in the concentrations of the ginsenoides but also in the presence of different compounds in the individual species. The main components elute between 20 and 40 minutes and are evidently the common ginsenosides Re, Rg_1, Rb_1, Rb_2, Rc and Rd as well as the special ginsenoides Rf and F_{11} in the ion trap MS base peak chromatogram (figures 2 and 3). Despite the good resolution of the 1.8-µm particle column, their resolution can be improved depending on the temperature. For instance, at a column temperature of 50 °C the genisenosides Re at m/z 946.5 $[M+H]^+$ and ginsenoside Rg_1 at m/z 823.5 $[M+Na]^+$ are not resolved (figure 2). Nevertheless, they are clearly separated at 80 °C and are contained in both ginseng samples (figure 3). To distinguish the subspecies of Asian and American ginseng by LC/MS the isomeric ginsenosides Rf and the pseudoginsenoside F_{11} are very useful. The analysis of Asian ginseng shows the ginsenoside Rf in protonated and sodiated form at m/z 801.5 and m/z 823.5, respectively. The protonated form of the isomeric pseudoginsenoside F_{11} at m/z 801.5 is only detectable as a trace compound (figure 3). In

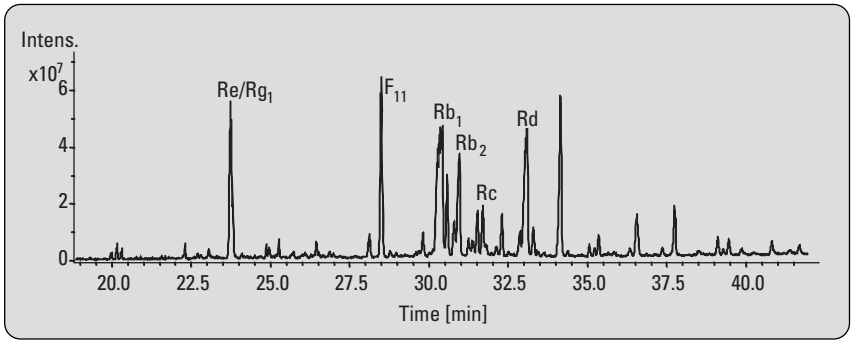


Figure 2
Base peak chromatogram of the separation of compounds contained in a crude extract from American ginseng (*Panax quinquefolius*) on a RRHT column at 50 °C.

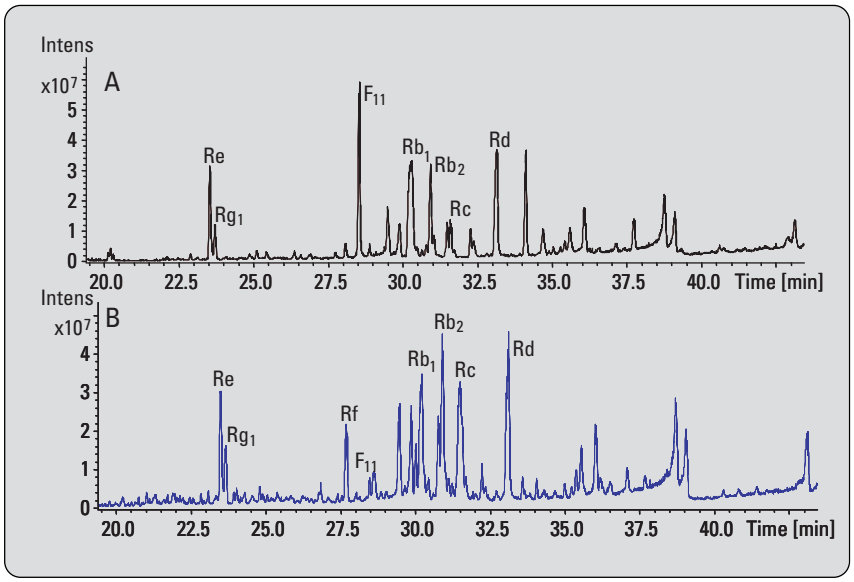


Figure 3
Base peak chromatograms from the separation of compounds in a crude extract from
A) American ginseng (*Panax quinquefolius*)
B) Asian ginseng (*Panax ginseng*) sub species on a RRHT column at 80 °C.

contrast, the American ginseng species only contains the pseudoginsenoside F_{11} in a larger amount and not the ginsenoside Rf. Both isomeric compounds have the same empirical formula ($C_{42}H_{72}O_{14}$). The compounds are constitutional isomers only differentiable by their structural formula. To distinguish between both isomeric forms of these species-typical compounds, it is necessary to perform MS/MS experiments. The ion trap MS/MS experiments of the American ginseng sample show the typical sequence of a loss of five water molecules at m/z 475.4, 457.4, 439.3, 421.3 and 403.3 after the cleavage of the glucose-rhamnose disaccharide moiety at m/z 309.1, which is characteristic for the molecular constitution of pseudoginsenoside F_{11} obtained at a retention time of 28.5 min

(figure 4). Ultimately the distinctive furan ring fragment is released at m/z 143.1. To confirm the structural identity of the proposed fragments, the experiment was repeated with a high resolution LC/MS oaTOF instrument for high mass accuracy measurement and empirical formula confirmation (figure 5). The measurement of the accurate mass of m/z 801.4997 confirmed the identity of the molecular ion with 0.41 ppm mass accuracy. Four of the empirical formulas for the fragments resulting from the typical consecutive loss of five molecules of water resulting in the ions at m/z 475.3782, 457.3676, 439.3569 and 421.3464 were confirmed with mass accuracies less than 2 ppm. The cleaved disaccharide fragment was confirmed by the accurate mass at m/z 309.1176 with 3.10 ppm accuracy and the small furan ring fragment at m/z 143.1065 with 4.90 ppm. The table in figure 5 summarizes the measured masses and the calculated accuracies for all obtained fragments derived from pseudoginsenoide F_{11}.
In comparison, the compound

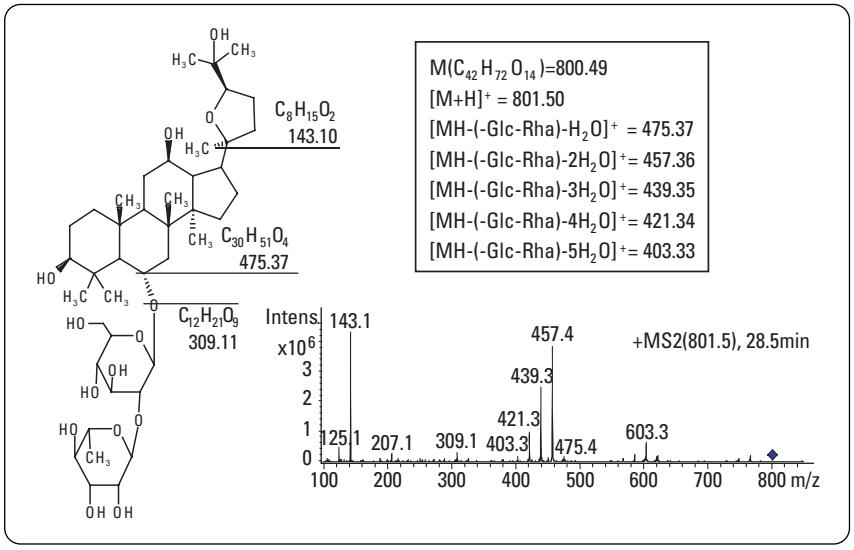
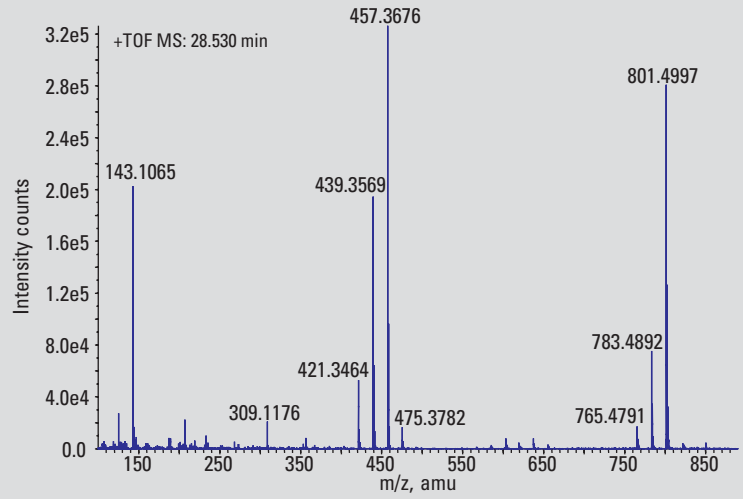


Figure 4
MS/MS of 24(R) pseudoginsenoside F_{11} typical for American ginseng (*Panax quinquefolius*).

obtained at a retention time of 27.7 minutes which is typical for Asian ginseng, is ginsenoside Rf. This compound also contains a protonated molecular ion of m/z 801.5 in the LC/MS analysis. The fragments of this molecule are typical and can be used to distinguish it from ginsenoside F_{11}. After the cleavage of the diglucose chain at m/z 325.0, a consecutive loss of four molecules of water is identified by MS/MS analysis (figure 6). The mass of the resulting ions at m/z 459.4, 441.4, 423.4 and 405.4 is different from the pattern obtained for the molecule F_{11}. The fragment at m/z 143.1 was not observed. To confirm the identity, a LC/MS oaTOF analysis was performed. The TOF spectrum shows the molecular ion at m/z 801.4999 with a high mass



Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
801.4997	801.5000	$C_{42}H_{73}O_{14}$	-0.35	0.41
783.4892	783.4895	$C_{42}H_{71}O_{13}$	-0.30	0.34
765.4791	765.4789	$C_{42}H_{69}O_{12}$	0.20	-0.25
475.3782	475.3787	$C_{30}H_{51}O_{4}$	-0.50	1.12
457.3676	457.3682	$C_{30}H_{49}O_{3}$	-0.60	1.25
439.3569	439.3574	$C_{30}H_{47}O_{2}$	-0.70	1.60
421.3464	421.3470	$C_{30}H_{43}O_{1}$	-0.60	1.52
309.1176	309.1186	$C_{12}H_{21}O_{9}$	-1.00	3.10
143.1065	143.1072	$C_{8}H_{15}O_{2}$	-0.70	4.90

Figure 5
HR-LC ESI oaTOF from an extract of American ginseng (*Panax quinquefolius*) 24(R) pseudoginsenoside F_{11}.

accuracy of 0.16 ppm (figure 7). The molecules obtained after the loss of water at m/z 459.3834, 441.3726, 423.3620 and 405.3515 were confirmed with accuracies between 0.9 and 1.6 ppm. The mass of the cleaved glucose disaccharide was measured at m/z 325.1134 with 0.22 ppm mass accuracy. The table in figure 6 summarizes the measured masses and the calculated accuracies for all obtained fragments derived from ginsenoside Rf. Other ginsenosides were detected by ion trap MS/MS and/or accurate mass

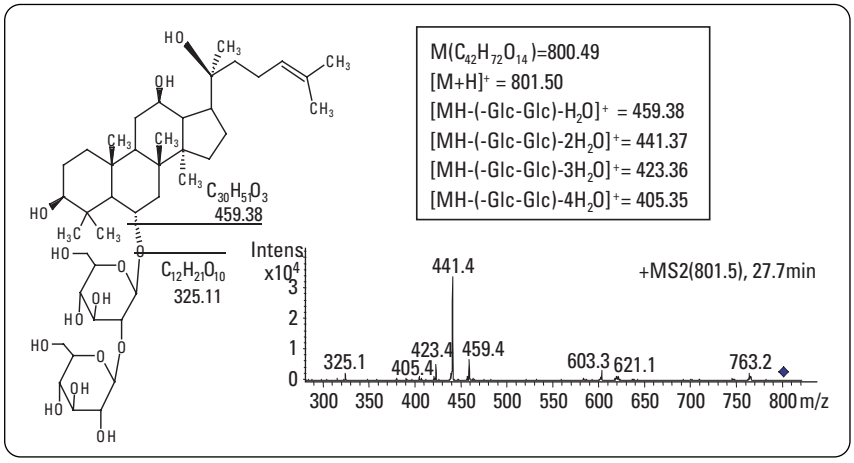
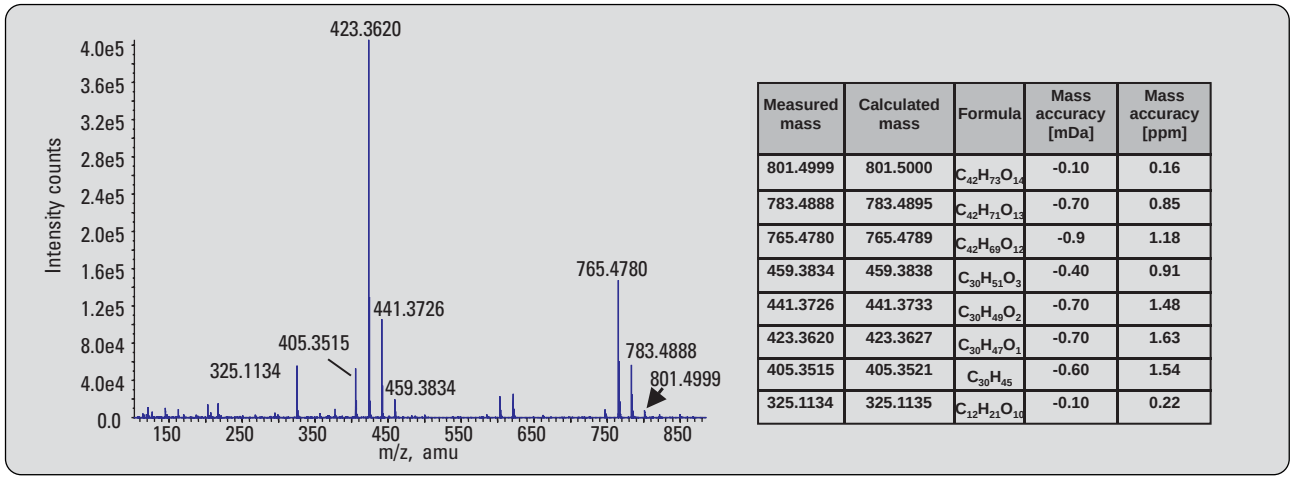


Figure 6
MS/MS of ginsenoside Rf typical for Asian ginseng (*Panax ginseng*).



Measured mass	Calculated mass	Formula	Mass accuracy [mDa]	Mass accuracy [ppm]
801.4999	801.5000	$C_{42}H_{73}O_{14}$	-0.10	0.16
783.4888	783.4895	$C_{42}H_{71}O_{13}$	-0.70	0.85
765.4780	765.4789	$C_{42}H_{69}O_{12}$	-0.9	1.18
459.3834	459.3838	$C_{30}H_{51}O_3$	-0.40	0.91
441.3726	441.3733	$C_{30}H_{49}O_2$	-0.70	1.48
423.3620	423.3627	$C_{30}H_{47}O_1$	-0.70	1.63
405.3515	405.3521	$C_{30}H_{45}$	-0.60	1.54
325.1134	325.1135	$C_{12}H_{21}O_{10}$	-0.10	0.22

Figure 7
HR-LC ESI TOF of an extract of Asian ginseng (*Panax ginseng*) ginsenoside Rf .

measurement with empirical formula calculation of the molecular ions by means of ESI oaTOF as indicated in figure 3. The detection of the typical ginsenosides Rf and F_{11} by LC/MS on either ion trap or ESI oaTOF can be used to determine the species of a ginseng plant and therefore the origin of a pharmaceutical ginseng preparation. This was performed with a black, syrup-like ginseng extract purified for pharmaceutical use, which was purchased from a Korean vendor. The analysis

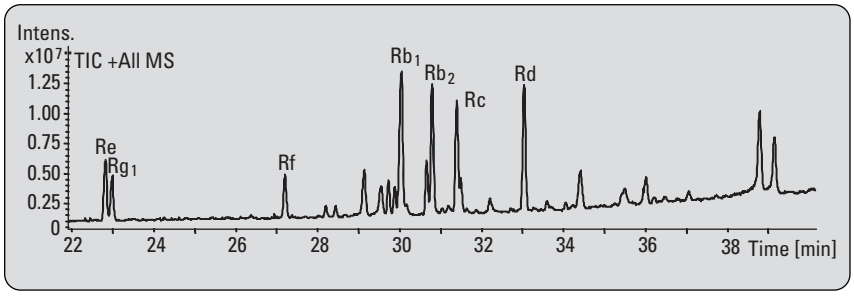


Figure 8
Ion trap MS analysis of a purified pharmaceutical ginseng extract from Asian ginseng (*Panax ginseng*) from Korea.

shows the pattern of ginsenoides Rb_1, Rb_2, Rc and Rd typical for the Asian ginseng sub species Panax ginseng (figure 8). The peaks for the ginsenosides Re and Rg_1 are clearly separated by RRHT

column chromatography. In the end the appearance of the characteristic ginsenoside Rf supplies the evidence for the Asian ginseng species. It is possible to use the ion trap MRM on one or more specific masses of typical ginsenoides to look only for characteristic ginsenoides in a sample[6].

Conclusion

The Agilent 1200 Series Rapid Resolution LC System together with the Agilent 6330 ion trap and the Agilent 6210 TOF are powerful analytical tools for the detection of structurally complex compounds contained in crude natural product extracts also found in the purified pharmaceutical products manufactured thereof. In this application a highly complex root extract from different ginseng species was analyzed using an Agilent 1200 Series Rapid Resolution LC/Trap systemn and an Agilent 1200 Series Rapid Resolution LC/TOF system. With the resulting highly resolved compounds of the extract, it was possible to identify compounds which are typical for the regional species of ginseng as well as the different compositions of the common ginsenosides in ginseng root extracts and pharmaceutical products. Complex structures of two species-typical ginsenosides could be elucidated by the interpretation of the obtained MS/MS ion trap data and could be confirmed by accurate mass measurement using the ESI oaTOF. The detailed knowledge about the different fragmentation was used to determine the regional origin of a pharmaceutical ginseng extract.

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