Agilent MassHunter – Fast, computer-aided analysis of LC/ESI-TOF data from complex natural product extracts

Part 2: Comparison of Agilent 6210 TOF data from different biological origin with the Mass Profiler in MassHunter software

Application Note

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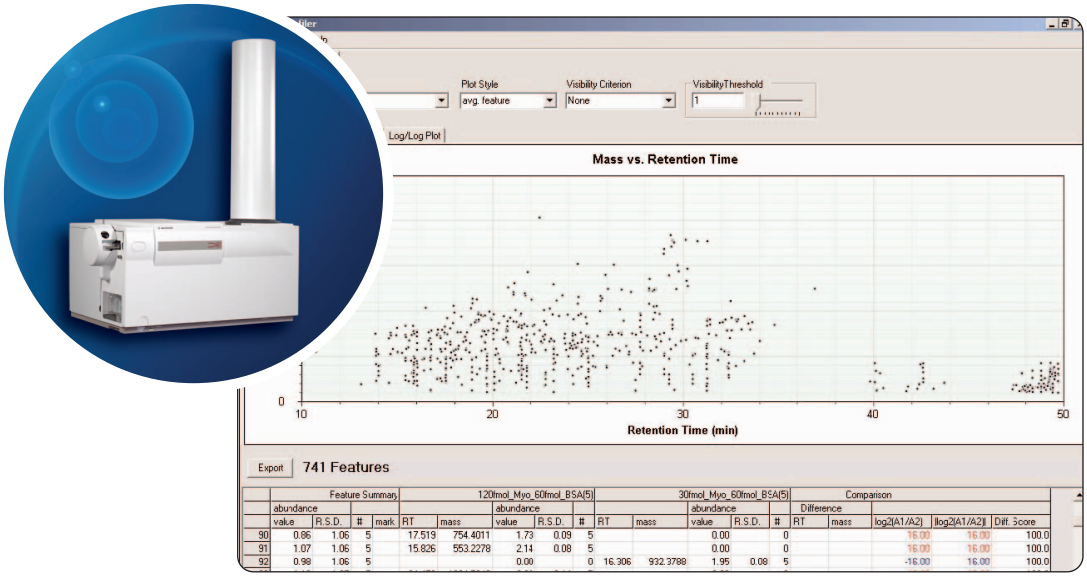

Abstract

This Application Note describes:

- Fast computer-aided identification of differences in complex natural product extracts
- Accurate mass measurement with LC/ESI-TOF for the determination of complex natural compound structures
- The use of the Agilent 1200 Series Rapid Resolution LC (RRLC) system with Rapid Resolution High Throughput (RRHT) columns for separation of ingredients in a complex ginseng root extract, and the use of the Agilent 6210 ESI-TOF mass spectrometer for accurate molecular mass measurement
- Processing of TOF data with the Mass Profiler of the Agilent MassHunter Workstation software to identify statistically significant differences in a set of samples from two different ginseng subspecies
- Identification of the different compounds by empirical formula calculations based on highly accurate mass measurement

Agilent Equipment:
1200 Series Rapid Resolution LC system
6210 Time-of-Flight MS
MassHunter Workstation software

Application Area:
Analysis of complex natural products in drug discovery

Agilent Technologies

Introduction

Herbal extracts have been used since prehistoric times for the treatment of disease. The effects of these extracts on humans were determined by simple trial and error over generations. A good example for the efficiency achieved is Traditional Chinese Medicine (TCM). In Western medicine, drugs of natural origin are gaining importance due to their therapeutic potential. However, Western pharmaceutical quality standards require a deeper know-ledge of the ingredients in medicines based on natural products. A famous Asian herb, which has been used in herbal medicine for more than 5000 years, is the Asian ginseng root (*Panax ginseng*).

Apart from the Asian subspecies, other subspecies of the ginseng plant are available such as the American subspecies (*Panax quinquefolius*). The pharmaceutically active compounds of each subspecies have different patterns of occurrence and concentration[1]. Therefore, it is important to know the composition of the plant extract, depending on its biological origin, to anticipate its medical activity before use. The main active compounds of ginsenosides are triterpene saponins, of which more than 80 have been isolated and characterized during the past years.

Much 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 such as the ginseng root is high-performance liquid chromatography (HPLC)[2]. For determination of the complex and similar structures of ginsenosides, modern LC/MS equipment is currently used such as LC/ESI-TOF for accurate mass measurement and LC/ion trap or LC/triple quadrupole systems for structural elucidation by MS/MS and MS^n[3]. However, the time-consuming bottleneck remains the examination of the acquired MS data.

This Application Note describes the analysis of TOF data with the Mass Profiler of the Agilent MassHunter Workstation software for fast and easy identification of differences in the extracts from different ginseng subspecies. This note also describes the use of the Agilent 1200 Series Rapid Resolution LC (RRLC) system with Rapid Resolution High Throughput (RRHT) columns for the separation of the ingredients in complex ginseng root extracts together with the Agilent 6210 ESI-TOF mass spectrometer for accurate measurement of their molecular masses.

Processing of the TOF data with the Molecular Feature Extractor (MFE) of the Agilent MassHunter Workstation software to generate molecular features for the identification of natural product compounds is described in part 1 of this study[4]. An example for an automated approach to analyze complex natural product extracts is described in part 3 of this study[5].

Experimental

Equipment

- Agilent 1200 Series binary pump SL with degasser. This pump is capable of performing high resolution HPLC analysis on 1.8 µm particle size columns for best resolution performance.
- Agilent 1200 Series high performance autosampler SL with thermostat. This autosampler is designed specifically to be used with the binary pump SL.
- Agilent 1200 Series thermostatted column compartment. This column compartment is supplied ready for use with the binary pump SL. Also available are heat exchangers for post-column cooling under optimized delay volume conditions, as well as a 2-position/10-port valve for alternating column regeneration.
- Agilent 1200 Series diode array detector SL. This detector is capable of acquiring data at a sampling rate up to 80 Hz.
- Agilent 6210 Time-of-Flight mass spectrometer. This orthogonal acceleration TOF MS has a dual sprayer interface for mass calibration and acquisition of molecular masses with highest accuracy, and is capable of acquiring data at 40 Hz and with positive/negative switching
- Column: ZORBAX SB C18, 2.1 x 150 mm, 1.8 µm particles.
- Software: TOF instrument control software Agilent MassHunter Workstation revision A.02.00 for data acquisition, Agilent Analyst Software for data review, and Agilent Mass Profiler software for data processing.

In this LC/TOF system setup, the binary pump SL is configured for low-delay volumes and connected to the high performance autosampler SL with a 0.17 mm ID stainless steel capillary. To reduce delay volume, the seat capillary in the high performance autosampler SL has an ID of 0.12 mm. The same type of capillary is used to connect the low delay volume (1.6 µL) heat exchanger, which is connected to the column, in the thermostatted column compartment. For UV detection, a 2 µL cell is built into the diode array detector SL. The outgoing capillary is connected directly to the sprayer in the electrospray source of the mass spectrometer. This instrument setup is optimized to achieve the highest possible resolution.

Sample preparation

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

Method

- 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 sample 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 thermostated column compartment was adjusted to 50 °C and equipped with the low delay volume heat exchanger.

- The Agilent 1200 Series DAD SL was operated at 80 Hz for data acquisition at a wavelength of 220 nm/4 nm, reference 360 nm/100 nm with the 2 µL flow cell, 3 mm path length flow cell.

- The Agilent 6210 TOF MS 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
Skimmer:	60 V
Capillary:	3000 V

Results and discussion

The ingredients of the Asian and American ginseng root extracts were separated with the Agilent 1200 Series RRLC system using an RRHT column (1.8 µm particle size) with subsequent ESI-TOF mass spectrometry (Agilent 6210 TOF). The high resolution LC facilitated excellent separation of the major and minor ingredients of the natural product extracts (figure 1). For the data analysis with Mass Profiler, five repeated injections of each extract sample were measured by the same LC/MS TOF method as described in the experimental section. The acquired TOF data were extracted by the Molecular Feature Extractor software as described[4]. In this process, the identified ions were clustered to molecular features comprising isotope compounds and adducts. The files obtained were grouped according to the biological origin of the measured extract samples into two respective groups for Asian and American ginseng and loaded into the Mass Profiler software. All 671 identified molecular features were displayed in a plot of mass against the retention time plot in the Mass Profiler software to inspect the quality of the data (figure 2). The molecular features for the known ginsenoside Rb_1 ($C_{54}H_{92}O_{23}$ at M = 1108.6029 and RT = 11.90) were enlarged.

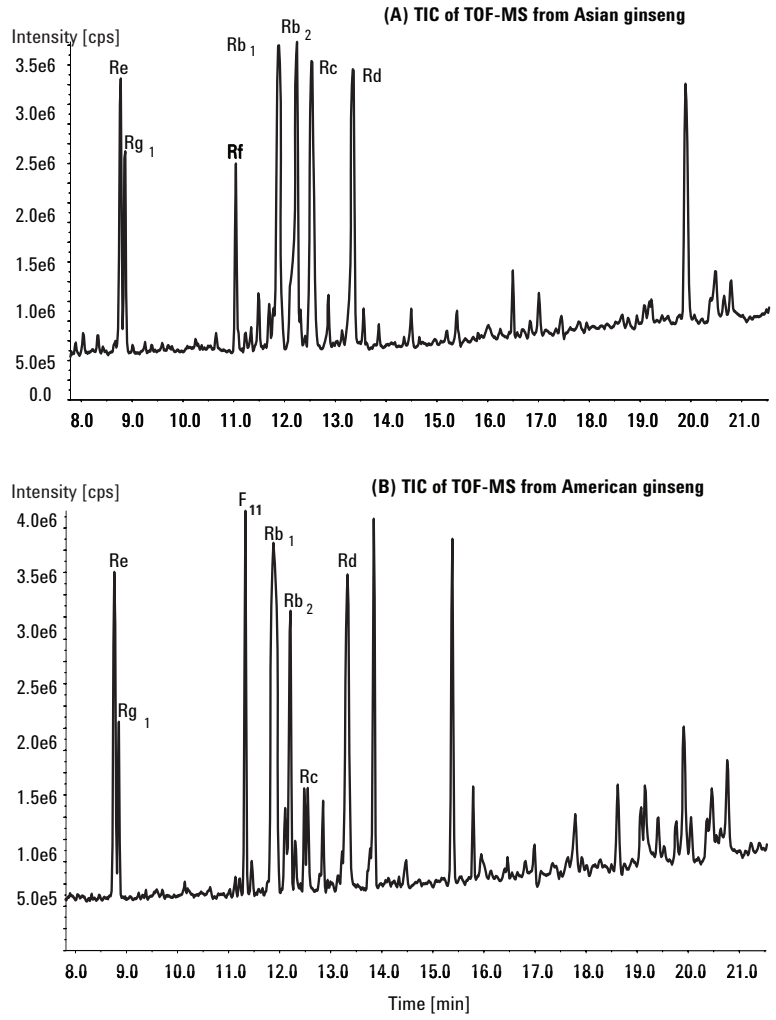

Figure 1
TIC of TOF-MS from an Asian (A) and an American (B) ginseng root extract between 8 and 21 minutes showing the main ginseosides.

The standard deviations for mass and retention time, the relative standard deviation of the abundance in each group as well as the high relative mass accuracy of 1.38 ppm for this particular compound demonstrate the high quality of the data.

For the differential analysis of both groups, the features of each group are displayed in a logarithmic (log2) plot showing the abun-

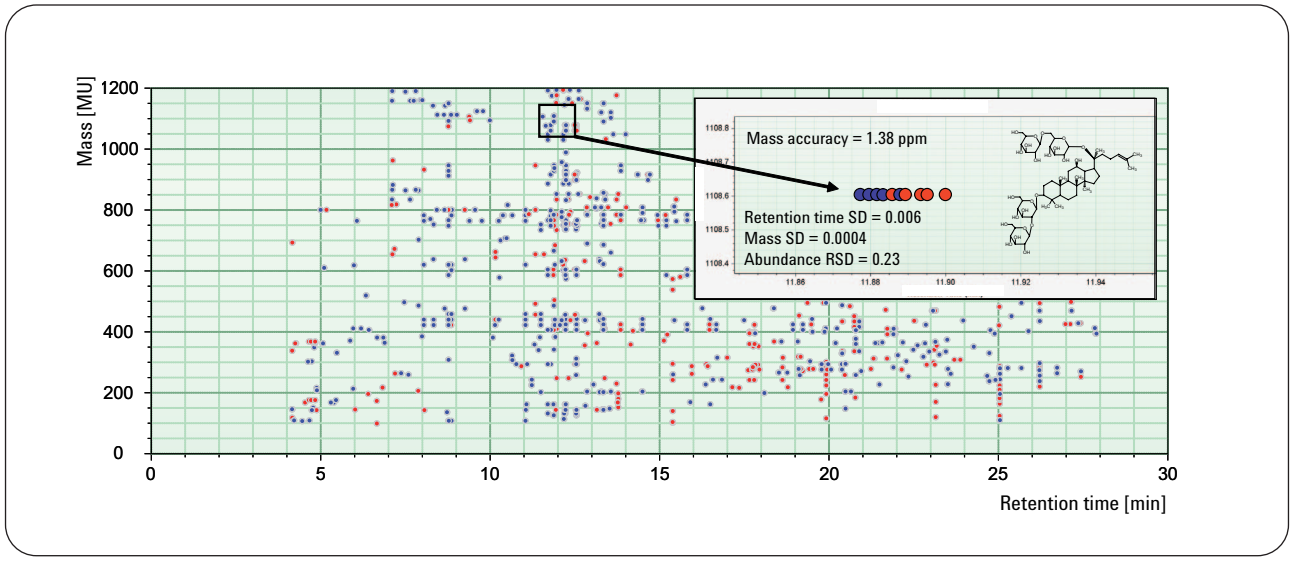

Figure 2
Plot of mass against retention time shows all 671 molecular features found in the Asian (red) and American (blue) ginseng extracts and molecular features of ginsenoside Rb_1 ($C_{54}H_{92}O_{23}$ at M = 1108.6029 and RT = 11.90)

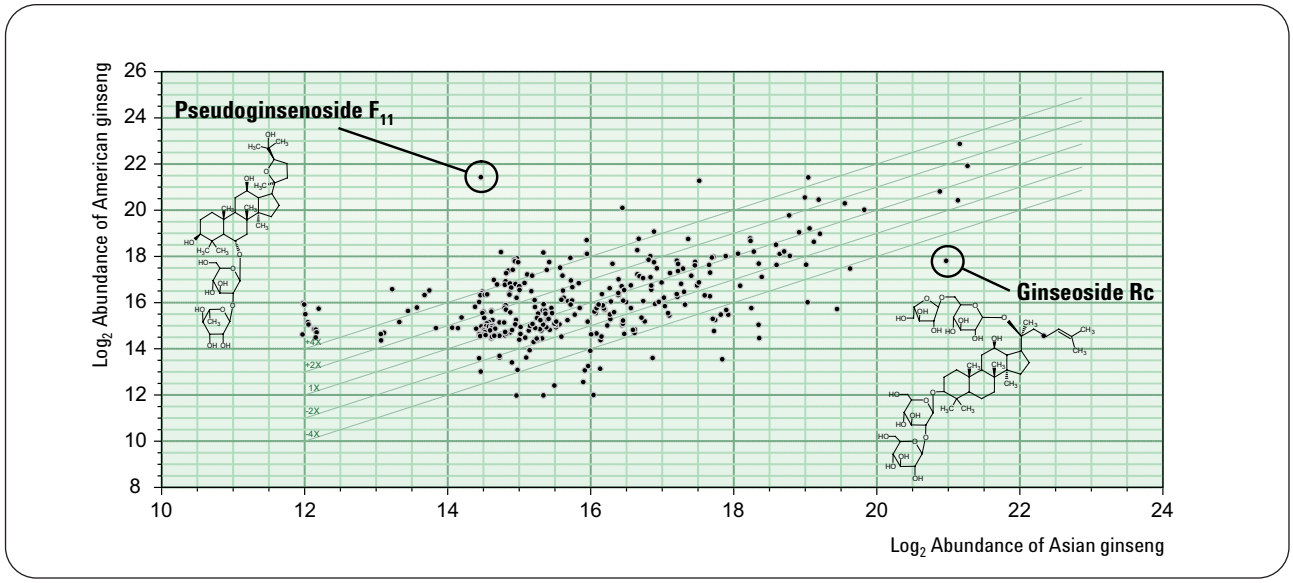

Figure 3
Differential analysis of Asian and American ginseng, showing pseudoginseoside F_{11} exclusively in American ginseng and higher concentration of ginsenoside Rc in Asian ginseng.

dance ratio of Asian and American ginseng extracts (figure 3). In the plot there are five lines for selected levels of abundance difference in the two sample groups. Molecular features lying on the line in the middle (1x) are equal in both groups, molecular features within the 2x margins are up to twice the abundance in one group and within the 4x margins up to fourfold. Beyond these margins a feature is nearly unique or exclusively present in one group. An example for a compound which has a higher concentration in the Asian ginseng sample group is the ginsenoside Rc (figures 1 and 3). The comparison of the abundances in each sample of the two groups clearly shows a significant eight-fold higher occurrence

ID	Name	RT	Mass	Abundance	Mass error [mDa]	Rel. mass error [ppm]	Av. rel. mass error [ppm]
1	American Ginseng_1	12.550	1078.5909	143,961	-1.50	1.36	0.99
2	American Ginseng_2	12.505	1078.5912	243,837	-1.20	1.09	
3	American Ginseng_3	12.557	1078.5921	240,121	-0.30	0.25	
4	American Ginseng_4	12.491	1078.5919	266,422	-0.50	0.44	
5	American Ginseng_5	12.493	1078.5904	252,872	-2.00	1.83	
6	Asian Ginseng_1	12.538	1078.5905	2,092,954	-1.90	1.73	1.71
7	Asian Ginseng_2	12.534	1078.5912	2,023,553	-1.20	1.09	
8	Asian Ginseng_3	12.545	1078.5898	2,058,617	-2.60	2.38	
9	Asian Ginseng_4	12.545	1078.5906	2,052,396	-1.80	1.64	
10	Asian Ginseng_5	12.534	1078.5905	2,042,638	-1.90	1.73	

Table 1
Retention times, abundancies and mass accuracies of ginsenoside Rc ($C_{53}H_{90}O_{22}$ at M = 1078.5924) in Asian and American ginseng.

ID	Name	RT	Mass	Abundance	Mass error [mDa]	Rel. mass error [ppm]	Av. rel. mass error [ppm]
1	American Ginseng_1	11.328	800.4900	2,835,414	-2.20	2.70	1.53
2	American Ginseng_2	11.347	800.4910	2,836,847	-1.20	1.50	
3	American Ginseng_3	11.337	800.4924	2,815,218	0.20	-0.24	
4	American Ginseng_4	11.333	800.4909	2,797,743	-1.30	1.60	
5	American Ginseng_5	11.335	800.4900	2,864,285	-2.20	1.60	
6	Asian Ginseng_1	11.337	800.4914	23,132	-0.80	1.00	1.26
7	Asian Ginseng_2	11.337	800.4930	22,875	0.80	-1.00	
8	Asian Ginseng_3	11.344	800.4927	20,865	0.50	-0.60	
9	Asian Ginseng_4	11.346	800.4942	23,578	2.00	-2.48	
10	Asian Ginseng_5	11.342	800.4912	22,556	-1.00	1.25	

Table 2
Retention times, abundancies and mass accuracies for Pseudoginsenoside F_{11} ($C_{42}H_{72}O_{14}$ at M = 800.4922), present nearly exclusively in American ginseng samples.

of ginsenoside Rc in Asian ginseng (table 1). The calculated relative mass errors are in the low single digit ppm range. The average for the Asian ginseng sample group is 1.71 ppm and the average for the American ginseng sample group is 0.90 ppm. The molecular feature, which is easily recognizable as almost exclusively present in the American ginseng samples, is the special the compound pseudoginsenoside F_{11} (figure 3). This compound has more than 100-fold higher abundance in the samples from the American ginseng root extract (table 2). For this compound, the average relative mass accuracy for the Asian ginseng sample group is 1.71 ppm and the average for the American ginseng sample group is 1.26 ppm.

Conclusion

The ingredients of highly complex natural products can be separated with very low standard deviations of retention times using the Agilent 1200 Series RRLC system and 1.8 µm particle size columns. Connection to the Agilent 6210 ESI-TOF MS facilitates acquisition of highly accurate and repeatable mass data. With these system prerequisites, the data can be processed by the Mass Profiler software for statistical evaluation of the differences in the abundance of the molecular features in the different sample groups. This facilitates identification of differences in concentration and abundance of single compounds in very complex samples such as natural product extracts.

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